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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	**EVE:** 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	**FOLIOS**: 6
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
HUMBERTO RUBIO CAMACHO

Asunto: Radicación: 11-27711- -15
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 15697
Folios: 6

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2009/053142				
Fecha de Presentación Internacional	07/08/2009				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado N°11-027711-00000-0000	07/03/2011
Reivindicaciones:	Radicado N°11-027711-00000-0000	07/03/2011
	Radicado N°11-027711-00001-0000	07/03/2011
	Radicado N°11-027711-00006-0000	05/10/2011
Prioridad:	US 61/188,307	08/08/2008

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

Título de la solicitud: "COMPUESTOS DE TETRACICLINA SUSTITUIDOS CON FLÚOR EN C7"

El problema planteado en esta solicitud consiste en la necesidad de diseñar nuevos análogos de tetraciclina sustituidos con Flúor en C7, con actividad antibacteriana mejorada.

Para resolver este problema técnico la solicitud en estudio proporciona un compuesto de fórmula estructural y su composición, según radicado N°11-027711-00006-0000.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07C 237/26, C07D 205/04.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d).

El objeto de las reivindicaciones 3 no es patentable de acuerdo con el Art citado, porque se refiere al método de tratamiento realizado con el compuesto reclamado en la reivindicación 1.

5. Determinación del Estado de la Técnica

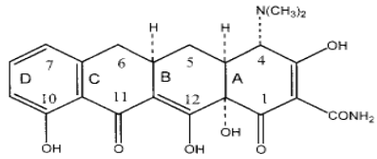
El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: agosto 08 de 2008, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	EP0582789	Novel 7-(substituted)-8-(substituted)-9-(substituted glycyl) amido-6-demethyl-6-deoxytetracyclines	Febrero 16 de 1994
D2	*Artículo	Tetracycline Antibiotics: Mode of action, Applications, Molecular Biology, and epidemiology of Bacterial Resistance	Junio de 2001

D1[1] divulga nuevos compuestos derivados 6 demetil, 6 deoxy tetraciclinas sustituidos 7,8 y 9 y sus composiciones, los cuales tienen efecto como antibióticos frente a microorganismos resistentes a las tetraciclinas.

[1] http://worldwide.espacenet.com/searchResults?compact=false&PN=EP0582789&ST=advanced&locale=en_EP&DB=EPODOC

D2[2] divulga diferentes miembros de las clases de tetraciclinas, cuyo grupo farmacóforo es:



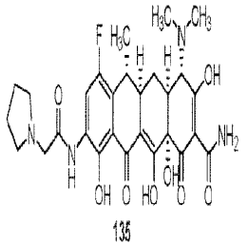
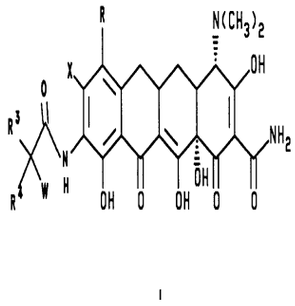
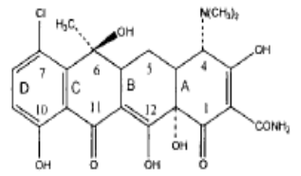
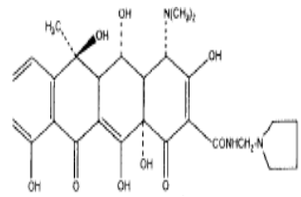
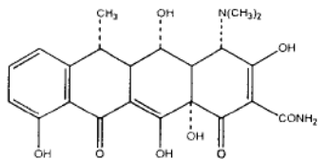
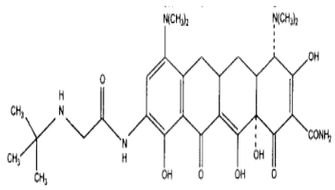
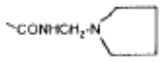
y tienen actividad antibacterial frente a organismos resistentes a tetraciclinas.

6. Examen de Patentabilidad (Art 14 D486)

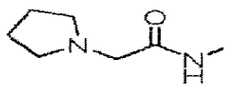
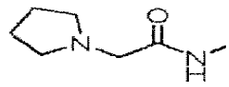
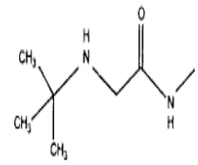
Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en un compuesto de formula estructural según radicado N°11-027711-00006-0000.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	**D1**	**D2**
 135	 I Fórmula I	    Tabla 2
1-CO	1-CO	1-CO
2-(CO)NH2	2-(CO)NH2	 2-(CO)NH2;
3-(OH)	3-(OH)	3-(OH)
4-N(CH3)2	4-N(CH3)2	4-N(CH3)2
5-(H)	5-(H)	5-(H)
6 metilo	H	6 metilo
7-F	R=F	Cl, H

[2] Adjunto al concepto.

8-H	X=Halógeno	8-H
H,H	R3 y R4 =H	
9- 	9- CH2-W=pirrolidinil 	9-H, 
10-C(OH)	10-C(OH)	10-C(OH)
11-C(O)	11-C(O)	11-C(O)
12--C(OH)	12--C(OH)	12--C(OH)

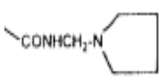
Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1.

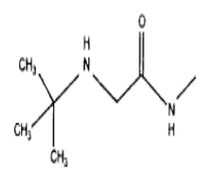
Porque D1 divulga compuestos derivados 6 demetil, 6 deoxy tetraciclinas sustituidos 7,8 y 9 de fórmulas (I y II) y sus composiciones, con actividad antimicrobiana frente a un amplio espectro de microorganismos incluyendo los altamente resistentes a las tetraciclinas (pág. 1,2).

La diferencia entre la invención, definida en la reivindicación 1, y el compuesto de D1 (fórmula I) consiste en la presencia un grupo metilo en la posición 6 y que no está sustituido en la posición 8.

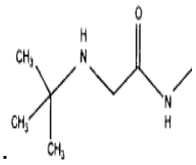
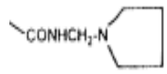
No obstante la diferencia anterior, no es posible evidenciar un efecto técnico sorprendente.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar el compuesto conocido en D1 para lograr una tetraciclina alternativa a las ya conocidas, con actividad antibacterial sobre bacterias resistentes a dichas tetraciclinas".

Adicionalmente, la presencia de un grupo metilo en la posición 6 y la no sustitución en las posición 8, ya estaba divulgada en los compuestos del documento D2, que se refiere a derivados de tetraciclinas, que en la posición 7 tienen un cloro (bioisosterismo)[3] en lugar del flúor en la posición 7 del compuesto reclamado, el grupo protector  en la posición 2 en lugar de la posición 9 del compuesto reclamado, sin embargo dichos compuestos presentan actividad antibiótica sobre microorganismos resistentes a dichas tetraciclinas (Tabla 2, profilaxis terapia en humanos, pág.249).

Igualmente, es importante mencionar que en la descripción de la presente solicitud se evidencia la misma actividad farmacológica para los compuestos 134 y 135 (pág.123, pág.136) de la invención frente a los mismos organismos ensayados, y que también el compuesto 134 está totalmente sugerido en el arte previo citado, cuando en la posición 9 del anillo D esta sustituido por  ,está presente el grupo metilo en la posición 6 y la no sustitución en la posición 8, de manera que se puede concluir que la actividad del compuesto 135 reclamado, que

[3] Introducción a la química terapéutica 2a Edición, Antonio Delgado C, Cristina Minguillón LL, Jesús Joglar Tamargo; pág. 139 tabla 7.1

es igual a la del compuesto 134, lo cual indica que los grupos protectores en la posición 9 como:  y  , no cambian la actividad de los compuestos), ni tampoco muestran un efecto sorprendente y por lo tanto el compuesto reclamado,135, no tiene nivel inventivo.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el grupo metilo en la posición 6 y no sustituiría la posición 8 de acuerdo a lo divulgado en los compuestos del documento D2, en el compuesto de D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Puesto que la reivindicación dependiente 2 no menciona características inventivas adicionales, tampoco se puede considerar inventiva.

7. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	EP0582789	1-2	Nivel inventivo
D2	*Artículo	1-2	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-08-27

Elaboró: Lyna Del Carmen Mordecay Dunoyer
Revisó: Gloria Jacqueline Alfonso Rozo

Microbiology and Molecular Biology Reviews

Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance

Ian Chopra and Marilyn Roberts
Microbiol. Mol. Biol. Rev. **2001, 65(2):232. DOI: 10.1128/MMBR.65.2.232-260.2001.**

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MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, June 2001, p. 232–260 Vol. 65, No. 2
1092-2172/01/$04.00+0 DOI: 10.1128/MMBR.65.2.232–260.2001
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Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance

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INTRODUCTION

The tetracyclines, which were discovered in the 1940s, are a family of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. Tetracyclines are broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites. The favorable antimicrobial properties of these agents and the absence of major adverse side effects has led to their extensive use in the therapy of human and animal infections. They are also used

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TABLE 1. Principal members of the tetracycline class

Chemical name	Generic name	Trade name	Yr of discovery	Status	Therapeutic administration
7-Chlortetracycline	Chlortetracycline	Aureomycin	1948	Marketed	Oral
5-Hydroxytetracycline	Oxytetracycline	Terramycin	1948	Marketed	Oral and parenteral
Tetracycline	Tetracycline	Achromycin	1953	Marketed	Oral
6-Demethyl-7-chlortetracycline	Demethylchlortetracycline	Declomycin	1957	Marketed	Oral
2-*N*-Pyrrolidinomethyltetracycline	Rolitetracycline	Reverin	1958	Marketed	Oral
2-*N*-Lysinomethyltetracycline	Limecycline	Tetralysal	1961	Marketed	Oral and parenteral
N-Methylol-7-chlortetracycline	Clomocycline	Megaclor	1963	Marketed	Oral
6-Methylene-5-hydroxytetracycline	Methacycline	Rondomycin	1965	Marketed	Oral
6-Deoxy-5-hydroxytetracycline	Doxycycline	Vibramycin	1967	Marketed	Oral and parenteral
7-Dimethylamino-6-demethyl-6-deoxytetracycline	Minocycline	Minocin	1972	Marketed	Oral and parenteral
9-(*t*-butylglycylamido)-minocycline	Tertiary-butylglycylamidominocycline	Tigilcycline	1993	Phase II clinical trials	

prophylactically for the prevention of malaria caused by mefloquine-resistant *Plasmodium falciparum*. Furthermore, in some countries, including the United States, tetracyclines are added at subtherapeutic levels to animal feeds to act as growth promoters. Although the tetracyclines retain important roles in both human and veterinary medicine, the emergence of microbial resistance has limited their effectiveness. Undoubtedly the use of tetracyclines in clinical practice has been responsible for the selection of resistant organisms. Nevertheless, as we enter the new millennium, the use of tetracyclines and other antibiotics as animal growth promoters is becoming increasingly controversial because of concerns that this practice may be contributing to the emergence of resistance in human pathogens. The increasing incidence of bacterial resistance to tetracyclines has in turn resulted in efforts to establish the mechanisms by which genetic determinants of resistance are transferred between bacteria and the molecular basis of the resistance mechanisms themselves. The improved understanding of tetracycline resistance mechanisms achieved by this work has provided opportunities for the recent discovery of a new generation of tetracyclines, the glycylcyclines (see below). Further research, already under way, is also identifying approaches by which inhibitors of tetracycline resistance mechanisms might be developed for use in conjunction with earlier tetracyclines to restore their antimicrobial activity (185, 186).

The tetracyclines have been extensively reviewed both by the present authors (41, 43, 44, 100, 227–229) and others (56, 73, 263, 275). Nevertheless, in view of continuing interest in this group of antibiotics for both infectious and noninfectious diseases (95), we have decided to write a review that focuses on recent developments in the field.

DISCOVERY AND DEVELOPMENT OF THE TETRACYCLINES

Chlortetracycline and oxytetracycline (Tables 1 and 2), both discovered in the late 1940s, were the first members of the tetracycline group to be described. These molecules were products of *Streptomyces aureofaciens* and *S. rimosus*, respectively. Other tetracyclines were identified later, either as naturally occurring molecules, e.g., tetracycline from *S. aureofaciens, S. rimosus*, and *S. viridofaciens* and demethylchlortetracycline from *S. aureofaciens*, or as products of semisynthetic approaches, e.g., methacycline, doxycycline, and minocycline. Despite the success of the early tetracyclines, analogs were sought with improved water solubility either to allow parenteral administration or to enhance oral absorption. These approaches resulted in the development of the semisynthetic compounds rolitetracycline and lymecycline (Tables 1 and 2). The most recently discovered tetracyclines are the semisynthetic group referred to as glycylcyclines, e.g., 9-(*N,N*-dimethylglycylamido)-6-demethyl-6-deoxytetracycline, 9-(*N,N*-dimethylglycylamido)-minocycline, and 9-*t*-(butylglycylamido)-minocycline (Tables 1 and 2). These compounds possess a 9-glycylamido substitutent (Table 2). The antibiotics in Tables 1 and 2 can be referred to as first-generation (1948 to 1963), second-generation (1965 to 1972), and third-generation (glycylcycline) tetracyclines. The 9-*t*-butylglycylamido derivative of minocycline (tigilcycline; formerly known as GAR-936) commenced phase I studies in October 1999 and is currently undergoing phase II clinical trials (113). Some of the earlier compounds, e.g., clomocycline, are no longer marketed, and others, e.g., rolitetracycline, lymecycline, and chlortetracycline, are not available in all countries (73, 137).

STRUCTURE-ACTIVITY RELATIONSHIPS

The structural features that confer antibacterial activity to the tetracyclines are well established (56, 179, 250) and will only be briefly discussed. More recently, however, new aspects of structure-activity relationships have emerged. This has followed efforts to extend the therapeutic utility of this antibiotic class to encompass bacteria expressing resistance to first- and second-generation compounds through ribosomal protection- and efflux-based mechanisms. This aspect is considered in more detail below.

Tetracycline molecules comprise a linear fused tetracyclic nucleus (rings designated A, B, C, and D [Table 2]) to which a variety of functional groups are attached. The simplest tetracycline to display detectable antibacterial activity is 6-deoxy-6-demethyltetracycline (Fig. 1) and so this structure may be regarded as the minimum pharmacophore (179). Features important for antibacterial activity among the tetracyclines are maintenance of the linear fused tetracycle, naturally occurring (α) stereochemical configurations at the 4a, 12a (A-B ring junction), and 4 (dimethylamino group) positions, and conser-

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TABLE 2. Structures of the principal members of the tetracycline class

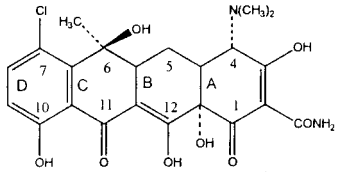
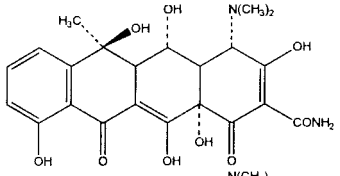
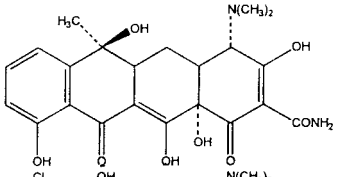
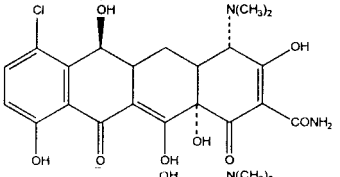
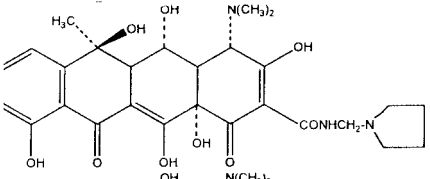
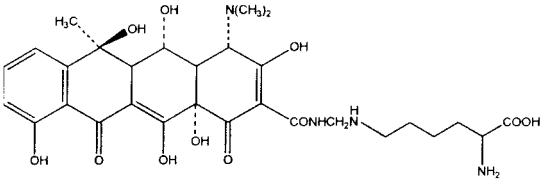
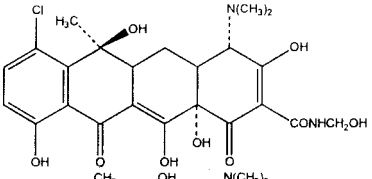
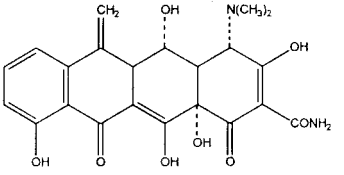
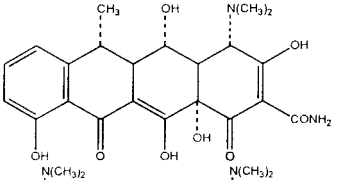
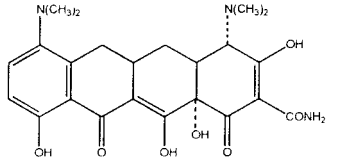
Chemical name	Structure
7-Chlortetracycline	
5-Hydroxytetracycline	
Tetracycline	
6-Demethyl-7-chlortetracycline	
2-*N*-Pyrrolidinomethyltetracycline	
2-*N*-Lysinomethyltetracycline	
N-Methylol-7-chlortetracycline	
6-Methylene-5-hydroxytetracycline (methacycline)	
6-Deoxy-5-hydroxytetracycline (doxycycline)	
7-Dimethylamino-6-demethyl-6-deoxytetracycline (minocycline)	

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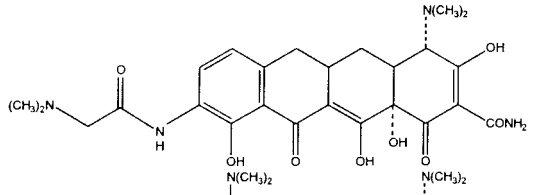
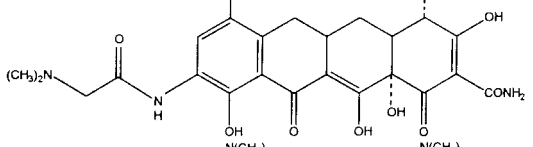
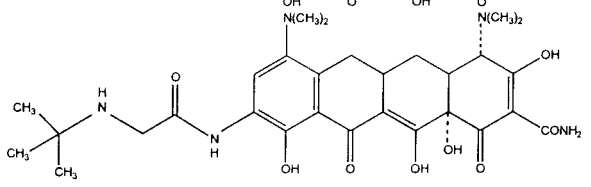
TABLE 2—*Continued*

Chemical name	Structure
9-(*N,N*-Dimethylglycylamido)-6-demethyl-6-deoxytetracycline	
9-(*N,N*-Dimethylglycylamido)-minocycline	
9-(*t*-Butylglycylamido)-minocycline	

vation of the keto-enol system (positions 11, 12, and 12a) in proximity to the phenolic D ring. The tetracyclines are strong chelating agents (20, 44) and both their antimicrobial and pharmacokinetic properties are influenced by chelation of metal ions (see below). Chelation sites include the β-diketone system (positions 11 and 12) and the enol (positions 1 and 3) and carboxamide (position 2) groups of the A ring (20, 44). The newly discovered glycylcyclines, like other tetracycline derivatives, also form chelation complexes with divalent cations (278). Replacement of the C-2 carboxamide moiety with other groups has generally resulted in analogs with inferior antibacterial activity (179), probably because bacteria accumulate these molecules poorly (42). However, the addition of substituents to the amide nitrogen can impart significant water solubility, as in the case of rolitetracycline and lymecycline (Table 2). Dissociation of the prodrugs in vivo liberates free tetracycline (179, 250). Consistent with the above observations, substitutions at positions 1, 3, 4a, 10, 11, or 12 are invariably detrimental for antibacterial activity (179). Nevertheless, a number of other substitutions at different positions on the B, C, and D rings are tolerated, and molecules possessing these substituents have given rise to the tetracyclines in clinical use today, as well as the new glycylcycline molecules that are currently undergoing clinical trials (Table 2).

The extensive structure-activity studies referred to above

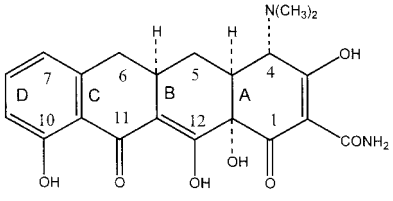


FIG. 1. Structure of 6-deoxy-6-demethyltetracycline, the minimum tetracycline pharmacophore.

revealed that with one exception, each of the rings in the linear fused tetracyclic nucleus must be six membered and purely carbocyclic for the molecules to retain antibacterial activity. For instance, the nortetracyclines, derivatives in which the B ring comprises a five-membered carbocycle, are essentially devoid of antibacterial activity (250). Nevertheless, 6-thiatetracycline, which possesses a sulfur atom at position 6 of the C ring, is an apparent exception to the rule that a purely carbocyclic six-membered ring structure is required for activity, since molecules in this series have potent antibacterial properties (43, 250). Nevertheless, it has now been established that the thiatetracyclines and a number of other tetracycline analogs, referred to collectively as atypical tetracyclines (43, 44), exhibit a different structure-activity relationship from the majority of tetracyclines. These molecules, which also include the anhydrotetracyclines, 4-epi-anhydrotetracyclines, and chelocardin, appear to directly perturb the bacterial cytoplasmic membrane, leading to a bactericidal response (43, 198, 199). This contrasts with the typical tetracyclines, which interact with the ribosome to inhibit bacterial protein synthesis and display a reversible bacteriostatic effect. The membrane-disrupting properties of the atypical tetracyclines are probably related to the relative planarity of the B, C, and D rings so that a lipophilic, nonionized molecule predominates. On interaction with the cell, the atypical tetracyclines are likely to be preferentially trapped in the hydrophobic environment of the cytoplasmic membrane, disrupting its function. These molecules are of no interest as therapeutic candidates because they cause adverse side effects in humans (250), which are probably related to their ability to interact nonspecifically with eukaryotic as well as prokaryotic cell membranes (43). A few other tetracycline molecules have been examined but have not moved on to further study (95, 185, 300).

There has been widespread emergence of efflux- and ribosome-based resistance to first- and second-generation tetracy-

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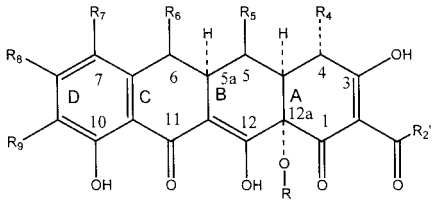

Rings (A-B-C-D) = linear-fused rings

Rings (D-C-B) C-10, C-11, C-12 = phenolic keto-enol system

Ring (A) C-1, C-2,C-3 = tricarbonyl-type keto-enol system

$R_{2'}$ = NH_2 or NHR where R is aminoalkyl

R_4 = (α) dimethylamino group superior

C-4a = (α)-proton

R_5 = H (tetracyclines) or OH (oxytetracyclines)

C-5a = (α)-proton

R_6 = combination of (β)-OH and/or (α)-CH_3 or unsubstituted, or methylene group

R_7 = H (tetracyclines), Cl (chlortetracyclines) or dialkylamino (minocycline-type) or NO_2

R_8 = H

R_9 = glycylamido

R_{12a} = H

FIG. 2. Stereochemical and substitution requirements for optimum antibacterial activity within the tetracycline series.

TABLE 3. Mechanisms of resistance for characterized *tet* and *otr* genes[a]

Genes
Efflux
tet(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(I), *tet*(J), *tet*(Z), *tet*(30)[b] *tet*(31)[b]
tet(K), *tet*(L)
otr(B), *tcr3*[c]
*tet*P(A)
tet(V)
tet(Y)[d]
Ribosomal protection
tet(M), *tet*(O), *tet*(S), *tet*(W)
tet(Q), *tet*(T)
otr(A), *tet*P(B),[e] *tet*[c]
Emzymatic, *tet*(X)
Unknown[f]
tet(U), *otr*(C)

[a] Grouped according to McMurry and Levy (173).
[b] First numbered genes (150).
[c] These genes have not been given new designations (150).
[d] Relatedness to groups 1 to 6 is unclear, since the gene has not been studied extensively.
[e] *tet*P(B) is not found alone, and *tet*P(A) and *tet*P(B) are counted as one gene (164, 273).
[f] *tet*(U) has been sequenced but does not appear to be related to either efflux or ribosomal protection proteins; *otr*(C) has not been sequenced (207, 220).

clines (1, 2, 19, 38, 44, 70, 85, 103, 120, 122, 150, 165, 227–229, 307). To restore the potential of the tetracyclines as a class of useful broad-spectrum agents, a systematic search was undertaken during the early 1990s to discover new analogs that might possess activity against organisms resistant to older members of the class while retaining activity against tetracycline-susceptible organisms (295). This resulted in the discovery of the 9-glycinyltetracyclines (glycylcyclines) (15, 286, 287, 295) (Tables 1 and 2). Previous attempts to introduce substituents at position 9 of the molecule, e.g., 9-nitro, 9-amino, and 9-hydroxy, led to analogs with poor antibacterial activity (179, 250). However, during the 1990s, a team at Lederle Laboratories (now American Home Products) noted that 9-acylamido derivatives of minocycline exhibited antibacterial activities typical of earlier tetracyclines but without activity against tetracycline-resistant organisms (15). Nevertheless, when the acyl group was modified to include an *N,N*-dialkylamine moiety, e.g., as in the 6-demethyl-6-deoxytetracycline and minocycline derivatives (GAR-936) shown in Table 2, not only was antibacterial activity retained but also the compounds displayed activity against bacteria containing *tet* genes (Tables 3 to 5) responsible for both efflux of earlier tetracyclines [Tet(A)- to Tet(D) and Tet(K)] and ribosomal protection [Tet(M)] (15, 286, 287, 295). These findings were extended to the 9-*t*-butylglycylamido derivative of minocycline (Tables 1 and 2) (208). These data suggest that new structure-activity relationships may have been defined for activity against strains expressing efflux or ribosomal protection mechanisms that encompass the previous requirements for activity against tetracycline-susceptible strains but in addition require an *N*-alkyl glycylamido substitution at the 9 position of the molecule.

Figure 2 presents a summary of the features that confer optimum antibacterial activity to the tetracycline nucleus.

MODE OF ACTION

It is well established that tetracyclines inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome (44, 263). Therefore, to interact with their targets these molecules need to traverse one or more membrane systems depending on whether the susceptible organism is gram positive or gram negative. Hence, a discussion of the mode of action of tetracyclines requires consideration of uptake and ribosomal binding mechanisms. Also pertinent to this discussion are explanations of the joint antibacterial-antiprotozoal activity of the tetracyclines and the microbial selectivity of the class as a whole. Most of these issues have been considered at length in recent years (44, 67, 78, 263), so the focus here will be on new information.

Tetracyclines traverse the outer membrane of gram-negative enteric bacteria through the OmpF and OmpC porin channels, as positively charged cation (probably magnesium)-tetracycline coordination complexes (44, 263). The cationic metal ion-antibiotic complex is attracted by the Donnan potential across the outer membrane, leading to accumulation in the periplasm, where the metal ion-tetracycline complex probably dissociates to liberate uncharged tetracycline, a weakly lipophilic molecule able to diffuse through the lipid bilayer regions of the inner (cytoplasmic) membrane. Similarly, the electroneutral, lipophilic form is assumed to be the species transferred across

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the cytoplasmic membrane of gram-positive bacteria. Uptake of tetracyclines across the cytoplasmic membrane is energy dependent and driven by the ΔpH component of the proton motive force (192, 263). Within the cytoplasm, tetracycline molecules are likely to become chelated since the internal pH and divalent metal ion concentrations are higher than those outside the cell (263). Indeed, it is probable that the active drug species which binds to the ribosome is a magnesium-tetracycline complex (44, 144). Association of tetracyclines with the ribosome is reversible, providing an explanation of the bacteriostatic effects of these antibiotics (44).

Several studies have indicated a single, high-affinity binding site for tetracyclines in the ribosomal 30S subunit, with indications through photoaffinity labeling and chemical footprinting studies that protein S7 and 16S rRNA bases G693, A892, U1052, C1054, G1300, and G1338 contribute to the binding pocket (44, 180, 196, 263). However, Schnappinger and Hillen (263) have pointed out that these apparent sites for drug interaction in the ribosome may not necessarily reflect the actual binding site. Indeed, interpretation of the probing studies referred to above is complicated by the observation that binding of tetracycline (which measures approximately 8 by 12 Å) to the ribosome appears to cause wide-ranging structural change in 16 S rRNA (193). Furthermore, photoincorporation methods are subject to the limitation that upon irradiation, tetracycline photoproducts are generated which may react further with the ribosomes (196). Nevertheless, naturally occurring tetracycline-resistant propionibacteria contain a cytosine-to-guanine point mutation at position 1058 in 16S rRNA (251) (see below), which does at least suggest that the neighboring bases U1052 and C1054 identified by chemical footprinting (180) may have functional significance for the binding of tetracyclines to the 30S subunit.

The absence of major antieukaryotic activity explains the selective antimicrobial properties of the tetracyclines. At the molecular level, this results from relatively weak inhibition of protein synthesis supported by 80S ribosomes (302) and poor accumulation of the antibiotics by mammalian cells (78). However, tetracyclines inhibit protein syntheses in mitochondria (221) due to the presence of 70S ribosomes in these organelles. It has been recognized for some time that the spectrum of activity of tetracyclines encompasses various protozoan parasites such as *P. falciparum, Entamoeba histolytica, Giardia lamblia, Leishmania major, Trichomonas vaginalis*, and *Toxoplasma gondii* (28, 44, 67, 137, 214). The antiparasitic activity is explained in some cases by the finding that certain organisms, e.g., *P. falciparum*, contain mitochondria (67). However, a number of other protozoa which lack mitochondria nevertheless remain susceptible to tetracyclines. At present there is no satisfactory molecular explanation for these findings (67).

RESISTANCE TO TETRACYCLINES

Introduction

Prior to the mid-1950s, the majority of commensal and pathogenic bacteria were susceptible to tetracyclines (144), as illustrated by the finding that among 433 different members of the *Enterobacteriaceae* collected between 1917 and 1954, only 2% were resistant to tetracycline (106). Studies of naturally occurring environmental bacteria, representative of populations existing before the widespread use of tetracyclines by humans (52), also support the view that the emergence of resistance is a relatively modern event that has followed the introduction of these agents for clinical, veterinary, and agricultural use. Indeed, resistance to tetracyclines has now emerged in many commensal and pathogenic bacteria due to genetic acquisition of *tet* genes. Subsequent parts of this section describe the genetic and biochemical mechanisms of tetracycline resistance, the regulation of resistance gene expression, and the distribution of *tet* genes in pathogenic and commensal bacteria. The impact of resistance on the use of tetracyclines for human medicine is examined later in the review.

Genetic and Biochemical Mechanisms of Tetracycline Resistance

Nomenclature of resistance determinants. Mendez et al. (176) in 1980 first examined the genetic heterogeneity of tetracycline resistance determinants from plasmids from members of the *Enterobacteriaceae* and *Pseudomonadaceae*. They used restriction enzyme analysis, DNA-DNA hybridization, and expression of resistance to tetracycline and various analogs to categorize the tetracycline-resistant (Tc^r) plasmids. Currently, two genes are considered related (i.e., of the same class) and given the same gene designation if they have ≥80% of their amino acid sequences in common. Two genes are considered different from each other if they have ≤79% amino acid sequence identity (150). This comparison can now be done using GenBank sequence information, since with few exceptions [*tet*(I) and *otr*(C)], representatives of all *tet* and *otr* genes have been sequenced and are available (150, 151) (Table 3).

For most genes, only one representative from each class has been sequenced, making comparisons easier to perform. One exception is the *tet*(M) gene, which has been sequenced from a number of gram-positive and gram-negative species (228). To determine the distribution of any particular *tet* or *otr* gene, it is now customary to prepare specific oligonucleotide probes that hybridize with the specific gene of interest but not to related genes. For example, if one is screening for the presence of the *tet*(M) gene, the oligonucleotide probes would not hybridize to *tet*(O) or *tet*(S) genes, which have approximately 78% sequence identity to the *tet*(M) gene (228). The number of *tet* genes has reached the end of the Roman alphabet, and numbers are being assigned to accommodate new *tet* genes (150). The first two genes with a number designation have now been assigned (Table 4). Stuart Levy has agreed to coordinate the assignment of proposed numbers for a new tetracycline resistance gene to prevent two distinct *tet* genes from being assigned the same numbers or two related genes (≥80% identity) being assigned different numbers (150).

Twenty-nine different tetracycline resistance (*tet*) genes and three oxytetracycline resistance (*otr*) genes have been characterized. There is no inherent difference between a tetracycline and an oxytetracycline resistance gene. The oxytetracycline genes were first identified in oxytetracycline-producing organisms, and thus the nomenclature reflects the organisms first shown to carry the particular gene. We have shown that the *tet* genes are found in the producing *Streptomyces* spp. and the *otr*

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TABLE 4. Distribution of *tet* resistance genes among gram-negative bacteria[a]

Efflux				Ribosomal protection[b] and/or efflux			
One gene		Two or more genes		One gene		Two or more genes	
Genus	Gene	Genus	Genes	Genus	Gene	Genus	Genes
Actinobacillus	*tet*(B)	*Edwardsiella*	*tet*(A), *tet*(D)	*Eikenella*	*tet*(M)	*Butyrivibrio*[c]	*tet*(O), *tet*(W)
Erwinia	*tet*(B)	*Providencia*	*tet*(B), *tet*(E), *tet*(I)	*Kingella*	*tet*(M)	*Mitsuokella*[c]	*tet*(Q), *tet*(W)
Moraxella	*tet*(B)	*Plesiomonas*	*tet*(A), *tet*(B), *tet*(D)	*Neisseria*	*tet*(M)	*Selenomonas*[c]	*tet*(Q), *tet*(W)
Pantoea	*tet*(B)	*Enterobacter*	*tet*(B), *tet*(C), *tet*(D)	*Campylobacter*	*tet*(O)	*Porphyromonas*[c]	*tet*(Q), *tet*(W)
Treponema[d]	*tet*(B)	*Mannheimia*	*tet*(B), *tet*(G), *tet*(H)	*Capnocytophaga*[c]	*tet*(Q)	*Bacteroides*[c]	*tet*(M), *tet*(Q), *tet*(X)
Yersinia	*tet*(D)	*Proteus*	*tet*(A), *tet*(B), *tet*(C), *tet*(J)	*Prevotella*[c]	*tet*(Q)	*Fusobacterium*[c]	*tet*(L), *tet*(M), *tet*(W)
Alcaligenes	*tet*(E)	*Pseudomonas*	*tet*(A), *tet*(C), *tet*(E), *tet*(G)			*Haemophilus*	*tet*(B), *tet*(K), *tet*(M)
Eubacterium[c]	*tet*(K)	*Serratia*	*tet*(A), *tet*(B), *tet*(C), *tet*(E)			*Veillonella*[c]	*tet*(L), *tet*(M), *tet*(Q)
Agrobacterium	*tet*(30)[e]	*Citrobacter*	*tet*(A), *tet*(B), *tet*(C), *tet*(D)			*Pasteurella*	*tet*(B), *tet*(D), *tet*(H), *tet*(G), *tet*(M)
		Klebsiella	*tet*(A), *tet*(B), *tet*(C), *tet*(D)				
		Shigella	*tet*(A), *tet*(B), *tet*(C), *tet*(D)				
		Salmonella	*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(G)				
		Aeromonas	*tet*(A), *tet*(B), *tet*(D), *tet*(E), *tet*(31)[e]				
		Vibrio	*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G)				
		Escherichia	*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(I), *tet*(Y)				

[a] Based on information from references 8, 9, 11, 14, 19, 40, 45, 55, 58–61, 81, 94, 97, 104, 111, 115, 116, 120, 122, 132, 139, 143, 149, 151, 152, 155, 163, 168–170, 182, 189, 200, 201, 206, 224, 227, 232, 233, 234, 241, 248, 256, 262, 267, 280, 282, 296, 314, 317, and 319, and M. Roberts, unpublished results).
[b] Ribosomal protection genes have not yet been found in enteric genera, and when these genes are cloned into *E. coli* the level of resistance conferred is relatively low.
[c] Anaerobic species.
[d] *T. denticola* anaerobic species, but not all species in the genus are anaerobes.
[e] Beginning of the number designations.

genes are found in the nonproducing *Mycobacterium* spp. (Table 5). Eighteen of the *tet* genes and one of the *otr* genes code for efflux pumps, and seven of the *tet* genes and one of the *otr* genes *otr*(A) code for ribosomal protection proteins (Table 3). The presence of both *tet* and *otr* genes with similar efflux or ribosomal protection mechanisms of resistance is consistent with the hypothesis of lateral gene transfer from the tetracycline-producing streptomycetes to other bacteria (16) (Table 5). The *tet*(P) gene is unusual because it consists of the *tetA*(P) gene, which encodes a functional efflux protein, linked to the *tetB*(P) gene, which appears to encode a ribosomal protection protein. *tet*(P) is counted as one gene in this review, although each component gene is listed in Table 3. *tetA*(P) has been found without *tetB*(P), but *tetB*(P) has not been found alone (164). The *otr*(C) gene has not been sequenced, while the *tet*(U) DNA sequence is unrelated to tetracycline efflux, tetracycline ribosomal protection proteins, or enzymatic protein (220, 228). The *tet*(I) gene has not been sequenced, but phenotypic studies suggest it encodes an efflux pump. It is therefore included in Table 3. An uncharacterized gene has also been described in a gram-negative species (116), and four different ribosomal protection genes, from streptococci, have been cloned using degenerate PCR primers (47). Whether these genes are related to some of the newer *tet* genes is not clear. The new ribosomal protection genes did not hybridize with *tet*(M), *tet*(O), *tet*P(B), *tet*(Q), *tet*(S), or *tet*(T), suggesting that at least some of the four could be novel, although hybridization with *tet*(W) was not examined. These five genes have not been characterized in detail and have not been added to Tables 3 to 5. The *tet*(X) gene encodes an enzyme which modifies and inactivates the tetracycline molecule (281). However, it does not seem to have much clinical relevance since it requires oxygen to function and is found only in strict anaerobes, where oxygen is excluded (281). Thus, it is unlikely that the *tet*(X) gene functions in its natural host (*Bacteroides*). No work has been done to determine whether *tet*(X) is associated with any aerobic species (229). The *otr* genes were first described in the antibiotic-producing *Streptomyces* species (63, 66, 218) but more recently have also been found in clinical *Mycobacterium* spp. (207) and may have a wider distribution among environmental species (Table 5).

There are reports of phenotypically Tc^r isolates, which did not hybridize with any of the *tet* probes examined at the time (59–61, 222, 226, 228, 235). Some of these isolates may carry subsequently characterized *tet* genes that were not identified at the time of the original work or not thought to be relevant for screening, e.g., determining whether *tet* genes from gram-positive species were present in gram-negative species. We now know that increasing numbers of gram-negative bacteria carry what have been labeled gram-positive *tet* genes, such as *tet*(K), *tet*(L), *tet*(O), and *tet*(M), although these are not often examined when dealing with gram-negative isolates. Similarly, the *tet*(Q) gene, which was first described in the gram-negative genes *Bacteroides*, has a low G+C content, can be expressed in both gram-positive and gram-negative species, and is often associated with conjugative transposons. Therefore it should be considered whenever Tc^r isolates are examined and not just when gram-negative anaerobes are screened (45, 46, 206) (Table 5). Another possibility is that these Tc^r isolates carry novel genes that have yet to be described. Certainly, new *tet* genes are

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TABLE 5. Distribution of tetracycline resistance genes among gram-positive bacteria, *Mycobacterium, Mycoplasma, Nocardia, Streptomyces*, and *Ureaplasma*[a]

One determinant		Two determinants		Three or more determinants	
Genus	Gene	Genus	Genes	Genus	Genes
Abiotrophia	*tet*(M)	*Actinomyces*	*tet*(L), *tet*(M)	*Eubacterium*[b]	*tet*(K), *tet*(M), *tet*(Q)
Bacterionema	*tet*(M)	*Aerococcus*	*tet*(M), *tet*(O)	*Bacillus*	*tet*(K), *tet*(L), *tet*(M)
Gemella	*tet*(M)	*Bifidobacterium*[b]	*tet*(M), *tet*(W)	*Listeria*	*tet*(K), *tet*(L), *tet*(M), *tet*(S)
Mycoplasma[c]	*tet*(M)	*Gardnerella*	*tet*(M), *tet*(Q)	*Staphylococcus*	*tet*(K), *tet*(L), *tet*(M), *tet*(O)
Ureaplasma[c]	*tet*(M)	*Lactobacillus*	*tet*(O), *tet*(Q)	*Clostridium*[b]	*tet*(K), *tet*(L), *tet*(M), *tet*(P), *tet*(Q)
Nocarida	*tet*(K)	*Mobiluncus*[b]	*tet*(O), *tet*(Q)	*Peptostreptococcus*[b]	*tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(Q)
		Corynebacterium	*tet*(M), *tet*(Z)	*Enterococcus*	*tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S), *tet*(U)
				Streptococcus	*tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(T)
				Mycobacterium[d]	*tet*(K), *tet*(L), *tet*(V), *otr*(A), *otr*(B)
				Streptomyces[e]	*tet*(K), *tet*(L), *otr*(A), *otr*(B), *otr*(C), *tcr3*[f], *tet*[f]

[a] Based on information from references 12, 13, 26, 27, 33–38, 45, 47, 48, 62–66, 69, 74–76, 84, 85, 93, 96, 98, 103, 105, 108, 138a, 139, 140, 143, 150–153, 162, 164, 183, 184, 188, 200, 201, 206, 207, 209, 210, 212, 213, 220, 222, 225–230, 235–238, 242–245, 255, 273, 290, 297, and 307; and M. Roberts, unpublished results.
[b] Anaerobic species.
[c] Cell-wall-free bacteria with a gram-positive metabolism.
[d] Acid-fast bacteria.
[e] Multicellular bacteria.
[f] *tet* and *tcr* have not been given number designations.

being identified, e.g., *tet*(Y) in *Escherichia coli, tet*(31) in *Aeromonas, tet*(W) in anaerobic species, and *tet*(Z) in *Corynebacterium* (Tables 4 and 5) (14, 150, 267).

Efflux proteins. The efflux proteins are the best studied of the Tet proteins. The genes encoding then belong to the major facilitator superfamily (MFS), whose products include over 300 individual proteins (205). All the *tet* efflux genes code for membrane-associated proteins which export tetracycline from the cell. Export of tetracycline reduces the intracellular drug concentration and thus protects the ribosomes within the cell. Efflux genes are found in both gram-positive and gram-negative species (Table 4 and 5). Most of these efflux proteins confer resistance to tetracycline but not to minocycline or glycylcyclines. In contrast, the gram-negative *tet*(B) gene codes for an efflux protein which confers resistance to both tetracycline and minocycline but not glycylcyclines (44, 295). However, laboratory-derived mutations in *tet*(A) or *tet*(B) have led to glycylcycline resistance, suggesting that bacterial resistance to this group of drugs may develop over time and with clinical use (90; M. Tuckman, P. J. Petersen, and S. Projan, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C98, p. 97, 1998).

Each of the efflux genes codes for an approximately 46-kDa membrane-bound efflux protein. These proteins have been divided into six groups based on amino acid sequence identity (173). Group 1 contains Tet(A), Tet(B), Tet(C), Tet(D), Tet(E), Tet(G), Tet(H), Tet(Z), and probably Tet(I), Tet(J), and Tet(30) (173, 291). The tetracycline resistance proteins in this group have 41 to 78% amino acid identity. Their tetracycline repressor proteins have 37 to 88% amino acid identity. In this group the proteins have 12 predicted transmembrane α-helices with long central nonconserved cytoplasmic loops connecting transmembrane helices 6 and 7. Among group 1, only Tet(Z) is found in gram-positive species; the others are found only in gram-negative isolates. Tet(Z) is the first gram-positive efflux protein to be described where regulation is controlled by a repressor protein (291). Most of the efflux proteins appear to reside in the lipid bilayer, with the hydrophilic amino acid loops protruding into the periplasmic and cytoplasmic space. The efflux proteins exchange a proton for a tetracycline-cation complex against a concentration gradient (318). The efflux genes from gram-negative bacteria have two functional domains, α and β, which correspond to the N- and C-terminal halves of the protein, respectively (253). Mutations in either half of the protein eliminate resistance, suggesting that residues dispersed across the protein are important for function. More recently, combined mutagenic and labeling approaches have been used to probe topology and structure-function relationships in the gram-negative MFS tetracycline transporter family. Mutations apparently affecting energy coupling have been located in cytoplasmic loops 2–3 and 10–11 of the efflux protein (174). Therefore, within the efflux proteins from gram-negative bacteria, these loops may interact functionally as the proton pump (175). The boundaries of membrane-embedded domains have been defined (126, 134), and the proposed topology of the proteins developed by earlier modeling methods has been confirmed experimentally (127). Furthermore, charge interactions between key residues such as arginine-70 and aspartate-120 in the Tet(B) protein have been identified as a requirement for correct positioning of transmembrane segments in the cytoplasmic membrane (279). Mutation studies have also been used to try to identify the antibiotic binding site within the efflux proteins from gram-negative bacteria. At least part of this site appears to reside in transmembrane helix 4 (110). Binding of the substrate to this region appears to affect the conformation of other regions in the protein, since substrate-induced conformational changes have been detected in transmembrane helices 1 and 11 (129). In a series of studies by Yamaguchi and coworkers, evidence has been obtained for a water-filled transmembrane channel in the Tet(B) efflux protein, flanked by transmembrane helices 2 and 5 and part of helix 4 (110, 128, 129).

Tetracycline efflux proteins have amino acid and protein structure similarities with other efflux proteins involved in multiple-drug resistance, quaternary ammonium resistance, and chloramphenicol and quinolone resistance, including methylenomycin A (MetA) from *Streptomyces coelicolor*, aminotriazole transport (Atr1) from *Saccharomyces*, and arabinose

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transport (AraB) from *Escherichia coli* (147, 269). Homology between the Tet and other efflux proteins has also been found with a new protein (EfpA) cloned from *Mycobacterium tuberculosis* (65).

The gram-negative efflux genes are widely distributed and normally associated with large plasmids, most of which are conjugative. They come from a number of different plasmid incompatibility groups (115, 176). These plasmids often carry other antibiotic resistance genes, heavy metal resistance genes, and/or pathogenic factors such as toxins (70). Thus, selection for any of these factors selects for the plasmid. This phenomenon of cross-selection has contributed to the dramatic increase in the number multiple-drug-resistant bacteria over the last 40 years (146, 226).

Group 2 includes Tet(K) and Tet(L), with 58 to 59% amino acid identity; these proteins are found primarily in gram-positive species. Group 2 has 14 predicted transmembrane α-helices. These genes code for proteins which confer resistance to tetracycline and chlortetracycline. Their presence is indicated when gram-positive bacteria are resistant to tetracycline but not to minocycline or glycylcyclines (287, 295). The *tet*(K) and *tet*(L) genes are generally found on small transmissible plasmids, which on occasion become integrated into the chromosome of staphylococci (84) or the chromosome of *Bacillus subtilis* (255) or into larger staphylococcal plasmids (184, 266). Large staphylococcal plasmids carrying the *tet*(K) genes are relatively uncommon, whereas small plasmids carrying the *tet*(K) genes are common. The small plasmids represent a family of closely related plasmids, which range in size from 4.4 to 4.7 kb (213). Plasmid pT181 is the prototype of the family and has been completely sequenced (124). The pT181 family of plasmids can carry antibiotic resistance genes other than *tet*(K) (124).

The large plasmid pJ3358 from *Staphylococcus aureus*, which codes for mupirocin resistance, carries a complete copy of plasmid pT181 flanked by directly repeating IS*257* insertion sequences (184). Chromosomally integrated copies of plasmid pT181 also occur and are also flanked by IS*257* elements (84, 252). More recently, we have examined four large staphylococcal plasmids from four species. In each case, a copy of pT181 was found within the large plasmid and was flanked by IS*257* sequences (266). In one plasmid, part of the pT181 sequence has been deleted, while in other plasmids the complete pT181 plasmid sequence appears to be present as judged by Southern blot hybridizations. The *tet*(K) gene is most commonly found in *S. aureus* but is present in other *Staphylococcus* species (266). Most staphylococcal species also tend to carry *tet*(K), with the exception of *Staphylococcus intermedius*, which preferentially carries *tet*(M) (266).

A small number of plasmid-borne *tet*(L) genes have been sequenced and shown to have, in general, 98 to 99% sequence identity (265). One exception is the chromosomal *tet*(L) gene of *B. subtilis* (283). This gene has only 81% amino acid sequence identity to the other sequenced *tet*(L) genes and is just at the limit of what would be considered part of the *tet*(L) gene. The *tet*(K) and *tet*(L) genes can be found together in single isolates of streptococci (27) and *C. difficile* (243) but cannot be distinguished by their resistance phenotype to different tetracyclines.

Group 3 includes Otr(B) and Tcr3, both found in *Streptomyces* spp. These proteins have topology similar to group 2 proteins, with 14 predicted transmembrane α-helices. Group 4 includes Tet A(P) from *Clostridium* spp., with 12 predicted transmembrane α-helices, while group 5 includes Tet(V) from *Mycobacterium smegmatis*. Group 6 includes unnamed determinants from *Corynebacterium striatum* (which are not included in Table 3) and includes one protein which is believed to use ATP rather than a proton gradient as the energy source. More information on efflux proteins can be found in the recent chapter by McMurray and Levy (173).

Ribosomal protection proteins. Nine ribosomal protection proteins are listed in Table 3. These are cytoplasmic proteins that protect the ribosomes from the action of tetracycline and confer resistance to doxycycline and minocycline. They confer a wider spectrum of resistance to tetracyclines than is seen with bacteria that carry tetracycline efflux proteins, with the exception of Tet(B). The ribosomal protection proteins have homology to elongation factors EF-Tu and EF-G (259, 292). The greatest homology is seen at the N-terminal area, which contains the GTP-binding domain. The Tet(M), Tet(O), and OtrA proteins reduce the susceptibility of ribosomes to the action of tetracyclines. The *Streptomyces* Otr(A) protein has greatest overall amino acid similarity to elongation factors. The mechanism of ribosomal protection works in vivo and in vitro, unlike the action of efflux proteins; which require intact membranes to function. Binding of the Tet(M) protein is not affected by tetracycline but is inhibited by thiostrepton, which also inhibits the binding of the EF-G protein (53). EF-G and the Tet(M) proteins compete for binding on the ribosomes, with Tet(M) having a higher affinity than EF-G. This suggests that these two proteins may have overlapping binding sites and that Tet(M) must be released from the ribosome to allow EF-G to bind (53).

The Tet(M) and Tet(O) proteins are the most extensively characterized of the ribosomal protection group (29–31, 292–294). They have been shown to have ribosome-dependent GTPase activity (173, 298). However, the Tet(M) protein could not replace the function of the EF-G protein in an *E. coli* isolate with a temperature-sensitive EF-G protein, at the nonpermissive temperature. The Tet(M) protein did not replace either the EF-G or EF-Tu proteins in an in vitro protein synthesis assay (31). The Tet(O) protein binds GDP and GTP. Site-directed mutations in the Tet(O) protein, which reduce the binding of GTP, were correlated with reduction in the susceptibility to tetracycline in isolates. This suggests that the GTP binding is important to the function of the Tet(O) protein (294).

Burdett (31) found that the Tet(M) protein allows the aminoacyl-tRNA to bind to the acceptor site of the ribosome in the presence of tetracycline concentrations that would normally inhibit translation. In the presence of the Tet(M) protein, tetracycline is apparently released from the ribosomes. In the presence of either the Tet(M) or the Tet(O) protein, tetracycline binding to the ribosomes is reduced when GTP but not GDP is present (298). Burdett (31) found that energy from GTP hydrolysis released the tetracycline from the ribosome when a nonhydrolyzabe GTP analog was used. In contrast, in related experiments with the Tet(O) protein, Trieber et al. (298) found that the data were more consistent with a role for GTP hydrolysis in the dissociation of the Tet(O) protein from

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the ribosomes. It is not clear if true differences between the Tet(M) and Tet(O) proteins exist, as suggested by these experiments from two different laboratories, or whether the results are due to differences in experimental details.

Although only two of the proteins from this group have been extensively examined, it has been assumed that the other proteins in the "ribosomal protection group" [Tet(S), Tet(T), Tet(Q), TetB(P), Tet(W), and Otr(A)] have GTPase activity and interact with tetracycline and the ribosomes in similar ways to those described for the Tet(M) and Tet(O) proteins, because of the similarities at the amino acid sequence level. The ribosomal protection proteins can be divided into groups based on amino acid sequence comparison. The first group includes Tet(M), Tet(O), Tet(S), and the newly described Tet(W) (14). The second group includes the Otr(A) and the TetB(P) proteins, while the third group includes the Tet(Q) and Tet(T) proteins (47, 273).

Like the *tet*(M) gene, *tet*(Q) is often associated with a large conjugative transposon which carries the *erm*(F) gene (encoding an rRNA methylase that confers erythromycin resistance) upstream from the *tet*(Q) gene (45, 46). Many of the different genera, including gram-positive, gram-negative, aerobic, and anaerobic bacteria, carried both *tet*(Q) and *erm*(F) in addition to an open reading frame (ORF) upstream of the *erm*(F) gene and two genes, *rteA* and *rteB*, downstream from the *tet*(Q) gene (45, 46). The *rteA* and *rteB* genes have been thought to play a role in the transfer of the conjugative element in *Bacteroides* (257). More recently, these genes have been found in a variety of gram-positive and gram-negative genera including *Clostridium, Actinobacillus, Prevotella, Selenomonas*, and *Veillonella* (45, 46).

The determinant TetP from *Clostridium* is unique because its gene consists of two overlapping genes; the first, *tetA*(P), encodes a classical efflux protein, and the second, *tetB*(P), encodes a protein which is related to the tetracycline ribosomal protection proteins (Table 5). No other gene with this organization has yet been described. *tetA*(P) is functional when separated from *tetB*(P). However, it is not clear whether the *tetB*(P) gene codes for a functional protein because cloned *tetB*(P), in both *Clostridium perfringens* and *E. coli*, expressed only a low-level resistance to tetracycline (273), which was lower than normally found when other ribosomal protection genes were cloned into these organisms and may not be functional in its natural host (26, 273).

The *E. coli miaA* gene encodes an enzyme that catalyzes the first step in the modification of A37 on tRNAs that read codons starting with U (173, 294). This is located near the anticodon and with modification decreases the rate of elogation, increases the number of errors at the first position of the codon, and decreases the number of errors at the third position. Mutations in *miaA* in the presence of Tet(M) reduce the level of tetracycline resistance in *E. coli*. However, this was not seen when the Tet(O) protein was examined in strains with this mutation (294). Mutations in the *rpsL* gene, which encodes the S12 ribosomal protein, also decrease tetracycline resistance in the presence of the Tet(M) and Tet(O) proteins.

The current data suggest that the ribosomal protection proteins bind to the ribosome. This causes an alteration in ribosomal conformation which prevents tetracycline from binding to the ribosome, without altering or stopping protein synthesis. The hydrolysis of GTP may provide the energy for the ribosomal conformational change. The ribosomal protection proteins also need to disocciate from the ribosome to allow EF-G to bind, since they have overlapping binding sites on the ribosome. A model for the way chromosomal mutations in the *miaA* and *rpsL* genes interfere with the function of the ribosomal protection proteins and reduce resistance to tetracycline can be found in reference 298.

Enzymatic inactivation of tetracycline. The *tet*(X) gene (281) encodes the only example of tetracycline resistance due to enzymatic alteration of tetracycline. Two closely related anaerobic *Bacteroides* transposons containing the *tet*(X) gene have been described (281). The *tet*(X) gene was found because it is linked to *erm*(F), which codes for a rRNA methylase gene. The *erm*(F) gene was cloned into *E. coli*, and the clones were found to confer tetracycline resistance in *E. coli* when grown aerobically. The *tet*(X) gene product is a 44-kDa cytoplasmic protein that chemically modifies tetracycline in the presence of both oxygen and NADPH. Sequence analysis indicates that this protein has amino acid homology with other NADPH-requiring oxidoreductases and should not be able to function in the natural anaerobic *Bacteroides* host (281). It has not been found outside *Bacteroides*. However, to date no surveys have been conducted to assess the distribution of the *tet*(X) gene. Thus, even though the transposon carrying *tet*(X) and linked *erm*(F) is thought to be of gram-positive aerobic or facultative origin, a putative ancestor has not been identified.

Other/unknown mechanisms of resistance. The *tet*(U) gene confers low-level tetracycline resistance (220). This gene encodes a 11.8-kDa protein containing 105 amino acids, which is smaller than the efflux proteins (45 kDa) and the ribosomal proteins (72 kDa) (see above). There is 21% similarity over the 105 amino acids between the Tet(U) and Tet(M) proteins, beginning close to the carboxy terminus of the latter. These similarities do not include the consensus GTP-binding sequences, which are thought to play a role in resistance in the Tet(M) and related proteins. However, the sequence is not really similar to either the efflux or ribosomal protection genes, and the mechanism is thus listed as unknown in Table 3.

The mechanism of resistance of the *otr*(C) gene from *Streptomyces* has not been determined because it has not yet been sequenced. It has been speculated that the *otr*(C) gene does not code for either an efflux or ribosomal protection protein. Whether *otr*(C) encodes an inactivation enzyme, similar to *tet*(X), or whether it has a novel mechanism of resistance like *tet*(U) has not yet been determined.

Regulation of Resistance Gene Expression

Efflux genes. The gram-negative efflux determinants consist of two genes, one coding for an efflux protein and one coding for a repressor protein. Both genes are regulated by tetracycline. The two genes are oriented divergently and share a central regulatory region with overlapping promoters and operators (99). In the absence of tetracycline, the repressor protein occurs as a homodimer, which binds two α-helix–turn–α-helix motifs to the two tandemly orientated *tet* operators (99, 130). This blocks transcription of the structural genes for both the repressor and the efflux protein. Induction in the system occurs when a tetracycline-Mg^{2+} complex enters the cell and

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binds to the repressor protein. Drug binding changes the conformation of the repressor so that it can no longer bind to the DNA operator region. Only nanomolar concentrations of tetracycline are needed for binding to the repressor protein. This system is the most sensitive effector-inducible transcriptional regulation system yet described. After the repressor binds the tetracycline-Mg^{2+} complex, transcription of the efflux structural and repressor genes occurs. This is a relatively rapid process (99, 144). The *tet* gene in Tn*10* is differentially regulated so that the repressor protein is synthesized before the efflux protein is expressed. The repressor protein will rebind to the DNA only when there is insufficient tetracycline (smaller than nanomolar amounts) present in the cell. This type of regulation most probably occurs with all the gram-negative efflux genes, *tet*(A), *tet*(C), *tet*(D), *tet*(E), *tet*(G), and *tet*(H), and probably also for the *tet*(I) gene. Crystallography has shown that the three α-helices at the N-terminal region of the repressor protein form the DNA-binding domain in the repressor molecule and that conformational changes in the repressor protein occur in the presence of tetracycline complexed with Mg^{2+} (130, 203). The tetracycline-binding pocket and the interaction between tetracycline and the repressor protein have also been characterized (99). The structural basis of *tet*(B) regulation is summarized in reference 203.

Three different strains of *Haemophilus parainfluenzae* were shown to carry constitutively expressed Tn*10* (97). Subsequently it was shown that a truncated nonfunctional repressor protein due to a frameshift mutation in the repressor gene was present (97). This resulted in the constitutive expression of the Tet(B) protein. However, when a functional repressor was added to the cell, the *tet*(B) gene was inducible and regulated normally. The incidence of defective repressors in nature outside the genus *Haemophilus* has not been examined.

No repressor proteins have been found in gram-positive *tet*(K) or *tet*(L) genes. Upstream of the plasmid tetracycline *tet*(L) gene is a putative leader peptide with a potential stem-loop mRNA structures with two ribosome-binding sites (RBS), one that overlaps the leader peptide and one downstream that hides the RBS for the structural gene. This suggests that regulation is by translational attenuation when, in the absence of tetracycline, the ribosome binds to the first RBS (RBS1) and a short leader peptide is translated, which ends before the second RBS (RBS2). In the presence of tetracycline, a second stem-loop structure in the mRNA forms which uncovers the RBS2 site and allows the efflux protein to be translated, resulting in the cell becoming phenotypically resistant to tetracycline (265). This is a model similar to that found in regulation of the *erm*(C) gene, where significant work has been done on the regulation mechanism (inducible versus constitutive). Clinical isolates with either inducible or constitutively regulated *erm*(C) genes can be isolated. In one isolate, a tandem 26-bp direct repeat was found in the leader sequence, while in a second isolate, a deletion of the 107-bp segment of the leader region was found. In both cases these changes were thought to have converted an inducibly regulated leader region into a nonfunctional leader region, which resulted in constitutive production of the Erm(C) protein (310a).

Induction of the chromosomal *tet*(L) by tetracycline does not involve unmasking of an RBS and does not occur by the type of translational attenuation described for the plasmid *tet*(L) gene above. The normal induction from the RBS, for the leader peptide, is more efficient than induction of the RBS from the structural gene. Induction seems to involve an mRNA stem-loop, which overlaps part of the leader peptide sequence just upstream of the structural gene. A model for a tetracycline-promoted stalling of the ribosomes during translation of the early codons of the leader peptide has been proposed (283). This allows stabilization of the larger stem-loop structure, which than guides the ribosomes from the leader sequence to reinitiate translation at the RBS for the structural gene. Tetracycline induction also occurs at the transcriptional level but has not been well studied (173). Naturally occurring constitutive *tet*(L) plasmid genes have truncated leader peptides (173). This is consistent with both the translational attenuation and reinitiation models. The *tet*(L) gene also appears to be regulated by elevation in the pH and the presence of Na^+ and K^+. The sequences upstream of the *tet*(K) gene suggest regulation of protein synthesis by translational attenuation. Production of the Tet(K) protein is inducibe by tetraycline. However, like the Tet(L) protein, it may also be regulated by other factors (pH, Na^+, or K^+) as described above (173).

Ribosomal protection. The expression of both Tet(M) and Tet(O) proteins appears to be regulated. Wang and Taylor (306) have suggested that the 400-bp region directly upstream from the coding region of the *tet*(O) gene was needed for full expression of the gene; however, the function of the region is not understood (292). Burdett (29) reported that the amount of Tet(M) protein increased when streptococci carrying the determinant were exposed to tetracycline. Similarly, Nesin et al. (188) found that preexposure to subinhibitory concentrations of tetracycline in *S. aureus* strains carrying the *tet*(M) gene resulted in both an increase in tetracycline resistance and an increase in the level of mRNA transcripts for *tet*(M). Su et al. (285) reported a stem-loop structure in the upstream region of the structural gene from Tn*916*, and both short and long transcripts were found by Northern blot analyses, similar to descriptions for attenuation of mRNA transcription of gram-positive proteins. Based on the DNA sequences from the upstream region in the *Ureaplasma urealyticum tet*(M) sequence, a similar stem-loop structure to that described for Tn*916* would be possible (228). However, we have not looked at the transcripts from this gene.

We have sequenced the 4.9-kb *Hin*cII fragment containing the *tet*(M) gene from *U. urealyticum* and Tn*916* has also been completely sequenced (75). We have compared the upstream regions of seven *tet*(M) genes using the *U. urealyticum* gene as the standard. We found that the seven upstream regions examined had between 96 and 100% sequence identity to the *U. urealyticum* upstream DNA sequences (228). In contrast, when downstream regions were compared, sequence identity was more variable and ranged from 81 to 99%, with the *S. aureus* sequences being the most divergent (188). Finding little variability in the upstream sequences and more variability in the downstream regions is consistent with the hypothesis that the upstream regions are important for regulation, while no role has been described for sequences directly downstream of the structural gene. The sequences of both the upstream and downstream regions, as well as the structural genes, have G+C contents of <40%. This is despite the fact the genes were isolated from bacteria which have chromosomal G+C contents

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that varied from a low of 28% (*U. urealyticum*) to a high of 50% (*Neisseria* spp.). This is consistent with the hypothesis that the *tet*(M) genes have come from gram-positive bacteria and illustrates the spread of gram-positive genes into gram-negative species (227). This information, along with that of Wang and Taylor (306), is consistent with the hypothesis that *tet*(O) and *tet*(S) genes may also be regulated. However, the regulation of *tet*(Q) expression is unclear since most of the work has been done to determine the regulation of self-transfer and mobilization of both coresident plasmids and unlinked integrated elements, rather than expression of the Tet(Q) protein (152, 256, 257). There are also no direct data for regulation in *tet*(S). There have not been adequate downstream sequences (<100 bp) available in GenBank, and thus we could not compare downstream regions of *tet*(O), *tet*(S), and *tet*(Q) with the *tet*(M) downstream sequences.

Incidence of Tetracycline Resistance

Overview for pathogenic and opportunistic organisms. Most work on bacterial resistance has either been conducted with pathogenic bacteria, which usually cause disease when present, or opportunistic bacteria, which occasionally cause disease (145, 146, 157, 160, 223, 229, 230, 232–244). Opportunistic bacteria are often part of the host's normal flora and can cause disease when they leave their normal sites. The majority of *tet* genes in bacteria have been associated with mobile plasmids, transposons, conjugative transposons, and integrons (gene cassettes) (176, 216, 224–228, 230, 232). These mobile units have enabled the *tet* genes to move from species to species and into a wide range of genera by conjugation (Tables 4 and 5). The gram-negative *tet* genes, first described in the *Enterobacteriaceae* and *Pseudomonadaceae*, are now also found in *Neisseria, Haemophilus, Mannheimia, Treponema*, and *Vibrio* (Table 4) (121, 132, 144, 149, 168, 182, 232, 233, 271). The *tet*(B) gene has the widest host range of the gram-negative *tet* genes and has been identified in 20 gram-negative genera (Table 4), while *tet*(M) is found in 26 genera including gram-negative and gram-positive bacteria (Tables 4 and 5). We have speculated that some genes, such as *tet*(E), may have a more limited host range because they are located on nonmobile plasmids, which reduces opportunities for transfer to other species and genera (61, 280). Some *tet* genes in some species or genera may confer low levels of resistance, which would be unlikely to protect the bacterial cell exposed to tetracyclines in clinical or environmental settings.

In 1953, the first tetracycline-resistant bacterium, *Shigella dysenteriae*, was isolated (70, 307). The first multiple-drug resistant *Shigella* was isolated in 1955. This later isolate was resistant to tetracycline, streptomycin, and chloramphenicol (4, 70, 155) and represented 0.02% of the isolates tested. By 1960, multiple-drug-resistant *Shigella* represented almost 10% of the strains tested in Japan (4, 70, 155), a dramatic increase in 5 years. The increase in multiple-drug resistant *Shigella* strains has continued to the present. The study by Lima et al. (155) showed that over 60% of the *S. flexneri* strains isolated between 1988 and 1993 were resistant to tetracycline, streptomycin, and chloramphenicol, which is the same combination of antibiotic resistance determinants found in the 1953 *S. dysenteriae* isolate. It was demonstrated that these antibiotic-resistant bacteria could transfer all their antibiotic-resistant phenotypes to susceptible isolates by cocultivation. This transfer was dependent on direct contact of the viable growing bacteria (308). We now know that the Japanese studies were the first reports of tetracycline resistance genes carried on conjugative R-plasmids. These tetracycline resistance genes conferred efflux of tetracycline from the cell and encoded the first of the three different types of tetracycline resistance mechanisms to be found in bacteria (228).

Isolation of *Salmonella enterica* serovar Typhimurium DT104 has become more common in recent years in both human and animal sources, and the isolates have been genetically characterized. Many of the isolates are multiple-drug resistant and carry a class 1 integron containing a variety of different antibiotic resistance genes including those encoding resistance to tetracycline (104, 189, 296). In a recent Canadian study (189), 10 human and 8 nonhuman isolates carried the *tet*(G) gene, along with genes conferring resistance to one or more other antibiotics including ampicillin, chloramphenicol, streptomycin, spectinomycin, and sulfonamide. One human isolate carried the *tet*(A) gene, and three carried *tet*(B) in place of the *tet*(G) gene. Multiple-drug resistance, which includes Tc^r, has been identified in an increasing number of gram-negative pathogens and opportunistic bacteria.

Gram-positive species have also acquired Tc^r, especially those that are multiple-drug resistant. A 1994 study (86) found that approximately 90% of the methicillin-resistant *Staphylococcus aureus*, 70% of *Streptococcus agalactiae*, 70% of multiple-drug resistant *Enterococcus faecalis*, and 60% of the multiple-drug resistant *Streptococcus pneumoniae* strains were now Tc^r.

"Gram-negative *tet* genes" are those which have been found only in gram-negative bacteria. These genes have higher G+C contents (>40%) than those of gram-positive origin. All of the gram-negative *tet* genes encode efflux proteins and do not express well if moved into gram-positive hosts. Most of the gram-negative *tet* genes are regulated by a repressor, which is transcribed in the opposite direction from the structural gene. "Gram-positive *tet* genes" are those which are usually found in gram-positive species but, more importantly, have relatively low G+C contents (<35%). These genes are found in an increasing number of gram-negative species, including anaerobes (Table 4). This is especially true with the *tet*(M) gene, which has been identified in clinical isolates from 8 gram-negative genera and 18 gram-positive genera (Tables 4 and 5). The *tet*(M) gene has been conjugally transferred in the laboratory to an even larger group of species and genera than has been found in natural isolates (18, 35, 48, 74, 209, 274).

Previous work by Levy (146) found that long-term use of tetracycline selects not only for tetracycline-resistant gram-negative bacteria but also for multiple-drug-resistant gram-negative species. Tc^r genes in both gram-positive and gram-negative species are often found on the same units (plasmids, transposons, or integrons) as other antibiotic resistance genes. For example, all chloramphenicol-resistant (Cm^r) *Haemophilus influenzae* strains isolated in the 1970s and 1980s were also Tc^r (111, 168, 282, 304). One hypothesis was that the first *H. influenzae* strains to obtain Cm^r had acquired the Tc^r Cm^r transposon, which was then passed between strains and species. In contrast, some *H. influenzae* strains obtained tetracy-

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cline resistance transposons without the chloramphenicol gene and thus passed on only the Tc^r. One plasmid had the tetracycline transposon inserted within one of the two inverted repeats of the chloramphenicol transposon (111). In another, the ampicillin transposon was integrated into the inverted repeats of the tetracycline transposon (111). Unfortunately, little work on antibiotic resistance plasmids from *H. influenzae*, or other *Haemophilus* spp., has been done recently.

Clewell and coworkers hypothesized that the transposon carrying the *tet*(M) gene, as typified by Tn*916*, was the original gram-positive conjugative transposon (75). It is suggested that over time other antibiotic resistance genes were inserted directly into this family of transposons, creating larger units carrying two to four different antibiotic resistance genes (35, 48, 108). This could be one explanation why the *tet*(M) gene is often linked to the *erm*(B) gene, which codes for an rRNA methylase and confers resistance to macrolides, lincosamides, and B streptogramins (MLS_B). The combination of *tet*(M) and *erm*(B) genes is common in gram-positive streptococci, staphylococci, and enterococci (48). Similarly, a chloramphenicol acetyltransferase gene and an aminoglycoside phosphotransferase-encoding kanamycin resistance (Kn^r) gene, *aph A-3*, are often linked to *tet*(M) in the same transposon (35). The presence of these genes in common transposons may explain why Cm^r and/or Kn^r *Streptococcus pneumoniae* strains have been isolated in North America recently, even though the use of chloramphenicol and kanamycin has essentially been discontinued in much of the industrialized world, including North America (64, 164). In addition, multiple conjugative transposons, which have one complete transposon inserted within another transposon, have been described in some of the cocci. These can transfer as a single unit, or the inserted transposon can be transferred separately, giving flexibility for transfer of antibiotic resistance genes (48, 140). Selection for any antibiotic on these multiple-drug-resistant units normally selects for the entire unit and may explain why the isolation of Tc^r *S. pneumoniae* in children occurs even though tetracycline is not used in this age group (162, 289).

Obligatory intracellular pathogens such as *Chlamydia* and *Rickettsia* have not yet acquired tetracycline resistance. Since these bacteria grow only inside cells, it would require that cells be infected with two genera to allow gene exchange into the obligate intercellular pathogen. Mutations to increased tetracycline resistance would be more likely to occur in such intracellular bacteria. A few reports have described "heterotypic" tetracycline resistance in *C. trachomatis* when grown at high density ($>5 \times 10^4$ IFU/ml), but there was no clear correlation of the phenotype to isolates from patients who did not respond to tetracycline therapy (277; R. B. Jones, B. Van der Pol, and B. F. Batteiger, Program Abstr. 30th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 679, p. 199, 1990). On further examination, the phenotype was not stably transferred and was most probably an artifact of the growth conditions (Jones et al., 30th ICAAC). Lefevre et al. (142) described a *C. trachomatis* strain for which the tetracyline MIC was >64 μg/ml, but <1% of the population showed this resistance. Unfortunately, this isolate has not been further examined, and it is unclear whether the apparent resistance is also due to growth conditions or a permanent change, such as a mutation. More recently, Somani et al. (277) described what appear to be stable multidrug-resistant urogenital solates of *C. trachomatis* that included doxycycline-resistant strains expressing levels of resistance up to 4 μg of doxycycline/ml. These strains were responsible for treatment failures with antibiotics and caused relapsing or persistent infections. This appears to be the first report of clinically significant infection with *C. trachomatis* resistant to a member of the tetracycline class.

Overview for commensal microorganisms. The commensal flora consists of microorganisms which are present in and on surfaces of a host and are not thought to cause disease. These organisms are often beneficial to the host, providing nutrients and inhibiting the growth of potential pathogens by preventing them from becoming established (10). Not surprisingly, these bacteria have the same *tet* genes, plasmids, transposons, conjugative transposons, and integrons as their disease-producing counterparts among the opportunistic and pathogenic bacteria. Many oral viridans streptococci (74, 93, 201) have acquired *tet*(M), *tet*(O), *tet*(L), or *tet*(K), as have the pathogenic streptococcal species *S. pneumoniae* and *S. pyogenes*. Most people today carry Tc^r viridans streptococci in their mouth regardless of use of tetracycline therapy or age, while Tc^r *S. pneumoniae* and *S. pyogenes* are significantly less common in most populations (162). This differs from isolates recovered before the introduction of tetracycline therapy, when the majority of bacteria were susceptible to tetracycline (12).

Another observation is that over time the gram-positive commensal bacteria have converted from carrying single *tet* genes to carrying multiple *tet* genes (13, 226, 236). The different *tet* genes can have either the same mode of action (efflux or ribosomal protection), or different modes of action (efflux and ribosomal protection), just like the pathogenic and opportunistic species do (230). The carriage of multiple *tet* genes of different classes is commonly found in individual gram-positive isolates (37, 236, 266, 267, 307) and in *Mycobacterium* spp. and *Streptomyces* spp. (207) but is uncommon in facultative gram-negative bacteria, especially enteric species (8, 60, 61, 81, 115, 168–170, 176). The reason for this is unknown, but a similar situation exists for the carriage of other antibiotic resistance genes (155). Differences in carriage of antibiotic resistance genes are found among different age groups within the same population and different areas of the world. This is why surveillance is needed for antibiotic resistance in commensal bacteria. In genera such as *Neisseria*, the commensal bacteria often carry more antibiotic resistance genes and acquired them earlier than the pathogenic *N. gonorrhoeae* and *N. meningitidis* (223, 234).

To stimulate the study of commensal bacteria, a new group recently came together to focus on antibiotic-resistant commensal bacteria and was named the Reservoirs of Antibiotic Resistance or (ROAR) project. The purpose of the group is to promote the study of carriage of antibiotic-resistant bacteria in humans, during food production and agricultural processes, and in the environment. The ROAR project is providing a source of information on resistance in commensal bacteria and can be found at. http://www.roar.antibiotic.org. Another Web site, which can be accessed from the ROAR site or directly, is The Alliance for the Prudent Use of Antibiotics (APUA) (http://www.APUA.org) (231). This is a nonprofit organization whose purpose is to promote proper antibiotic use and curb antibiotic resistance worldwide.

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In general, it has been found that most disease-producing species of gram-negative genera carry the same tetracycline resistance genes as do the commensal species within the same genus. Many examples exist in the literature for the gram-negative genera *Haemophilus* (168), *Neisseria* (132), and *Bacteriodes* (45, 57, 143) and for gram-positive genera such as *Streptococcus*, which all carry the same *tet* genes as their pathogenic and opportunistic related species (45, 74, 140) Commensal *Neisseria* species carry an incomplete *tet*(M) transposon integrated into their chromosomal DNA while the pathogenic *N. gonorrhoeae* and *N. meningitidis* carry an incomplete *tet*(M) transposon integrated on a 25.2-MDa plasmid of gonococcal origin (132, 223). However, in the laboratory the 25.2-MDa plasmid can be transferred and maintained in the commensal *Neisseria* spp. (223). This is an exception, since in most other genera examined the *tet* genes are located in the same place (plasmid or chromosome) and often carry the same or related plasmids, transposons, or conjugative transposons (222, 230). In view of the above observations, it has been proposed that commensal bacteria act as a reservoir for *tet* and other antibiotic resistance genes found in, human pathogens and are thus very important in our understanding of how antibiotic resistance genes are maintained and spread through bacterial populations (222, 230).

Environmental bacteria such as *Bacillus subtilis* carry the same *tet* genes as clinical gram-positive bacteria (255, 283). Similarly, gram-negative bacteria isolated from aquaculture, including bacteria from fish (both suface and intestinal floras), water, marine sediment, and plants, all have the same *tet* genes as do clinical isolates (8, 59–61). Clinical isolates of streptomycetes, which may or may not be producing disease, have the same *otr* genes as do industrial strains (207).

Distribution and Mobility of *tet* Genes

Overview. The *tet* genes are found in a variety of bacteria isolated from humans, animals, and the environment (Tables 4 and 5). The majority of the *tet* genes are associated with either conjugative or mobilizable elements, which may partially explain their wide distribution among bacterial species (115, 176, 216). The gram-negative *tet* efflux genes are found on transposons inserted into a diverse group of plasmids from a variety of incompatibility groups (115, 176). Gram-positive efflux genes are associated with small plasmids (124, 265, 266). The ribosomal protection genes *tet*(S) and *tet*(O) can be found on conjugative plasmids, or in the chromosome, where they are not self-mobile (36, 37, 162). The *tet*(M) and *tet*(Q) genes are generally associated with conjugative chromosomal elements, which code for their own transfer (48, 152, 256, 257). These conjugative transposons transfer mobilizable plasmids to other isolates and species and even unlinked genomic DNA (26, 48, 152, 166, 184, 272). Genes in the *tet*(Q) operon have been identified which mediate excision and circularization of discrete nonadjacent segments of chromosomal DNA in *Bacteroides*. The transfer origin (*oriT*) region of one of the *Bacteroides* conjugative transposons has been located near the middle of the conjugative transposon (152). *Bacteroides* conjugative transposons range from 65 to over 150 kb; most elements carry both *tet*(Q) and *erm*(F) and belong to a family of elements with the prototype being Tc^r Em^r DOT (257). A 16-kb region of the transposon is required and sufficient for conjugal transfer of the element and for mobilization of both coresident plasmids and unlinked integrated elements. DNA transfer is tetracycline regulated and mediated by at least three regulatory genes including a putative sensor (*rteA*), a putative regulator (*rteB*), and a third gene, *rteC*, which seems to stimulate transfer in an unknown fashion (152). This differs from the mechanism proposed for tetracycline regulation of conjugation of the Tn*916* family of elements, where Manganelli et al. (166) suggest that tetracycline increases the number of circular intermediates present in the cell, which leads to more transconjugants. A gene can be induced to increase conjugal transfer by the presence of low doses of tetracycline. This has been illustrated in vitro with *tet*(M) and gram-positive cocci and rods (69, 222, 272) and in the gram-positive *Listeria* spp. (69). Transfer of the *tet*(Q) gene is also inducible by tetracyclines in *Bacteriodes* spp. (256, 257).

Movement of the Tn*916*-like and *Bacteroides* conjugative elements is hypothesized to involve a Rec-independent excision event which produces a nonreplicative circular intermediate that can insert at a different site within the cell or transfer to a new host by a conjugative plasmid-like process (48, 268). Integration of the *Bacteroides* conjugative transposon into a new host is relatively site specific, while the Tn*916*-like transposons can be relatively site specific or more randomly integrated into the host chromosome, depending on the host (121, 268). Integration into plasmids can occur (224), as can integration within conjugative transposons to create composite elements (48). These composite gram-positive elements are ≥50 kb and have been found in streptococci and enterococci. The prototype of the composite element is Tn*3701*, first described in *S. pyogenes* (140). In some of these composite elements, the central Tn*916*-like element can be removed and the nonhomologous segment can undergo conjugative transposition independently, as occurs in Tn*5253* from *S. pneumoniae* (48). The structural organization of these composite elements varies. Both the composite and Tn*916* families of elements can carry antibiotic resistance genes which confer resistance to chloramphenicol, erythromycin, and kanamycin, in addition to the *tet*(M) gene, which confers resistance to both tetracycline and minocycline (48, 140).

The *Bacteroides* conjugative transposons can mobilize resident plasmids either in *trans* or in *cis*. In *trans* the transposon provides all the proteins needed for mating and the plasmid provided the proteins that nick the plasmid and initiate plasmid transfer. In *cis*, the transposon provides the proteins needed for transfer (257). The Tn*916* family can mobilize plasmids in *trans* (48, 183, 297). Low levels of tetracycline increase the transfer of these genes and also increase the ability of the bacterial host to spread antibiotic resistance genes to other isolates, species, and genera (48, 69, 297). With their transfer ability, one might hypothesize that the ribosomal protection genes would be found in virtually all tetracycline-resistant genera examined; however, this is not the case (Tables 4 and 5).

A few isolates of *Haemophilus* spp. and two clones of high-level tetracycline-resistant *Moraxella catarrhalis* carry the *tet*(B) gene in their chromosome (246, 247). The *tet*(B) gene is not conjugative in these isolates but can be moved by transformation using chromosomal DNA. More recently, we have found the *tet*(B) gene in *Treponema denticola*, an anaerobic spiro-

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chete thought to play an important role in periodontal disease (233). In the *T. denticola* isolates the *tet*(B) gene was nonmobile and not associated with a plasmid, suggesting that it was most likely to be located on the chromosome (233). This is the first description of a gram-negative efflux gene in a strictly anaerobic species. When a PCR product from the *T. denticola tet*(B) gene was sequenced, there was 90% DNA sequence identity between the gene from *T. denticola* and the *tet*(B) gene from Tn*10*. However, it has been difficult to assess whether the *tet*(B) gene confers tetracycline resistance in *T. denticola*, because of the organism's growth requirements. In contrast to the *tet*(B) gene, which is nonmobile, *T. denticola* does carry conjugative *erm* genes (233).

The *tet*(E) gene differs from the *tet*(A), *tet*(B), *tet*(C), and *tet*(D) genes because it is associated with large plasmids which are neither mobile nor conjugative (61, 280). This may explain its limited distribution and predominance in aquatic environments and its prevalence in polluted marine sediment (8) (Table 4). The *tet*(E) gene has also recently been associated with the chromosome (141). Jones et al. (115) found a correlation between the plasmid incompatibility group and the particular *tet* genes carried by the plasmid. They suggested that some of the *tet* genes may have become genetically linked to specific incompatibility and/or replication genes and thus the distribution of these genes could reflect the occurrence of particular incompatibility groups in particular genera or species. This hypothesis has not been thoroughly examined, and this relationship was not shown in an earlier study by Mendez et al. (176).

Gram-negative bacteria. Currently 39 genera of gram-negative bacteria (Table 4) and 23 genera of gram-positive bacteria and related genera (Table 5) have been described in which the mechanism of tetracycline resistance has been determined. Other uncharacterized *tet* genes probably exist since studies have identified tetracycline-resistant isolates which do not carry any of the known *tet* genes (58, 61, 115, 225, 235, 244). New *tet* genes are continuing to be described (14, 47, 62, 150, 220, 267), and new genera have been identified which carry acquired *tet* genes (9, 45, 206, 207).

The *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(I), *tet*(J), *tet*(Y), *tet*(30), and *tet*(31) genes are found exclusively in gram-negative genera (Table 4). Most of these genera belong to the facultative enteric group. The *tet*(B) gene has the widest host range among gram-negative species and has been identified in such diverse species as *Actinobacillus actinomycetemcomitans, H. influenzae, Moraxella catarrhalis*, and *T. denticola* (Table 4). *Actinobacillus, Haemophilus, Moraxella*, and *Treponema* species are all found in the oral area and the respiratory tract. The *tet*(B) gene is found on conjugative plasmids in *Actinobacillus* and *Haemophilus* (224, 248), and we have recently been able to show transfer of the *tet*(B)-carrying plasmid from *A. actinomycetemcomitans* to *H. influenzae* (248). The *tet*(B) gene is not mobile in the small number of *Moraxella* (246) and *Treponema* (233) isolates examined, but it would be interesting to determine whether either *A. actinomycetemcomitans* or *Haemophilus* spp. could transfer their *tet*(B) genes into either of these genera and if the transconjugants could then transfer the *tet*(B) gene.

Of the species which carry the *tet*(M) gene, some have complete conjugative elements, like *Veilbonella parvula* and *Fusobacterium nucleatum*, which are mobile, while another species, *H. ducreyi*, has a complete conjugative element integrated into a conjugative plasmid (224, 241). Other species carry nonconjugative incomplete elements in their chromosome, like *Neisseria* spp. and most *Listeria innocua* and *Gardnarella vaginalis* spp. (69, 105), or incomplete elements on conjugative plasmids, like *N. gonorrhoeae* (223, 239). In *N. gonorrhoeae*, the incomplete transposons are associated with 25.2-MDa conjugative plasmids (223). The 25.2-MDa conjugative plasmids have one of two different deletions of the *tet*(M) transposon (223, 317). One plasmid has a deletion downstream of a *Hin*cII site and is found in plasmids isolated from *N. gonorrhoeae, Kingella denitrificans*, and *Eikenella corrodens* (317). The second plasmid type has a deletion of over 800 bp upstream of the *Hin*cII site and is found in *N. gonorrhoeae, N. meningitidis*, and *K. denitrificans* isolates (317). Both types of plasmid confer resistance to $\geq$16 μg of tetracycline/ml and can transfer to other *N. gonorrhoeae* strains at frequencies ranging from 10^{-1} to 10^{-9} per recipient. Even though *N. meningitidis* has been found naturally with only one of the two plasmid types, both are readily transferred by conjugation into *N. meningitidis* (223). The 25.2-MDa plasmids can be stably transferred into a number of the commensal *Neisseria* spp. in the laboratory, but to date only two species, *N. perflava*/*sicca* and *N. mucosa*, have been found that naturally carry the *tet*(M) gene (223, 240). In all these isolates, an incomplete Tet(M) transposon located in the chromosome rather than on the 25.2-MDa plasmid has been found. This illustrates that plasmids or genes can be moved in the laboratory, resulting in stable transconjugants, but similar strains are not found in natural isolates.

Both 25.2-MDa plasmids can be transferred in the laboratory into *Haemophilus* spp., *K. denitrificans*, and *E. corrodens* but not into *Moraxella catarrhalis*, which carries nonmobile *tet*(B) rather than *tet*(M) (132, 240). Once the *Neisseria* 25.2-MDa plasmid has been transferred into *Haemophilus* spp., it is easily transferred among species within the genus *Haemophilus* but not back to *Neisseria* spp. (239). Similarly, an *H. ducreyi* plasmid which carries the complete Tet(M) transposon can be transferred among *Haemophilus* spp. but not outside the genus (224). This type of plasmid incompatibility may influence the spread of particular *tet* genes into new genera.

The *tet*(Q) gene was first described in human colonic *Bacteroides* spp. and is normally associated with conjugative elements (152, 256, 257). This gene is now found in a number of *Prevotella* spp. from the oral cavity and in other oral gram-negative genera such as *Capnocytophaga, Mitsuokella, Selenomonas*, and *Veillonella* (200, 201, 230). From other sites we found *tet*(Q) in gram-positive *Clostridium, Gardnerella*, and *Peptostreptococcus* spp. (45). The *tet*(Q) gene has been found in seven gram-positive genera (Table 5) and six gram-negative genera (Table 4); it is possible that the host range of the *tet*(Q) gene might be similar to that found for the *tet*(M) gene.

The number of different *tet* genes found in a particular gram-negative genus varies from one *tet* gene carrying either *tet*(B) (efflux), *tet*(M), or *tet*(Q) (both ribosomal protection), to a maximum of seven different *tet* efflux genes found in the genus *Escherichia* (Table 4). However, Jones et al. (115) and Mendez et al. (176) found that no *E. coli* plasmids carried more than one type of *tet* gene. In contrast, *Streptomyces* and gram-positive genera often have individual isolates that carry multi-

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ple genes coding for the same antimicrobial resistance including tetracycline (207, 307). The *tet*(G) (11, 319), *tet*(H) (94), and *tet*(I) genes (116) have not been extensively examined for their distribution in gram-negative bacteria. It is clear that many environmental (8, 52) and food and animal (14, 40, 46, 55, 60, 61, 101, 102, 133, 135, 141, 266, 276, 280) isolates are tetracycline resistant. However, these bacteria have not yet been as extensively examined as those associated with human disease. Little work has been done to elucidate why some genera carry only a single *tet* gene, while members of other genera can carry a variety of different *tet* genes. More work is required to better understand the factors, which influence not only whether a particular genus or species of bacteria will carry tetracycline resistance genes but also whether they will encode efflux, ribosomal protection, or both.

The majority of gram-negative isolates described in the literature carry a single type of *tet* gene, although it may occur on multiple plasmids. This was evident from the earliest studies of the distribution of *tet* genes, when it was found that only 3.5% of the lactose-fermenting coliforms carried two different *tet* genes (169). Similar results have been found in bacteria isolated from catfish and their environment (59–61), in *Shigella* spp. isolated in Mexico (170), and in *S. enterica* serovar Typhimurium isolated in Africa (120). In fact, the only study where large numbers of gram-negative species were reported to carry more than a single *tet* gene was the recent study of polluted marine sediments from Norway (8). In that study, 26% of tetracycline-resistant isolates carried both *tet*(D) and *tet*(E) genes.

The incompatibility groups of gram-negative plasmids, which determines their bacterial host range, has been studied (115, 176). Plasmid host ranges vary from very restrictive, such as with the large conjugative *Haemophilus* R-plasmids (224, 247), which do not readily survive outside their own genus, to broad, which allow the plasmid to survive in diverse host backgrounds (2). These differences in plasmid host range may influence the spread of particular *tet* genes associated with them.

Gram-negative anaerobic genera and some nonenteric gram-negative genera like *Neisseria, Eikenella*, and *Kingella* most commonly or exclusively carry ribosomal protection genes. One exception is the genus *Haemophilus*, especially *H. influenzae* and *H. parainfluenzae*, where all of the early isolates (from the 1970s) carried the *tet*(B) efflux gene (168). Unfortunately, with the use of the *H. influenzae* b vaccine, interest in this organism has faded and no recent studies have been performed to see whether current isolates also contain only *tet*(B). A few strains of *H. aphrophilus*, isolated from periodontal patients in the 1990s, carry the *tet*(K) gene (206), while *H. ducreyi* can carry either *tet*(B) or *tet*(M) (168, 224) (Table 4). A few isolates of *V. parvula* have been found which carry either *tet*(L) or *tet*(Q); however, most of the isolates examined carry the *tet*(M) gene (45, 206). Different ribosomal protection genes have been found in tetracycline-resistant *Bacteroides* spp. (45, 57, 152), in addition to the *tet*(X) gene (281). A variety of different *tet* genes have been found within the genus *Pasteurella* (40, 94) (Table 4).

Eight gram-negative genera currently carry *tet*(M) genes, seven carry *tet*(Q), five carry *tet*(W) genes, two carry *tet*(O), two carry *tet*(K), and one each carries *tet*(H), *tet*(I), *tet*(J), *tet* (Y), *tet*(30), and *tet*(31) (Table 4). Based on their low G+C content and their regulation (see "Regulation of resistance gene expression" above), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(P), *tet*(S), *tet*(T), *tet*(Q), *tet*(W), and maybe *tet*(Z) are thought to be gram-positive origin. This strengthens the hypothesis that antibiotic resistance genes from gram-positive species, especially the ribosomal protection genes, have been exchanged throughout the bacterial population without regard to species or genus and can be successfully integrated and expressed in a variety of bacterial host backgrounds (18, 24, 229).

Gram-positive bacteria. Seventeen Gram-positive genera, two cell wall-free genera (*Mycoplasma* and *Ureaplasma*), *Actinomyces, Nocadia, Mycobacterium*, and *Streptomyces* all carry known tetracycline resistance genes (Table 5). However, as shown above with gram-negative isolates, not all tetracycline-resistant gram-positive bacteria have been correlated with possession of specific known *tet* genes (244). A total of 18 genera carry the *tet*(M) gene, 11 carry *tet*(K), 10 carry *tet*(L), 7 carry *tet*(Q), 5 carry *tet*(O), 2 carry *tet*(S), and 1 carries *tet*(P). Recently, *Bifidobacterium* spp. have been shown to carry the *tet*(W) gene (267) and *Corynebacterium* carries either *tet*(M) or the newly described *tet*(Z) (150). The new *tet*(U) gene is found in *Enterococcus* (220), the *tet*(T) gene is found in *Streptococcus* (47), and the *tet*(V) gene is found in *Mycobacterium smegmatis* (62) (Table 5).

The *tet*(K) and *tet*(L) genes are widely distributed among gram-positive species associated with humans, animals, and the soil (Table 5) and have been found in rapidly growing *Mycobacterium, Norcardia*, and *Streptomyces* spp. (65, 207) isolated from patients (Table 5). This is the first time that acquisition of an antibiotic-resistance determinant has been documented in *Mycobacterium* and *Nocardia* spp., and it suggests that gene exchange between tetracycline-resistant gram-positive bacteria, *Mycobacterium* spp., *Nocardia* spp., and *Streptomyces* spp. has occurred. The hypothesis that *Streptomyces* spp. exchange antibiotic resistance genes with other genera is strengthened by the finding of the *otr* genes, from industrial *Streptomyces* spp., in clinical isolates of *Mycobacterium* and *Streptomyces* spp. (207). This is consistent with the hypothesis that some of the *tet* genes could be ancestrally related to genes found in the antibiotic-producing *Streptomyces* spp. (16). Work is still needed to determine the host range of the *otr* genes.

The *tet*(M) gene is often associated with a conjugative element of the Tn*916*-Tn*1545* family (48). This group of elements form nonreplicating circular intermediates, which are essential for both intracellular transposition and intercellular conjugative transfer (75). Both types of movements occur by an excision-integration process, where excision and formation of a covalently closed circular molecule precedes movement of the conjugative element. In both Tn*916* and Tn*1545*, imperfect inverted repeats (20 of 26 bp) are present at the ends of the transposon and integration occurs without duplication of the target DNA sequence (48, 268). More recently, Manganelli et al. (166) have shown that the number of circular intermediates varies in different *Enterococcus faecalis* strains. Their work indicates that the number of circular intermediates influences the frequency of conjugation between 5.1×10^{-8} and 2.8×10^{-6}, while Rice et al. (219) demonstrated that the number of circular intermediates increased when the strains were grown in the presence of tetracycline.

Clewell et al. (48) determined that the Tn*916* family of

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elements was found naturally or could be transferred in the laboratory into over 50 different species representing 24 bacterial genera. However, the host range of the Tn*916* family is even greater (Tables 4 and 5). The *tet*(M) gene can also be introduced into a significant number of other genera including gram-negative and gram-positive organisms and species lacking cell walls (18, 24, 69, 237, 274, 297). In a majority of gram-positive species, the *tet*(M) gene is found in the chromosome, most often on conjugative elements (48, 140, 226). Little has been done with the *tet*(S) gene other than to show transfer between *L. monocytogenes* and *E. faecalis* at frequencies ranging from 10^{-4} to 10^{-9} (37). The *tet*(O) gene is not associated with conjugative elements, is mobile only when found on conjugative plasmids, and can be transferred among streptococci and *Campylobacter* (27, 293). The *tet*(Q) genes have been identified in gram-positive species (Table 5), and we have recently shown that they are located on conjugative elements in these species, where they are often linked with the *erm*(F) gene (45, 46).

Distribution of Other Resistance Determinants

Efflux systems. Bacteria have a number of innate chromosomally encoded proteins, which transport molecules in and out of the cell. These have been divided into groups which include the MFS, resistance-nodulation-cell division (RND) family, the small multidrug resistance (SMR) family, and the ATP-binding cassette transport family (21, 204). Some, but not all of these efflux pumps confer resistance to tetracycline (190, 191). An external layer of peptidoglycan surrounds gram-positive bacterial cytoplasmic membranes, while gram-negative bacteria have both peptidoglycan and an outer membrane outside the cytoplasmic membrane. Consequently, exported molecules need to go through these layers. It is hypothesized that a membrane fusion protein (MFP) is connected to the outer membrane protein channel, enabling transport of small molecules across the outer membrane of gram-negative bacteria. The pumps of the RND, MFS, and SMR families use the proton motive force as the driving force for efflux. In contrast, the ATP-binding cassette transporters use ATP hydrolysis (205). Interest in these efflux pumps has increased dramatically over the last few years, and there are a number of recent reviews dealing with them (21, 190, 191, 205).

A number of the RND pumps have tetracycline as a substrate (191). RND efflux pumps have 12 predicted transmembrane segments and are unrelated to the MFS family, of which the above-described *tet* efflux pumps are members. The RND pumps are found mainly in gram-negative bacteria, and most are involved in pumping multiple ligands (antibiotics, toxic metal ions, etc.) out of the bacterial cell (191). Many of the RND efflux pumps are associated with a linker protein and an outer membrane channel. RND pumps which transport tetracycline comprise the Acr system found in *E. coli*, the multiple Mex systems found in *Pseudomonas aeruginosa*, the Mtr system found in *N. gonorrhoeae*, and the Mtr-like system recently found in *Stenotrophomonas maltophilia* (6). *P. aeruginosa* has a number of different RND efflux pump systems including MexA-MexB-OprM. Strains which overproduce the efflux operon are more resistant to tetracycline, chloramphenicol, and quinolones, while mutants with deletions in this operon are hypersensitive to these antibiotics. Another operon, MexC-MexD-OprM, also confers resistance to tetracycline, chloramphenicol, and quinolones (21, 87, 153).

A related MexA-MexB-OprM operon has been isolated from multiple-drug-resistant *Burkholderia cepacia* (32), while a similar system has been identified in multiple-drug resistant *Campylobacter jejuni* (39). An analogous system (the *mtr* system) has been found in *N. gonorrhoeae* (161). Production of the MtrCDE efflux proteins is controlled by transcription with both *cis*- and *trans*-acting factors involving the *mtrR* gene. The MtrR protein has amino acid similarities to the tetracycline repressors regulating the gram-negative efflux genes *tet*(A) to *tet*(I). Missense or deletion mutations within the *mtrR* coding region result in increased transcription of the *mtrCDE* genes, protein production, and increased resistance to antibiotics and other agents (161). The *N. gonorrhoeae mtr* system makes these isolates clinically resistant to a variety of antibiotics, and its prevalence has increased in *N. gonorrhoeae* populations since its discovery in the 1970s.

A chromosomal tetracycline efflux system associated with multiple-antibiotic resistance (the *mar* locus) has been described in *E. coli* (147). The mutation in the *marR* region of the chromosome enhances intrinsic resistance to a large group of antibiotics including tetracycline. The level of tetracycline resistance increases as the cells are grown continually in the presence of tetracycline. MarA is a transcriptional activator of a common group of promoters (5, 147). The *mar* locus appears to be widely established in bacteria (5, 147). Mutants expressing high levels of MarA show decreased accumulation of tetracycline (147). The efflux is dependent on the expression of *marA*. Inactivation of the region creates a susceptible phenotype (147). Weak acids, uncouplers, and antibiotics such as tetracycline induce expression of the *mar* operon. The regulator MarR negatively controls the expression of the *mar* operon. Overexpression of MarA causes decreased expression of the OmpF porin and increased expression of the multiple-drug efflux pump AcrAB, another operon in *E. coli* that affects antibiotic resistance (197). Spontaneous multiple-drug-resistant mutants of *K. pneumoniae*, which have increased resistance to a range of unrelated antibiotics including tetracycline, have also been described (82). Similar mutants have been found in *Serratia marcescens, Enterobacter* spp., and *Campylobacter* (39). These mutants exhibited efflux of tetracycline, similar to the *E. coli* Mar mutants. An *acrAB* homolog coding for a functional multiple-drug efflux pump has been described in *H. influenzae* (258), although disruption of the region did not produce detectable changes in tetracycline susceptibility. However, only one strain, with disruption in two different genes, was examined (258). Currently, none of the RND systems has been associated with natural transposable elements. The effect of this has been to limit the spread of these systems and hence, in general, to limit their importance in clinical tetracycline resistance. However, whether any of these will, or could, be picked up by integron-like elements is unknown. If this does happen, these new elements would allow for transfer between strains and species and more rapid spread through the bacterial population of these mutant genes.

The SMR family codes for proton-dependent efflux pumps with a multiple-drug proton antiport mechanism. The *E. coli emrE* gene produces a protein which confers resistance to monovalent cations and antibiotics including tetracycline (205).

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Point mutations. Recently 15 Tc^r clinical isolates (MICs, 2 to 64 μg of tetracycline per ml, 1 to 32 μg of doxycycline per ml) of cutaneous propionibacteria were found to have a cytosine instead of guanine at position 1058 in the 16S rRNA (251). This change was associated with increased tetracycline resistance. This region of the 16S rRNA, known as helix 34, is important for peptide chain termination and translational accuracy. The clinical significance of these strains is not clear at present. Mutations which alter the permeability of the outer membrane porins and/or lipopolysaccharides can also affect bacterial susceptibility to tetracycline and other agents (21, 154). How often these mutations occur and whether they are of clinical importance has not been established.

APPLICATIONS OF TETRACYCLINES

Administration and Pharmacokinetic Behavior in Humans

Tetracyclines are usually administered orally, although some are also available as parenteral products (Table 1) (73, 137). Rolitetracycline is available only as a parenteral product. The ability to use either oral or parenteral formulations of doxycycline has been used advantageously to permit switching programs from intravenous to oral administration (51).

The dosing regimens and pharmacokinetic properties of the tetracyclines have been extensively reviewed in previous publications (73, 137, 313). Therefore only a brief summary is provided here. Absorption following oral administration occurs largely in the stomach and proximal small intestine and is influenced by the presence of food, milk, or divalent cations, particularly, calcium, with which tetracyclines form nonabsorbable chelates. Levels achieved in serum after normal oral dosing are in the range of 2 to 5 μg/ml, and most tetracyclines have to be given four times daily to maintain therapeutic concentrations in the serum. However, the long elimination half-lives of doxycycline and minocycline permit once- or twice-daily dosing. Tetracyclines generally penetrate moderately well into body fluids and tissues and are excreted in the urine. For instance, levels in sputum about 20% of those in serum, can be achieved, which explains why the tetracyclines have a role in the treatment of respiratory tract infections. Tetracyclines also penetrate into the sebum and are excreted in perspiration, properties which contribute to their usefulness in the management of acne.

Human Therapy and Prophylactics

Although 9-(*t*-butylglycylamido)-minocycline, a third-generation compound, is currently undergoing clinical trials, it is now nearly 30 years since the last tetracycline, minocycline, was introduced (Table 1). During this period, as already discussed, there have been increases in the incidence of bacterial resistance to the tetracyclines and in availability of more active and better tolerated agents from different antimicrobial classes. Consequently, in recent years the clinical use of tetracyclines has significantly declined in most countries since they are no longer drugs of choice in many instances (44, 73, 79, 137). However, in other cases new applications have been identified. For instance, tetracycline has been used as part of a triple therapy for management of gastritis and peptic ulcer disease associated with *Helicobacter pylori*. Although omeprazole, clarithromycin, and amoxicillin (or metronidazole) are standard therapy, the role of tetracycline may increase as more clarithromycin- and methronidazole-resistant *H. pylori* isolates are encountered (303). Tetracyclines are active against malaria, and this has unexpectedly become important for prophlylaxis following the rapid increase of mefloquine-resistant *P. falciparum* strains (28, 211, 264).

Table 6 provides a summary of current anti-infective applications of the tetracyclines in humans. These antibiotics have also been evaluated for their potential in other situations. However, such applications may not necessarily gain widespread acceptance or become components of standard therapeutic regimens. For instance, on the basis of limited clinical evaluation, Ji et al. (119) consider that minocycline, in combination with ofloxacin, may have a role in the treatment of leprosy. Although larger clinical trials would be needed to establish benefit, it seems likely that a combination of minocycline and ofloxacin could provide the opportunity for supervised monthly administration of these antibiotics, thereby greatly improving patient compliance.

Veterinary Medicine

The tetracyclines have applications for the treatment of infections in poultry, cattle, sheep, and swine. In some cases, e.g., for therapeutic treatment of large numbers of poultry reared on commercial farms, the antibiotics are added directly to feed or water or can be administered in aerosols. The use of tetracyclines in the rearing of farm animals has been reviewed in recent years (44, 91) and readers are referred to these earlier papers for details. Tetracyclines are also used for treatment of infections in domestic pets (135, 146).

Animal Growth Promoters

Antibiotics represent one of the few classes of drugs that can be used in food animals both therapeutically to treat disease and subtherapeutically, usually over long periods, to improve their rate of growth and feed conversion efficiency. The practice of adding low concentrations of antibiotics, defined in the United States as < 200 g/ton of feed (50, 83, 109), to animals to improve growth and feed efficiency is referred to as growth promotion or growth enhancement (44, 50, 83, 91, 109, 113a). An obvious outcome of this practice is that animals need less food to reach marketable weight. The mechanisms responsible for growth promotion have not been fully elucidated but appear to include enhancement of vitamin production by gastrointestinal microorganisms, elimination of subclinical populations of pathogenic organisms, and increased intestinal absorption of nutrients (50).

The growth-promoting properties of tetracyclines were discovered in 1949, when it was observed that low levels of chlortetracycline in livestock rations beneficially affected the rate of growth and feed utilization by young chickens (284). The initial observations in chickens were confirmed and soon extended to swine and cattle, leading to the development of both chlortetracycline and oxytetracycline as animal growth promoters (91). In the United States these antibiotics were approved by Food and Drug Administration as feed additives in 1951 (chlortetracycline) and 1953 (oxytetracycline) (109). Increasing concerns about growth promoters followed the publication in the United Kingdom of the Swann report in 1969 (288). This

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TABLE 6. Current applications of the tetracyclines for therapy and prophylaxis of human infections[a]

Infection for which tetracyclines are:	
First choice	Acceptable alternative to other agents[b]
Respiratory	
Atypical pneumonia due to *Mycoplasma pneumoniae, Chlamydia pneumoniae, C. psittaci*	Community-acquired pneumonia[c] Infective exacerbations of chronic bronchitis[c] Legionellosis (doxycycline)
Bowel	
Cholera	
Prophylaxis of traveler's diarrhea	
Genital	
Nongonococcal urethritis	Syphilis
Cervicitis	Epididymitis
Lymphogranuloma venereum	Prostatitis
Pelvic inflammatory disease	
Granuloma inguinale	
Local and systemic	
Rocky mountain spotted fever	MRSA
Endemic and epidemic typhus	MRSE (minocycline) when vancomycin or other agents inappropriate
Q fever	Plague
Brucellosis (in combination with rifampin or streptomycin)	Tularerira
Lyme disease	Bartonellosis
Relapsing fever	Leptospirosis
Periodontal infection (topical therapy with tetracycline or minocycline)	Whipple's disease
Acne vulgaris (topical and systemic treatment)	Cutaneous infections caused by *Mycobacterium marinum* and in multiple-drug regimens for ocular infections caused by *M. cheloni*
Prophylaxis of mefloquine-resistant *Plasmodium falciparum* malaria	Gastritis caused by *Helicobacter pylori* (tetracycline in multiple-drug regimens).

[a] Based on information from references 3, 73, 79, 88, 107, 118, 131, 171, 275, 301, 303, 305, and 313.
[b] MRSA, methicillin-resistant *Staphylococcus aureus*; MRSE; methicillin-resistant *Staphylococcus epidermidis*.
[c] Except in situations where there is a high rate of resistance among pneumococci and/or *H. influenzae*.

report, suggesting that subtherapeutic application of tetracyclines and other antibiotics to farm animals might contribute to the development of resistant human isolates, led to a ban of the use of tetracyclines for growth promotion in Europe in the early 1970s (EC directive 70/524; see http://europa.eu.int/comm/dg24/health/sc/index_en.html). Although issues surrounding the use of growth-promoting antibiotics have been widely discussed in other countries, particularly the United States (50, 83, 91, 136, 146), and Australia (113a), no such ban has been imposed on the use of tetracyclines for growth promotion in these and many other countries.

Other Uses

Tetracyclines are used in aquaculture to control infections in salmon, catfish, and lobsters (59, 109, 146). They are also sprayed onto fruit trees and other plants to treat infection by *Erwinia amylovara*, injected into palm trees to treat mycoplasma infections (lethal yellow), and used to control infection of seeds by *Xanthomonas campestis* (black rot) (146; http://europa.eu.int/comm/dg24/health/sc/index_en.html). They also have applications in the treatment of insects of commercial value; e.g., oxytetracycline is used to treat foulbrood disease of the honeybee, which is caused by either *Bacillus larvae* or *Streptococcus pluton* (146).

QUANTITIES OF TETRACYCLINES USED

Introduction

Tetracyclines are one of the cheapest classes of antibiotic available, and their cost in real terms is declining due to improved manufacturing technology (159). The pricing structure makes them particularly attractive for use in developing nations (73). Furthermore, the HIV Meeting 2000 suggested the use of tetracyclines to reduce bacterial sexually transmitted diseases in the developing world. Nevertheless, the emergence of bacterial resistance to tetracyclines, the development of alternative agents that are better tolerated and more potent, and the introduction in some countries of legislation to prevent the use of tetracyclines as animal growth promoters are factors influencing the usage of tetracyclines. Although reliable data on production and consumption of antibiotics, including tetracyclines, are notoriously difficult to obtain (49, 109, 112), such data where available can assist analyses of trends in relation to the clinical and legislative factors mentioned above (109, 159). Furthermore, such data provide an indication of the extent to which continuing selection pressure for the emergence of tetracycline resistance is being imposed on human and veterinary pathogens and commensal bacteria (109). The following sections present data on human and animal consumption of tetracyclines. It has not been possible to obtain data relating to quantities used in aquaculture and agriculture.

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TABLE 7. Estimates of human and animal consumption of tetracyclines in a number of countries during the mid-1990s[a]

Region	Tetracycline consumption (kg/yr) by:	
	Human	Farm animals
United States	NA[b]	3,488,000[c]
Canada	NA	398,000[c]
Europe	NA	2,294,000[d]
Norway	2,191	NA
Australia	12,677	77,619[c]
New Zealand	1,343	2,311[c]

[a] Based on information from references 89 and 113a, http://europa.eu.int/comm/dg24/health/sc/index_en.html, and http://www.maf.govt.nz/ACVM/.
[b] NA, not available.
[c] Does not distinguish between therapeutic and subtherapeutic levels.
[d] Therapeutic use only.

Current data on the total quantities of tetracyclines used in human therapy and prophylaxis have, with the exception of Australia, New Zealand, and Norway (Table 7), been difficult to acquire. As discussed above, tetracyclines are of value primarily in the prophylaxis and treatment of community-acquired infections, with a more minor role for nosocomial infections. Therefore it is expected that the use of tetracyclines in humans (Table 7) primarily represents community use. A recent report on the use of antibiotics in Dutch hospitals certainly supports this view (112). The use of tetracyclines for community medicine is declining in many countries, with reduced prescription rates recently recorded for tetracyclines in Spain (23), the United States (172), and the United Kingdom (54). In the United Kingdom this followed an earlier period of decline between 1967 and 1984 (44). A similar downward trend in Norway has occurred, where annual consumption of tetracyclines for therapeutic use in humans fell from 3,185 kg in 1992 to 2,191 kg in 1996 (89).

Veterinary Medicine and Animal Growth Promotion

As discussed above, tetracyclines have applications both in veterinary medicine and as animal growth promoters. Current data on their use in farm animals are presented in Table 7. These data do not distinguish between therapeutic and subtherapeutic uses. However, in countries where use of tetracyclines at subtherapeutic levels in animal feed is still permitted, it can be estimated from earlier data (109) that approximately 90% of tetracyclines administered to cattle and swine are used at subtherapeutic concentrations whereas only 15% of usage in poultry reflects subtherapeutic administration. On the basis of animal consumption data, the use of tetracyclines in farm animals appears to be increasing in the United States, since approximately 2.6×10^6 kg was consumed in 1985 (109), rising to current levels of 3.5×10^6 kg (Table 7). This probably reflects an increase in the numbers of farm animals being raised following a switch from grain farms to pig and cattle farms due to the low prices of cereal crops.

RESISTANCE DEVELOPMENT AND ITS IMPLICATIONS

Introduction

Although bacterial resistance to tetracyclines has emerged in plant and fish pathogens as a consequence of using these antibiotics to control disease (144), it is the development of resistance in the context of human and animal use that has raised the greatest levels of concern. Since the tetracyclines have been used in humans and animals for some 50 years, it is not surprising that the selection pressure resulting from their use has resulted in the emergence of resistant bacterial variants, particularly those containing the *tet* genes described above. The emergence of resistance in human and veterinary pathogens has already had consequences for the use of tetracyclines as therapeutic agents. However, one of the biggest issues to emerge in recent years concerns the use of tetracyclines as animal growth promoters and the implications of this practice for human health. These topics are addressed in the following sections.

Resistance Following Human Therapy and Prophylaxis

The reported rates of bacterial resistance to tetracyclines have varied widely on the basis of geographical locale and year of isolation (137). However, by the mid-1970s resistance to tetracycline was common among the *Enterobacteriaceae*, staphylococci, streptococci, and bacteroides (144). In some locations resistance rates have been very high. For instance, in a Boston Hospital in 1969, 38% of *S. aureus*, 61% of *E. coli*, 62% of *Klebsiella* spp., 58% of *Enterobacter* spp., 91% of *Proteus* spp., and 97% of *Serratia* spp. were resistant to tetracyclines (254). Comparable, high rates of resistance were also recorded in *Bacteroides fragilis* and *H. influenzae* in the early 1980s, both in the United States and in Europe (137). The emergence of resistance to the tetracyclines in human clinical isolates has severely limited the further utility of these drugs and has undoubtedly been an important factor in the declining use of these antibiotics, in most countries, for the therapy of human infections (44, 73, 137). Fortunately, resistance has not yet become a problem for most situations (Table 6) where tetracyclines are still the drugs of choice, e.g., as demonstrated by the apparent absence of resistance in *Brucella melitensis* (3) and *Coxiella burnetii* (171) and low rates or only sporadic reports of resistance in periodontal bacteria (139), in *Helicobacter pylori* (58, 138, 177), and possibly *Chlamydia trachomatis* (142, 277). However, this situation may change in light of the recommendations for tetracyclines as first-line agents for acne (Table 6), since resistance rates as high as 25% have recently been reported for cutaneous propionibacteria (114). However, resistance in the propionibacteria is due to mutations rather than acquisition of *tet* genes (251).

For other antibiotic classes there is some relationship between use and the emergence of resistance in human isolates (261, 309; W. L. Nelson, J. N. Kuritsky, D. L. Kennedy, and C. S. Lao, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 455, p. 175, 1987). Since tetracyclines are still used quite extensively in certain developing countries, is there evidence that the incidence of resistance is higher in these countries than in regions where use has declined? Recent extensive data on the global antibiotic resistance patterns of community-acquired lower respiratory tract pathogens (71) permit such an analysis. In centers in Brazil, Mexico, and South Africa the incidence of resistance in *S. pneumoniae* for 1996 and 1997 is relatively high, ranging between 7.2 and 27.5%. Nevertheless, similar ranges in resistance

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rates, with possibly higher upper levels (5.3 to 39.4%) were recorded in the United States and many European countries (71), where sales of tetracyclines have been diminishing both during the last decade and in earlier periods. Comparison of data for global resistance rates in *H. influenzae* (71) also provides no indication of resistance trends in relation to tetracycline usage.

The above examples illustrate how little we know about the precise factors that lead to selection and maintenance of tetracycline-resistant organisms in bacterial populations. A factor contributing to this lack of knowledge is the absence of studies designed to evaluate changes in antibiotic resistance rates over time, with an emphasis in many cases on point prevalence studies (http://europa.eu.int/comm/dg24/health/sc/index_en.html). However, the observation that tetracycline resistance rates in *S. pneumoniae* are still high in countries where use of these drugs has declined illustrates the potential for stabilization of *tet* genes in bacterial populations even when selection pressure is reduced. Indeed, the persistence of resistance in pathogens such as *S. pneumoniae* might relate to a general ability of gram-positive pathogens to acquire and maintain multiple *tet* genes and the spread of multiple-drug-resistant clones. In addition, many of the transposons carry multiple-antibiotic resistance genes, any one of which can be selected, thereby maintaining all genes (see above).

In view of the extensive dissemination of *tet* genes into a wide range of bacteria and their apparent stabilization in these species, it is not surprising that tetracyclines are no longer regarded as reliable for use as initial therapy in many infections (195). Consequently, even if a reversal in the rates of tetracycline resistance could be achieved through decreased usage, there is unlikely to be renewed interest in the older tetracyclines by clinicians.

Resistance Following Applications in Veterinary Medicine and Use as Animal Growth Promoters

There is a substantial body of evidence that the use of antibiotics for veterinary therapy, prophylaxis, and animal growth promotion results in the selection of resistant animal pathogens and commensals (http://europa.eu.int/comm/dg24/health/sc/index_en.html; http://www.fda.gov/cvm/fda/mappgs/narms.html) (83, 125, 144, 146, 148, 158, 167, 316). The primary impact of resistance to antibacterials in terms of veterinary medicine is failure of empiric therapy of bacterial infection, which causes an increase in morbidity and mortality and hence prolonged suffering of infected animals; untreatable infections do occur but are rare and are not yet a significant problem in veterinary medicine (http://europa.eu.int/comm/dg24/health/sc/index_en.html). Antibiotic resistance in animals arises in a number of zoonotic pathogens such as *Salmonella* serovars, *Campylobacter* spp., and *Yersinia* spp and in commensals such as *E. coli* and enterococci, which exist in both human and animal ecosystems. Examples exist showing direct linkage between use of antibiotics in food animals and development of resistant infections in humans, indicating that transfer of antibiotic-resistant bacteria from animals to humans can occur (http://www.fda.gov/cvm/fda/mappgs/narms.html) (72, 83, 109, 146, 181, 316). Thereafter, these organisms might themselves cause infections that are difficult to treat, as in the case of food-borne diseases, or might transfer their resistance genes to other organisms that affect humans. These issues have been the subject of intense and continuous debate since the 1960s, when the Swann Committee in the United Kingdom concluded that antibiotics used in human chemotherapy or those that promote cross-resistance to other therapeutically valuable agents should not be used as growth promoters in animals (288). The antibiotics used as growth promoters in animals are considered to be associated with the greatest risk for selection of resistance because of the continuous subtherapeutic levels used in growth promotion regimens compared to the higher, short-term, therapeutic levels used to treat animal infections (50, 133, 217). Even though there is only circumstantial evidence that resistance in humans is exacerbated by the use of antibiotics in animal feed (http://europa.eu.int/comm/dg24/health/sc/index_en.html)(50, 83, 109), there are increasing concerns, and growing public awareness, that use of antibiotics in animal feed could constitute a human health hazard. In the European Union this has led to application of the precautionary principle whereby the European Commission banned, in animal feed, the use of bacitracin, spiromycin, virginiamycin, and tylosin on 1 July 1999 (Council Directive 2821/98) followed by olaquindox and carbadox on 1 October 1999 (Council Directive 2788/98). These decisions followed earlier ones banning the subtherapeutic use of tetracyclines in 1970 and avoparcin in 1997. Furthermore, the World Health Organization favors the elimination of antibiotics used to treat human diseases as growth promoters in food animals (www.who.ch/programmes/emc/zoo/oct97.pdf). Recently, the World Health Organization has placed on the internet, for public comment, their recommendations *Prudent Use of Antimicrobials in Food-Producing Animals* (http://www.who.int/emc/diseases/zoo/edg/draft.html.). Key factors of this proposal include proactive approaches by governments to reduce the need for antibiotics in animals, consideration of safety issues regarding the human health impact of resistance development in food animals, and surveillance of antimicrobial consumption by food animals. If such recommendations can be adopted, they will go a long way toward dealing with the agricultural issues of antibiotic use.

CONCLUSIONS

The tetracyclines are a class of antibiotics discovered more than 50 years ago. They are relatively inexpensive drugs with a broad spectrum of activity. Consequently, they have been extensively used in the prophylaxis and therapy of human and animal infections and as animal growth promoters. The selection pressures exerted by the use of tetracyclines in these various environments have resulted in the emergence of resistant organisms. The first tetracycline resistance R-factors were identified over 40 years ago in Japan (70, 146, 308). Since then, tetracycline resistance genes have spread in both gram-negative and gram-positive genera, primarily by conjugal transfer of plasmids and/or transposons. The dramatic increase in the number of species and genera that have acquired tetracycline resistance since the 1950s has led to a reduction in the efficacy and use of current tetracycline therapy for many diseases.

The gram-negative efflux genes have a common genetic organization and show ancestral relationships both in the efflux and regulation proteins. Genetic relationships are found with

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the gram-positive efflux proteins [Tet(K) and Tet(L)] and among the different ribosomal protection proteins [Tet(M), Tet(O), and Tet(S)]. Both efflux and ribosomal protection proteins are found in antibiotic-producing streptomycetes (33, 63, 206, 218).

The facultative gram-negative efflux genes are associated primarily with classic transposons and integrons, which are located on a diverse group of plasmids, as well as in the chromosome (19, 115, 176, 216). The gram-negative *tet*(B) gene has the widest host range of the efflux genes and is the only efflux gene which confers resistance to both tetracycline and minocycline. *Escherichia* spp., *Vibrio* spp., and *Streptomyces* spp. host the largest number of different *tet* genes (Table 4). Many of the *Vibrio* spp. are associated with fish diseases and/or water and have been subjected to extensive exposure to antibiotics because of fish aquaculture (8, 11, 59–61, 146, 280, 319). *E. coli* is also found in this environment (60, 61). Whether the environment can or does influence the ability of bacteria to acquire different *tet* genes or other antibiotic resistance genes is unknown. However, it is likely that an environment where there are large numbers of bacteria with many different species each carrying different *tet* genes provides an excellent climate for gene exchange. Why various gram-negative enteric genera carry only some of the gram-negative efflux genes is also not clear. We have speculated that the reduced host range of *tet*(E) may be due to its association with nonmobile and nonconjugative plasmids (61, 280). Perhaps there is a linkage between *tet* genes and particular incompatible-group plasmids, as suggested by Jones et al. (115). It is also possible that the ribosomal protection genes have not yet been found in enteric species because they confer relatively low-level tetracycline resistance in these hosts. However, given time and enough tetracycline exposure, this barrier may be breached in the future, as have so many in the past (7).

Gram-positive efflux genes [*tet*(K) and *tet*(L)] have recently been found in isolates of *Haemophilus* spp. and *Veillonella* spp., both of which can also carry the ribosomal protection *tet*(M) gene. More work must be done to look for gram-positive *tet* genes in natural gram-negative isolates. The ability to acquire *tet* genes from both gram-positive and gram-negative bacteria gives gram-positive species more options for acquiring different *tet* genes. The ribosomal protection genes, especially *tet*(M), are found in a number of gram-positive and gram-negative species, suggesting that they may have an advantage when it comes to being distributed among the different genera.

The same *tet* genes are found in bacterial pathogens, opportunistic microbes, and members of the normal flora. Significant numbers of normal flora bacteria from humans and animals, as well as bacteria isolated from food and the environment, are resistant to tetracycline (8, 69, 141, 146, 178, 266). The normal human flora may act as a reservoir for antibiotic resistance genes in general and for *tet* genes in particular (45, 46, 143, 225, 230, 236, 249). Similarly, the normal floras in animals, plants, and bacteria in the environment may also play import roles as reservoirs for antibiotic resistance genes (8, 46, 125, 230, 266, 319).

The widespread distribution of specific *tet* genes like *tet*(B) or *tet*(M) supports the hypothesis that the *tet* genes are exchanged by bacteria from many different ecosystems and between humans and both pet and food animals. Thus, bacteria exposed to antibiotics in the environment or in animals can ultimately influence antibiotic resistance in bacteria of human origin (125, 316). The presence of gram-positive *tet* genes in gram-negative species supports the hypothesis that gram-positive genes are being introduced and maintained in gram-negative species in nature and is not simply an in vitro artifact. It is very likely that this trend will continue, with more gram-positive genes becoming stably maintained in gram-negative hosts.

The use of tetracyclines to combat active infection in individual animals and herds should continue, not only because it reduces animal suffering but also because animals of considerable economic value can be saved (50; Miller, Abstract, 1999). However, the use of tetracyclines as animal growth promoters is a more contentious issue. The use of tetracyclines in food production is considerable and contributes to the worldwide exposure of bacteria to tetracyclines. There is no doubt that this practice results in the selection of resistant organisms and that in some cases these can be transmitted to humans. Furthermore, since *tet* genes can be located in integrons, the use of tetracyclines as growth promoters could result in selection and transfer to humans of resistance to unrelated antibiotics whose resistance gene cassettes are also incorporated within the integron. Although there is only poor scientific evidence that the use of growth-promoting antibiotics, including tetracyclines, poses a significant hazard to public health, the use of tetracyclines and other antibiotics as animal growth promoters has been banned in the European Union. Eliminating the use of tetracyclines and other antibiotics at subtherapeutic levels in animal feeds in other countries should also be a priority for feed manufacturers and policy makers, in the hope that these changes will help to reduce the incidence of resistant bacteria in the environment. It could be argued that further transmission of tetracycline resistance from animals to humans is of minor consequence because of the declining importance of the older tetracyclines in human medicine. Nevertheless, these agents still have valuable roles in the first-line treatment of certain infections, and for some applications improved formulations are being developed to further enhance their efficacy (260). These important medical uses of tetracyclines could be eroded if the human pathogens concerned acquired *tet* genes from the animal environment. In addition, the consequences of continued selection pressure with older tetracyclines in the animal setting for the success of the new glycylcyclines in human medicine are unknown. The observation that *tet* genes with the capacity to confer resistance to glycylcyclines apparently already exist in veterinary *Salmonella* isolates (M. Tuckman, P. J. Petersen, and S. Projan, Program Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C98, p. 97, 1998) is clearly a matter for concern.

FUTURE DIRECTION

The emergence of bacterial resistance to tetracyclines mirrors the situation with most antimicrobials currently in use. New derivatives of the tetracyclines, such as the glycylcyclines, dactylocycline, and other analogs with large hydrophobic groups, are all under examination for potential introduction as clinical agents to circumvent existing tetracycline resistance mechanisms (173, 300). There is also interest in discovering

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tetracycline efflux pump inhibitors that could be used in conjunction with older tetracyclines to restore their activity (185, 186). Among these agents, the glycycline analog tigilcycline (formerly known as GAR-936) is closest to introduction as a therapeutic agent since it is currently in phase II clinical trials (113).

Glycylcyclines display activity against strains expressing a variety of different *tet* genes, including those that encode ribosomal protection and efflux mechanisms. The glycylcyclines inhibit protein synthesis catalyzed by translation systems prepared from cells expressing Tet(M) and Tet(O) proteins (17, 215). Glycylcyclines compete with tetracycline for ribosomal binding but have a higher binding affinity for ribosomes than earlier tetracyclines (17). This is the most likely reason that ribosomal protection proteins are unable to confer resistance to glycylcyclines. The molecular basis of the activity of glycylcyclines against strains containing *tet* genes that encode efflux has also been examined. Activity could be due to the failure of the Tet efflux proteins to recognize glycylcyclines per se or to the inability of the Tet transporters to translocate glycylcyclines across the cytoplasmic membrane even though these proteins may recognize and bind the new analogs. The result of either mechanism would be failure to remove glycylcyclines from the bacterial cytoplasm so that inhibitor concentrations necessary to prevent protein synthesis would be maintained. Using everted membrane vesicles prepared from *E. coli* expressing the Tet(B) efflux transporter, Someya et al. (278) obtained data to suggest that 9-(*N,N*-dimethylglycylamido)-6-demethyl-6-deoxytetracycline (Table 2) is not recognized and hence not bound by the efflux protein. Structural studies with Tet(B) repressor protein, albeit from another class of tetracycline-interactive (water-soluble) proteins, demonstrate that the repressor does interact with glycylcyclines (202). Compared to tetracycline, the chelating nucleus of 9-(*N,N*-dimethylglycylamido)-6-demethyl-6-deoxytetracycline was unable to reach the normal inducer binding position within the repressor, as a result of a weakened hydrogen-bonding pattern within and around the tetracycline binding site.

Glycylcyclines possess the basic structural features associated with the broad spectrum of activity of the class, and, as already discussed, they are also active against strains containing *tet* determinants. A number of studies have been conducted on clinical isolates taken from patients with respiratory tract infections, skin and soft tissue infections, urinary tract infections, enterococcal infections, anaerobic infections, gonorrhoea, and cutaneous infections caused by rapidly growing mycobacteria, and they show promise for the glycylcyclines in these infections (25, 68, 77, 86, 92, 123, 194, 208, 270, 287, 295, 310–312, 315). In addition, 9-(*t*-butylglycylamido)-minocycline (tigilcycline; GAR-936) (Tables 1 and 2) could have therapeutic potential in cystic fibrosis patients infected with *P. aeruginosa* (113). More recently, 1,730 clinical isolates were examined in two studies with tigilcycline and older tetracyclines (22, 80). In general, the glycylcycline was 2- to 4-fold more active than minocycline and 2- to 16-fold more active than tetracycline against the *Enterobacteriaceae*. An exception was found with *Proteus mirabilis* and indole-positive *Proteus* spp. where the tigilcycline MIC against 90% of isolates was $\geq$8 μg/ml (80). Tigilcycline was active against virtually all gram-positive clinical isolates including those resistant to earlier tetracyclines (22). Surveillance studies have not so far identified any naturally occurring high-level glycylcycline-resistant strains among human clinical isolates (113). However, resistance to the investigational glycylcyclines 9-(*N,N*-dimethylglycylamido)-6-demethyl-6-deoxytetracycline and 9-(*N,N*-dimethylglycylamido)minocycline has been reported in two veterinary isolates, one of *S. enterica* serovar Typhimurium and one of serovar Choleraesuis (Tuckman et al. 38th ICAAC). Resistance was also found in African multiple-drug-resistant *Salmonella* and *Shigella* isolates (M. Roberts, unpublished results).

The outcome of the current phase II clinical trials with tigicycline will indicate whether this member of the latest class of tetracyclines has the potential for introduction as a chemotherapeutic agent. The observation that *tet* genes with the capacity to confer resistance to some glycylcyclines apparently already exist in veterinary *Salmonella* and *Shigella* isolates is clearly a potential issue.

Unfortunately, since resistance to new tetracycline deriviates may occur quickly, other strategies must also be considered to combat bacterial resistance, such as the development of new classes of drugs with unique antibacterial mechanisms. We also require a better understanding of the ecology of resistance to all antibiotics, including tetracyclines, that could lead to the design of new intervention strategies. We need to better understand the fate of tetracyclines in the environment. This is important because uneaten tetracycline-supplemented animal feeds go directly into the soil and water environment and it is not clear how long tetracyclines remain active in these environments. We need to develop methods to contain or detoxify antimicrobials, including tetracyclines. Antibacterial substances, such as triclosan, are being added to household products such as soap, children's toys, and clothing. How these substances affect the development of bacterial antibiotic resistance, including tetracycline resistance, is not clear. The experience gained with tetracycline highlights the need for a better understanding of bacterial resistance at the global level. We need a variety of approaches to reduce the amounts of antimicrobials being used worldwide in human and animal medicine and food production. This is the only way we will be able to keep tetracyclines and other antibiotics as resources for the next generation.

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