Bacterial Resistance to the Tetracyclines

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INTRODUCTION

The tetracyclines are a family of broad-spectrum antibiotics which inhibit protein synthesis by preventing the binding of amino acyl transfer ribonucleic acid to the bacterial ribosome (70). Although a large number of tetracycline derivatives are now known (15), relatively few have found extensive clinical use apart from tetracycline itself, chlortetracycline, oxytetracycline, and, more recently, doxycycline and minocycline (for the structures of these compounds, see reference 15). These tetracyclines provide safe, inexpensive, and effective treatment for many bacterial infections (159) and are also used for antimicrobial prophylaxis (131). However, the emergence of tetracycline-resistant bacteria has considerably reduced the usefulness of these antibiotics (159).

Studies on the mechanism of resistance to tetracycline (and antibiotics in general) may be expected (i) to contribute toward the more effective deployment of these drugs against bacteria and (ii) to lead to the development of new derivatives with improved antibacterial spectra and less susceptibility to bacterial resistance. Although the molecular basis of resistance to the tetracyclines has, until recently, been poorly understood, sufficient information has now accrued to warrant this review of the topic. Recent studies in this area have employed many of the advanced techniques now available in molecular genetics and membrane biochemistry.

Because resistance to the tetracyclines in the majority of clinical isolates probably results from acquisition of plasmids, this review will primarily be concerned with the mechanism of plasmid-mediated resistance to the tetracyclines. Because resistance results from changes in the cell envelope, it will be logical first to consider the passage of tetracyclines across this structure in sensitive bacteria. Subsequent sections deal with the nature of the membrane changes, gene dosage and expression of resistance, and, finally, the possible origin of the resistance genes.

MECHANISMS OF PENETRATION OF THE TETRACYCLINES INTO BACTERIA

General Features of Tetracycline Uptake

The energy dependence of tetracycline accumulation by bacteria was first reported in 1963 (4). However, as recently as 1973 Franklin (60) stated, "the active transport of the tetracycline

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antibiotics poses many more questions about the mechanism of this phenomenon than can yet be answered." Some of the puzzles noted by Franklin (60) now seem closer to resolution, but there is still no model for tetracycline accumulation which is consistent with all of the data. Because the envelope structures of gram-positive and -negative bacteria differ, the mechanisms of tetracycline uptake by the two bacterial types must be considered separately. However, in both cases the energy-dependent movement of tetracyclines across the cytoplasmic membrane is probably preceded by an energy-independent binding to the cell surface that most likely represents adsorption to the cytoplasmic membrane.

Passage of Tetracyclines Across the Outer Membrane of Gram-Negative Bacteria and Binding to the Surface of the Inner Membrane

The envelope of gram-negative bacteria (as typified by Escherichia coli) consists of three distinct regions (37): the outer membrane, the periplasmic space, and the inner (cytoplasmic) membrane. Most gram-negative bacteria are resistant to many antibiotics effective against gram-positive organisms, and this intrinsic insensitivity is attributable to the inability of the drugs to penetrate the outer membrane (113).

An approximate estimate of the ability of an antibiotic to penetrate the outer membrane can be derived from comparison of the minimum drug concentrations required to inhibit the growth of E. coli and Staphylococcus aureus (Table 1). On this basis the outer membrane can most readily exclude rifamycin, fusidic acid, and penicillin G, but is essentially permeable to d-cycloserine and streptomycin. The ratios of inhibitory activity for the tetracyclines are low and in some cases approach unity (Table 1), indicating that these molecules can readily penetrate the E. coli outer membrane.

Our understanding of the permeability of the outer membrane of gram-negative bacteria has recently been considerably extended, in particular by the work of Braun, Henning, Lugtenberg, Nakae, and Nikaido (for recent reviews, see references 18 and 44). The nomenclatures used by various research groups to describe the major outer membrane proteins of E. coli K-12 differ (18, 44, 77). In this article we shall refer to the proteins by the terminology of Henning (18, 44, 72, 78, 80). At least three of the major outer membrane proteins of E. coli (proteins Ia, Ib, and II') appear to form aqueous pores which span the membrane and allow diffusion of small hydrophilic molecules into the periplasmic space (9, 18, 44, 56, 103, 110, 119, 153, 154). Although we have been skeptical that these proteins form pores (30), there is considerable evidence to support the pore model (18, 44) which, with some reservations (29), we now accept. In addition to controversy concerning pores, a complete understanding of the organization of the outer membrane is still lacking as, for instance, there appears to be insufficient space in the outer half of the bilayer to accommodate the outer membrane proteins (compare data in references 80 and 136).

Because tetracyclines have molecular weights of less than 500 and the pores can accommodate molecules of up to 650 daltons (40), these antibiotics, in principle, could diffuse through the pores. This does occur for tetracycline (7, 29, 56, 113, 119), as might be expected for a hydrophilic molecule (Table 2). Tetracycline diffuses preferentially through pores formed from protein Ia (29, 56, 119), but those features of the molecule that consign it to this route are unknown because protein Ia pores are used by several other compounds (e.g., nucleotides, β-lactams, and copper ions) (56, 101, 119, 153, 154) which have no apparent structural similarity to tetracycline. Both chlorotetracycline and oxytetracycline also diffuse through protein Ia pores (56), which suggests that this may be the route of entry for all hydrophilic tetracyclines. Minocycline, however, is markedly more lipophilic than other tetracyclines (Table 2) and appears to penetrate the outer membrane by diffusing across the hydrocarbon interior of the bilayer (7, 29). Doxycy-
cine may also be too hydrophobic (Table 2) to enter through pores.

Because the tetracyclines are acids which can ionize in aqueous solution (Fig. 1 and Table 3), it is important to consider the nature of the species that diffuse across the outer membrane. Tetracycline hydrochloride and oxytetracycline hydrochloride each have four ionic or molecular forms (Fig. 1 and Table 3). At neutral pH the forms TH₄, TH⁻, ÖH₂, ÖH⁻ predominate (11, 84). Because a Donnan equilibrium exists across the outer membrane (negative charge inside; 142), diffusion of the anionic species is likely to be severely hindered. However, the formation of antibiotic-cation coordination complexes (2), for example with magnesium (Fig. 1), could produce cationic chelates which would respond to the Donnan equilibrium. Several studies (11, 84) indicate that only the products of the first dissociation (i.e., TH₂ and ÖH₂) are likely to penetrate gram-negative bacteria. Furthermore, the sensitivity of E. coli, Aerobacter aerogenes, and S. aureus to tetracycline is depressed in the presence of increasing concentrations of magnesium (11, 84, 141). This response has been attributed to a direct effect on the structure of tetracycline itself (141) and presumably reflects the formation of (TH-Mg)⁺ complexes which reduce the content of TH₂ species (11, 84). Passage of tetracycline and oxytetracycline through protein lα pores, therefore, presumably involves diffusion of TH₂ and ÖH₂ species down concentration gradients.

A dissociation scheme for the ionizable groups in minocycline apparently has not been reported. However, in view of the Donnan equilibrium across the outer membrane (142), minocycline is likely to diffuse through hydrophobic regions either in a molecular (uncharged) form or as a cationic chelate.

Because the pH in the periplasmic space is below that in the extracellular medium (142), tetracyclines entering the periplasmic space are likely to become protonated. Protonation may promote binding of antibiotic molecules to the surface of the membrane by interaction with anionic residues. These events could represent the initial energy-independent phase of uptake noted by several authors (43, 97, 98).

**Table 2. Partition coefficients of some of the tetracyclines**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Chloroform/water</th>
<th>Octanol/water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minocycline</td>
<td>30</td>
<td>1.1</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.48</td>
<td>0.60</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.09</td>
<td>0.036</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>0.007</td>
<td>0.025</td>
</tr>
</tbody>
</table>

* Based on data quoted in reference 8.

**Fig. 1. Dissociation schemes for (A) tetracycline hydrochloride and (B) oxytetracycline hydrochloride. Redrawn from reference 84 with permission.**

**Table 3. Ionization constants (K₁, K₂, K₃) of tetracycline and oxytetracycline hydrochlorides and stability constants K₅ of the complexes with magnesium**

<table>
<thead>
<tr>
<th>Tetracycline</th>
<th>pK₁</th>
<th>pK₂</th>
<th>pK₃</th>
<th>pK₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytetracycline</td>
<td>pK₁</td>
<td>pK₂</td>
<td>pK₃</td>
<td>pK₅</td>
</tr>
<tr>
<td>3.1–3.46</td>
<td>7.26–7.76</td>
<td>9.11–9.70</td>
<td>3.80–3.96</td>
<td></td>
</tr>
</tbody>
</table>

* Based on data quoted in reference 84.
a true active transport phenomenon. A simple explanation of the data (60) would be that transport of the tetracyclines requires protein carrier molecules dependent on membrane energization. We can consider this hypothesis by evaluating (i) data on the nature of energy coupling to transport, (ii) evidence which favors membrane carriers, and (iii) information on the nature of the carriers.

Recent studies clearly establish two major types of active transport systems in gram-negative bacteria (for reviews, see references 74, 76, and 134). One type is membrane bound and can be observed in membrane vesicles. The other type is sensitive to osmotic shock and requires periplasmic binding proteins to produce active transport. The membrane-bound systems derive energy from an electrochemical gradient of protons, the protonmotive force, which is the direct driving force for active transport. The transport systems involving binding proteins are more directly coupled to phosphate bond energy, probably as adenosine 5'-triphosphate. Although gram-positive bacteria have no periplasmic binding proteins, they can employ both adenosine 5'-triphosphate and the protonmotive force to drive transport (76).

Tetracycline uptake by E. coli is not impaired by osmotic shock (63), which suggests that a membrane-bound system powered by the proton-motive force could be involved in drug transport. However, tetracycline uptake by membrane vesicles was reported to be energy independent and apparently did not result in accumulation of antibiotic against a concentration gradient (59). The results of the vesicle experiments do not necessarily contradict the data obtained with whole cells. Franklin (59) did not check the transport capacity of his vesicles for any other solutes, and the use of Triton X-100 in some experiments (59) could have prevented the generation of a protonmotive force due to the formation of transmembrane pores by the detergent (129). More recently Levy et al. (98) reported that the sensitivity to tetracycline of an unc" adenosine triphosphatase" mutant (strain AN120) of E. coli K-12 was similar to that of the wild-type parental strain. Because adenosine 5'-triphosphate-driven, but not respiration-driven, transport is impaired in strain AN120 (134), these data (98) could support the model that tetracycline is transported across the inner membrane by a carrier driven by the protonmotive force. However, this interpretation is complicated by the fact that Levy et al. (98) did note that the kinetics of tetracycline accumulation differed between strain AN120 and its wild-type parent. The apparent discrepancy when strain AN120 was used might relate to the leakiness of this mutant, which can exhibit as much as 50% of the parental adenosine triphosphatase activity under certain conditions (134). Unfortunately, Levy et al. (98) did not estimate adenosine triphosphatase activity in their experiments with strain AN120, so that their results on the nature of energy coupling to tetracycline accumulation remain equivocal. Nothing is known of the nature of energy coupling to tetracycline transport in gram-positive bacteria.

If transport of tetracyclines is mediated by carriers, then it might be expected that the initial rates of uptake would be dependent on the external antibiotic concentrations up to the point when all of the available carrier sites are saturated with antibiotic. Although Franklin and Higginson (63) were unable to demonstrate saturation kinetics for the initial rate of uptake by whole cells, Franklin (59) showed that uptake by membrane vesicles was saturable. Data obtained more recently (45, 47, 158) provide convincing evidence that uptake of tetracycline and chlorotetracycline by several bacteria, including E. coli, S. aureus, and B. megaterium, demonstrates saturation kinetics. Furthermore, in B. megaterium there is evidence from biophysical studies that the membrane carrier for tetracycline is a protein (47). Data of another type also support the notion of a protein carrier for chlorotetracycline transport. Dockter and co-workers (46, 47) showed that energy-dependent chlorotetracycline accumulation in S. aureus and B. megaterium is influenced by the fluidity of membrane fatty acids. These results favor an active transport system for chlorotetracycline in which the movement of the protein carrier molecules becomes restricted when certain membrane fatty acids are in a crystalline state.

By analogy with the transport of other antibiotics and inhibitory analogs (3), the tetracyclines are presumably accumulated by illicit use of normal carrier systems. Several suggestions have been made concerning the identity of the postulated carriers. Franklin (60) proposed that tetracycline could be accumulated as an antibiotic-cation coordination complex via a mobile carrier involved in divergent cation transport. Indeed, chlorotetracycline may be transported across the staphylococcal membrane as an antibiotic-magnesium complex (45). Magnesium can be transported into E. coli by one or the other of two separate energy-dependent carrier systems (112, 116) which are presumably located in the cytoplasmic membrane. The first system is expressed constitutively and in addition to magnesium can transport other cations, such as cobalt (112, 116). The lack of total substrate specificity exhibited by this system indicates that it might transport magnesium-tetracycline com-
plexes. The second magnesium transport system is less likely to be a candidate for tetracycline accumulation because it is active only at low extracellular magnesium concentrations and is substrate specific (112, 116). Mutants lacking these magnesium transport systems accumulate tetracycline as readily as wild-type bacteria (7), and, therefore, at least in E. coli magnesium transport and tetracycline accumulation are not likely to be directly related. Transport of antibiotic into bacteria via the magnesium system is also unlikely for other reasons. The selective toxicity of the tetracyclines toward an infecting organism depends upon the failure of the antibiotic to be accumulated by mammalian cells (58). Because mammalian cells have an energy-dependent magnesium transport system (10), they might be expected to accumulate antibiotic-magnesium complexes. Therefore, the ability of bacterial cells to accumulate antibiotic presumably cannot depend on magnesium transport.

Tetracycline, chlortetracycline, and oxytetracycline could be accumulated in E. coli and S. aureus by the C_{4}-dicarboxylic acid transport system because oxaloacetate apparently competes with the antibiotics for uptake (108, 109, 118). In E. coli this transport system is inducible and requires cyclic adenosine 3',5'-monophosphate as part of a positive control system for its induction (100). Indeed, some tetracyclines (doxycycline and chlortetracycline, but apparently not tetracycline, oxytetracycline, or methacycline) may be transported into Salmonella typhimurium by systems that are cyclic adenosine 3',5'-monophosphate dependent (3). These studies in S. typhimurium (3) imply that separate carriers might be utilized by different tetracycline derivatives. Few data are available to clarify the situation, but other studies show that separate carriers could be involved in the transport of minocycline and tetracycline across the inner membrane of E. coli (41).

Although, as mentioned above, mammalian cells are impermeable to tetracyclines (58), isolated mitochondria can accumulate the antibiotics (22, 58) in a manner which depends upon the respiratory state of the mitochondria (22). These observations are at least consistent with the view that passage of tetracyclines across biological membranes could depend upon transport by dicarboxylate systems because mitochondria do possess such a transport system (86). The impermeability of mammalian cytoplasmic membranes presumably might reflect absence of dicarboxylate transport systems equivalent to those found in bacteria and mitochondria.

Although the preceding description favors a model involving membrane carriers for the active transport of tetracyclines into bacteria, some authors would probably disagree with this interpretation. It should be possible to clarify some of the questions concerning the mechanism of accumulation of tetracyclines in bacteria by the more extensive use of mutants impaired in energy transduction (134) and the C_{4}-dicarboxylic acid transport system (99). A model for the passage of tetracycline across the E. coli envelope will be presented (Fig. 2) after a discussion of the mechanism of plasmid-mediated resistance.

**PLASMID-MEDIATED RESISTANCE**

**Resistance Phenotypes**

Plasmids which confer resistance to the tetracyclines have been found in at least 25 bacterial species, including both gram-negative and gram-positive types (19). Early studies on the nature of plasmid-determined tetracycline resistance in E. coli and S. aureus established that full expression of resistance was obtained only after cells had been exposed to a sub-inhibitory concentration of the drug (90). These observations have subsequently been confirmed for a large number of plasmids (e.g., see references 31 and 55), and the only naturally occurring plasmid-linked tetracycline determinant to be expressed constitutively is that of plasmid pA21 found in Streptococcus faecalis (33). Expression of inducible resistance is likely to be under negative regulatory control, and proteins with repressor-like properties have been isolated from extracts of E. coli containing R222 (163) and pSC101 (144).

A wide variety in the characteristics of expression of resistance to the tetracyclines by naturally occurring R plasmids in E. coli has been

![Fig. 2. Model for tetracycline accumulation in E. coli and its blockade by plasmid-specified proteins. General features of envelope structure have been omitted for clarity. The diagram shows the possible sites of interaction in the envelope of proteins specified by the tetracycline resistance regions of Tn10 and pSC101. The organization of the inner membrane transport protein is based on the model of Singer (135). Four proteins are specified by the resistance region of pSC101, but only two have been assigned to the resistance region of Tn10. E, Kilodalton; — to, location at the site of membrane-bound polysomes is tentative; — →, located in the minicell envelope, but site (if any) in the whole cell envelope is unknown.](http://mmbr.asm.org/Downloaded from http://mmbr.asm.org)
found (23, 55). This prompts the question of whether the different phenotypic classes of \( R \) plasmid result from differences in the expression of a common tetracycline determinant on different plasmids. The tetracycline resistance genes of the plasmid R100-1 (a derepressed analog of R222; 51) are contained in an element called transposon 10 (Tn10; 34, 54, 88). This element can be translocated from one replicon to another by a recombination process which conserves the structure of the transposon and which occurs independently of the recA* product of the host cell (see references 34 and 87 for reviews). Dissemination of Tn10 could therefore account for the evolution of \( R \) plasmids specifying tetracycline resistance, and recent evidence (123) does indeed indicate that several plasmids contain a determinant related to Tn10. That altered tetracycline resistance phenotypes may arise from differences in the transcription of one set of resistance genes is supported by the studies of Tait and co-workers (104, 146, 147), who found differences in the expression of tetracycline resistance in derivatives of pSC101 which were obtained by insertion of fragments of deoxyribonucleic acid (DNA) into the EcoRI or HindIII cleavage sites of the plasmid. The EcoRI site in pSC101 probably lies within one of the structural genes for a polypeptide involved in resistance (104, 146), and some altered phenotypes have been attributed to disruption of a structural gene (104, 146). On the other hand, insertion of DNA at the EcoRI site also seems to affect transcription of an adjacent gene by altering ribonucleic acid polymerase attachment sites (147). Insertion of DNA at the HindIII site does not inactivate a structural gene but alters transcription because the HindIII site is located in a promoter for one or more of the structural genes involved in resistance (146).

Although, as mentioned above, one group of plasmids contains Tn10, other plasmids exist whose tetracycline resistance determinants are either only partially related or seemingly totally unrelated to Tn10. Chabbert and Scavizzi (23) noted that expression of plasmid-mediated resistance to minocycline and tetracycline in \( E. coli \) resembled plasmid-determined macrolide resistance in \( S. aureus \). Because macrolide resistance in the latter involves ribosomal modification, Chabbert and Scavizzi (23) suggested that plasmids which confer resistance to tetracycline and minocycline might do so by provoking alterations at the 30S ribosomal subunit rather than the membrane changes (see below) determined by plasmids containing Tn10. This situation certainly implies that some tetracycline resistance plasmids code for products not specified by Tn10. The plasmid pIP69 (formerly designated RIP69) belongs to that group postulated to confer resistance solely by ribosomal modification (23), but evidence for some degree of base similarity between the tetracycline resistance regions of this plasmid and R100 (containing Tn10) is provided by the finding of recombination between tetracycline-sensitive point mutants of these two plasmids (T. J. Foster, personal communication). Therefore, the tetracycline resistance region of pIP69 may have arisen by microevolution from Tn10 DNA. The products of pSC101 involved in tetracycline resistance appear to be totally unrelated to those specified by Tn10 because the resistance region of pSC101 has no homology with that in R6 (35), which is believed to contain Tn10 (123). Although tests (e.g., immunological cross-reactivity) to compare the similarity of proteins specified by the resistance regions of Tn10 and pSC101 have yet to be performed, both elements code for membrane proteins which exclude tetracycline from the cell (see below), and it would therefore seem that, although some tetracycline resistance regions may have evolved separately from one another, in each case their products act in a similar manner.

In \( S. aureus \) there are two phenotypic patterns of high-level resistance to the tetracyclines (5). One phenotype is determined by plasmid-located genes, and the other is determined by chromosomal genes. Because all of the plasmids which specify tetracycline resistance in \( S. aureus \) have a similar size (2.7 to 2.8 megadaltons; 19, 92) and confer similar levels of resistance to the antibiotic, it has been suggested (92) that one plasmid (i.e., a plasmid containing the same tetracycline resistance determinant) has spread throughout the staphylococci. The relationship between the two loci determining tetracycline resistance is unknown. However, the different phenotypes might be explained by assuming that the resistance genes are contained in a transposon, the expression of which is altered by integration into the chromosome. There is evidence that genes for resistance to some antibiotics are parts of transposons in \( S. aureus \) (117, 130), and in gram-negative bacteria transposition of Tn10 from a plasmid to a chromosomal locus has occurred under natural conditions (50).

Clearly, studies on the nature and function of the products of various tetracycline resistance determinants will contribute to the unravelling of the basis of the multiple phenotypes. Detailed studies have as yet been confined to the \( E. coli \) plasmids pSC101 and R100-1, but a little information is also available on the nature of plasmid-determined resistance in \( S. aureus \).
Proteins Associated with Plasmid-Determined Resistance in *Escherichia coli* and *Staphylococcus aureus*

Wojdani et al. (160) were the first authors to claim isolation of proteins specified by tetracycline resistance determinants. In *E. coli* they detected an envelope protein of apparent molecular weight 50,000, and in *S. aureus* they detected a membrane protein of apparent molecular weight 32,000. The two proteins were immunologically unrelated. It is difficult to assign either of these proteins as products of tetracycline resistance genes because the authors compared proteins produced by bacteria carrying plasmids with those from plasmidless organisms. Thus the proteins isolated could well represent plasmid products other than proteins associated with tetracycline resistance. Furthermore, induction of resistance in *S. aureus* caused no detectable increase in the content of the membrane protein (160), and the molecular weight of 50,000 reported for the *E. coli* protein is higher than that of any of the proteins now identified as products of R-factor tetracycline resistance determinants (see below). The most recent studies of Wojdani and his co-workers (139) do not provide totally convincing evidence that the 32,000-dalton staphylococcal protein is involved in tetracycline resistance. The remainder of the experiments summarized in this section either have involved comparison of strains which are isogenic apart from possession of tetracycline resistance determinants or have followed changes in protein content after induction of resistance. We can, therefore, be more confident that the proteins detected are products of the tetracycline resistance genes.

The *E. coli* plasmid pSC101 contains genetic information sufficient for its replication and the expression of tetracycline resistance (35). Resistance is inducible and results in the increased synthesis of three polypeptides of molecular weights 34,000, 26,000, and 14,000 (104, 144, 146). In minicells these proteins are located in the envelope (144), but their location in whole cells is unknown. A fourth protein, of molecular weight 18,000, is also associated with pSC101-mediated resistance, but its synthesis is constitutive (144). A protein of molecular weight 36,000 has been tentatively identified as the repressor of the pSC101 tetracycline resistance region (144). However, transcriptional control of the genes coding for the proteins of molecular weights 34,000, 26,000, and 14,000 is seemingly not achieved by regulating the synthesis of a single polycistronic messenger ribonucleic acid molecule, because the structural genes concerned are not contiguous (145).

As noted earlier, the genes conferring inducible resistance to tetracycline in the *E. coli* plasmid R100-1 are contained in Tn10. Levy and co-workers (163) detected in minicells two R222-coded proteins whose synthesis was stimulated after incubation in the presence of tetracycline. One of these proteins, of molecular weight 36,000 (163), is preferentially located in the minicell envelope (96, 163), whereas the second protein, of molecular weight 15,000, is located in the cytoplasmic fraction (163). These authors also demonstrated synthesis of the 36,000-dalton protein in a cell-free system for coupled transcription and translation directed by R222 DNA (163).

Until recently, the location of these proteins in whole cells of *E. coli* was unknown, and indeed our recent experiments (I. Chopra, P. R. Ball, and T. G. B. Howe, manuscript in preparation) have been directed toward clarification of the problem. In a strain containing a chromosomally integrated Tn10 element we have detected an inner membrane protein of molecular weight 15,000 whose synthesis is apparently inducible by tetracycline and which comprises about 4,000 molecules per membrane. This protein seems certain to be identical to that detected by Levy and co-workers (163) and, in view of the specific derepression of its synthesis by tetracycline, is unlikely to be a protein coded by a region of Tn10 not involved in expression of resistance. Levy et al. (98) reported that the protein of molecular weight 36,000 was located in the inner membrane of whole bacteria inhibited with rifamycin, but we have been unable to detect this protein in the envelope of resistant bacteria grown under physiological conditions, suggesting that normally it may only be present in very small amounts. As noted before, the tetracycline resistance region of pSC101 determines the synthesis of five proteins (including the repressor), but to date only two proteins (molecular weights, 36,000 and 15,000) have been identified as probable products of the resistance region of Tn10. Consideration of the coding capacity of the resistance region of Tn10 (Table 4) suggests that one or more as-yet-unidentified proteins (with a total molecular weight not exceeding about 27,000) could be involved in resistance. Indeed, studies on the location of ribosome binding sites within the central portion of Tn10 (i.e., excluding the terminal inverted repeat sequences) are consistent with the synthesis of a Tn10-specified polypeptide of approximate molecular weight 24,000 (21).

Franklin and Foster (61) showed that plasmid-determined tetracycline resistance in *E. coli* is
impaired by subjecting bacteria to osmotic shock. Because such treatment releases periplasmic proteins (79), the data of Franklin and Foster are consistent with a periplasmic location for one or more of the proteins involved in resistance. However, we have found (I. Chopra and S. J. Eccles, Proc. Soc. Gen. Microbiol., in press) that a protein IV (lipoprotein)-deficient mutant of E. coli, known to leak periplasmic proteins (69, 82), allows normal expression of Tn10-mediated resistance. Therefore, none of the proteins involved in resistance is likely to be periplasmic. On the other hand, expression of resistance is reduced in mutants lacking outer membrane protein Ia or containing deep rough lipopolysaccharide (Chopra and Eccles, in press), which possibly suggests an outer membrane location for a Tn10-specified protein.

Function of Proteins Associated with Plasmid-Determined Tetracycline Resistance

In view of their location in the cell envelope and earlier circumstantial evidence (60), the proteins could be directly involved in exclusion of tetracyclines from the cell. This view is consistent with the observation that plasmid-determined tetracycline resistance is associated with decreased accumulation of the antibiotic in E. coli (60) and other species (90, 132, 140, 150) and with the lack of evidence of enzymatic destruction of antibiotic by resistant bacteria (60, 140). Franklin (60) suggested that the data demonstrating reduced permeability in resistant cells could be explained if the cells actively excreted antibiotic. Young and Hubball (164) tested this hypothesis by studying tetracycline efflux from E. coli carrying a plasmid expressing temperature-conditional resistance to the drug. The efflux rates of tetracycline from these cells at permissive (30°C) and nonpermissive (42°C) temperatures were similar to or less than the efflux rates from preloaded plasmidless cells. The envelope proteins therefore do not promote drug efflux, but probably prevent influx.

Precise definition of the mode of action of the envelope proteins specified by tetracycline resistance determinants in E. coli is currently impossible. The protein of molecular weight 14,000 specified by pSC101 may block the energy-dependent accumulation of tetracycline (144). The inner membrane location of the 15,000-dalton protein specified by Tn10 is consistent with a similar function, and indeed the energy-dependent phase of tetracycline accumulation is also known to be modified in bacteria carrying R222 (97). These proteins could prevent binding of tetracycline to the uptake system by specific association with carrier molecules. The proteins of molecular weights 34,000 and 18,000 specified by pSC101 apparently block a final energy-independent phase of uptake (144) that could represent binding of antibiotic to membrane-associated ribosomes. Energy-independent binding of tetracycline may also be inhibited by a product of R222, although in this case a primary rather than final stage of uptake appears to be affected (97).

Levy and McMurry (97) have presented data which tentatively suggest that at least one of the R222 (Tn10)-coded proteins, probably that of molecular weight 15,000, acts at the level of the ribosome. Because we have detected this protein in the inner membrane, we are left with the intriguing possibility that this protein in vivo may be associated with both ribosomes and the membrane. The distribution of this protein upon cell fractionation might therefore resemble that of the E. coli phosphatidylserine synthetase which probably associates with both the membrane and ribosomes in vivo (84) and yet is not released from ribosomes during their fractionation from crude cell lysates (120). Evidence is gradually accumulating that at least some proteins in E. coli (and other bacteria) are synthesized on membrane-bound polysomes (121, 137, 138). Because protein synthesis on membrane-
bound polysomes may be particularly susceptible to inhibition by tetracycline (81), the 15,000-dalton protein may specifically protect these ribosomes from inhibition by the antibiotic. Comparison of the polypeptide composition of membrane-bound and free ribosomes in tetracycline-resistant bacteria might well provide a profitable approach toward an understanding of the role of this protein in resistance.

The principal difficulty in establishing the function of any of the proteins associated with tetracycline resistance stems from lack of detailed knowledge of the mechanism of tetracycline accumulation by sensitive bacteria. However, a scheme for the possible interaction in the E. coli envelope of the plasmid-determined proteins associated with resistance is presented in Fig. 2.

Membrane Architecture in Relation to the Binding and Function of Proteins Associated with Resistance

The membrane proteins must presumably integrate with and assume specific configurations within the envelope in order to function as resistance proteins. Several studies have suggested that fatty acid and phospholipid metabolism are altered in E. coli harboring plasmids that specify tetracycline resistance (48, 91, 125, 143). Such changes could be related to the formation of new lipid domains to accommodate the envelope proteins. However, we have been unable to find evidence to support this hypothesis either in E. coli (26, 28) or S. aureus (24). Thus, resistance is inducible in the absence of lipid synthesis (24, 26), and no differences in phospholipid or fatty acid content were noted in E. coli strains isogenic apart from possession of Tn10 (26, 28). The reported changes in the lipid content of tetracycline-resistant strains may therefore be due to plasmid products other than those of the tetracycline resistance regions. Although new lipid domains are not synthesized to accommodate the proteins, the physical state of the lipids at the sites of attachment to the envelope appears to influence the binding or function of the proteins. Thus, resistant organisms held at 5°C can no longer exclude the antibiotic (132), which presumably reflects the transition from a fluid to a crystalline lipid phase in those membrane regions occupied by the proteins.

Minicells derived from cultures of E. coli expressing high-level resistance to tetracycline are themselves only partially resistant to the antibiotic, as determined by inhibition of minicell protein synthesis (62, 95). This may indicate preferential localization of the plasmid-specified proteins in the equatorial regions of the whole cell envelope because minicells are derived from the polar regions of their parental cells (57). In S. aureus expression of high-level resistance to tetracycline is associated with a decrease in the content of membrane proteins of molecular weight about 22,000 (31), which could represent competition for insertion of plasmid-directed proteins and integral proteins into the same membrane domains.

Protein synthesis in minicells containing tetracycline resistance plasmids is more susceptible to inhibition by the antibiotic than is synthesis in whole cells containing the same R factors (62, 95). Although the sensitivity of protein synthesis in minicells could result from lack of synthesis of one or more resistance proteins (see above), it is clear that several of the proteins involved in resistance can be inserted into the minicell envelope (96, 144, 163). This implies that the activity of these proteins depends markedly on membrane architecture. Lack of expression of resistance in minicells could reflect (as noted above) absence of material from the equatorial regions of the cell envelope. Alternatively, because minicells do not replicate (95), effective placement of the envelope proteins could require integration into a growing cell membrane. However, as noted above, concomitant lipid and protein synthesis is not required for inducible expression of resistance in whole cells (24, 26). Little is known about the distribution of envelope components from parental cells to minicells, but research directed toward this problem might permit identification of those integral membrane components apparently required for some or all of the plasmid-specified proteins to function.

RELATION BETWEEN TETRACYCLINE GENE COPY NUMBER AND RESISTANCE LEVEL

Where R-plasmid resistance genes determine the synthesis of drug-metabolizing enzymes, there is usually a correlation between gene copy number (gene dosage) and enzyme activity per cell and, thence, resistance level (114, 151). Uhlin and Nordström (152) found that an increase in gene dosage in copy mutants of R1drd-19 was paralleled by an increase both in resistance to ampicillin and in beta-lactamase activity up to levels at least 10-fold higher than those characteristic of the unmutated plasmid. A similar relation between chloramphenicol acetyltransferase and streptomycin adenyllytransferase and gene dosage was found, although the levels of resistance to chloramphenicol and streptomycin reached plateaus of two- and threefold higher, respectively, when gene copy numbers were increased.

Efforts to demonstrate whether a similar relationship exists in the case of tetracycline re-
sistance have been hampered both by difficulty in the interpretation of tests for minimum inhibitory concentration of tetracycline (23) and by the lack, until recently, of suitable assay systems for the products of the resistance region. Furthermore, the interpretation of data relating to tetracycline gene copy number and resistance level is complicated by the association of the plasmid products with the cell envelope. Thus, an anticipated increase in resistance may not be realized if the number of binding sites within the membrane that can accommodate the plasmid-specified proteins is limited. This type of situation probably applies to the \textit{E. coli} outer membrane protein II*, where there is also evidence that free or nascent protein II* may inhibit its own synthesis (38). On the other hand, several \textit{E. coli} envelope proteins do show gene dosage responses (17, 122, 149) which can lead to very substantial enrichment of the envelope in specific proteins (149).

The first workers to consider the possibility of a tetracycline resistance gene dosage effect were Franklin and Rownd (64), who studied R100-mediated expression in \textit{E. coli} and \textit{Proteus mirabilis}. They found that a markedly higher level of resistance to chloramphenicol, streptomycin, and spectinomycin in \textit{P. mirabilis} when compared with \textit{E. coli} was not paralleled by an increase in tetracycline resistance level. This can be ascribed to the well-known dissociation of R100 into two separate DNA species in \textit{Proteus}, in which the replicon that includes the tetracycline resistance region does not undergo the same increase in copy number as does that comprising the other resistance genes.

Foster and Walsh (55), in a survey of resistance levels determined by a range of R plasmids, made several resistance measurements on strains carrying two compatible plasmids. Combinations of R6-S in separate experiments with R69, RP1, and R46 led to resistance levels in \textit{E. coli} J5-3 no higher than that conferred by one type of plasmid alone. Reeve and Robertson (124), however, obtained a conflicting result in a study of the R6-S plus R57 combination, finding that the growth rate of induced cells carrying both R plasmids, on challenge with 160 \mu g of tetracycline per ml, was 58\% that of an induced but unchallenged control, compared with a value of 39\% for one R plasmid alone. Although this discrepancy might be explained by differences in the host strains and plasmids used, neither group made any measurement of plasmid DNA content and thus there are no data on the gene copy numbers in the strains compared. Where such measurements have been made, there is strong evidence for a correlation between plasmid copy number and resistance level. \textit{E. coli} CR34 (pSC101) contains five copies of the tetracycline resistance-determining plasmid pSC101 and is resistant to 25 \mu g of tetracycline per ml on solid medium; when the same host strain contains pSC134, constructed by in vitro linkage of pSC101 to ColEl at the EcoRI cleavage sites, thus bringing the tetracycline resistance region under the relaxed replication control of ColEl, the plasmid copy number rises to 16, and the resistance level rises to 80 \mu g/ml. (20).

A similar relationship between resistance level and gene copy number is exhibited by pAMa1 in \textit{S. faecalis} (32, 33, 161, 162). \textit{S. faecalis} (pAMa1) is resistant to 250 \mu g of tetracycline per ml in liquid medium, but when the cells are grown in a sub-inhibitory concentration of the drug (150 \mu g/ml), the resistance level rises from 250 to 500 \mu g/ml and the 6-megadalton pAMa1 molecule is concomitantly replaced by a larger species of DNA; both properties return to the wild-type level on withdrawal of the drug. Heteroduplex studies and EcoRI digest preparations show that the enlarged DNA contains repeated sequences 2.65 megadaltons in length, each consisting of a tetracycline resistance determinant flanked by a small "recombination sequence" 380 nucleotide pairs in length which is presumably responsible for the reversible gene amplification. The ratio of plasmid to chromosomal DNA was similar in both states, indicating that the increase in resistance determinants per cell was balanced partially by a reduction in plasmid copy number. Likewise, the plasmid pRSD1 in \textit{E. coli} develops multiple copies of a 3.5-mega-
dalton sequence coding for tetracycline resistance when host cells are challenged with the drug (R. Mattes, H. J. Burkardt, and R. Schmitt, submitted for publication).

A much more complex situation is revealed by studies on a round of replication mutant of NR1 (105, 106, 148; H. Hashimoto, Y. Ike, C. F. Morris, and R. H. Rownd, submitted for publication; NR1 is probably identical to R100 and R222 [51]). Plasmid pRR12 (also called R12) is a copy mutant of NR1 that confers raised levels of resistance to streptomycin and chloramphenicol but lowered resistance to tetracycline relative to NR1. Most \textit{E. coli} cells that contain pRR12, when grown on tetracycline-containing agar, are found to contain pRR12 and one or more smaller plasmids; the latter are incompatible with pRR12 itself and can thus be recovered from clones that have lost pRR12. Such clones are fully sensitive to tetracycline. It can be shown by heteroduplex and restriction endonuclease analysis that pRR12 contains all of the sequences present in these small plasmids, and they are therefore presumed to originate from it. All contain the same origin of replication as the
resistance transfer factor of pRR12; yet, although they carry no tetracycline resistance determinant, they confer an increased level of resistance when present with pRR12 itself. Several other copy mutants of NR1 behave similarly (148). It has been suggested (Hashimoto et al., submitted for publication) that the decreased resistance conferred by pRR12 (3 µg/ml) relative to NR1 (100 µg/ml) in E. coli results from a higher level of tetracycline gene repressor owing to a repressor gene dosage effect in the copy mutant, and a similar increase in the F-plasmid transfer inhibition and entry exclusion has been explained in the same way. If this is so, it indicates that comparison of resistance levels between strains supposedly isogenic except for the number of tetracycline resistance genes must be interpreted with caution, unless tests are included to detect differences in the genetic regions controlling resistance expression (see above).

These effects may also influence the interpretation of experiments designed to test the dominance of particular alleles of the tetracycline resistance region, because such tests are generally infected gene dosage experiments involving two genetic regions. Foster (62) constructed strains of E. coli heterozygous for the resistance region, in which wild-type Tn10 was present on the chromosome and a reversible single-site tetracycline-sensitive mutant of Tn10 was present on R100; some of the heterozygotes failed to express resistance fully upon induction, but loss of the dominant sensitive allele by deletion restored the normal resistance level. These data are consistent with a loss of ability to synthesize resistance products, but not of repressor, in the single-site mutants. Similar tests on a series of constitutive mutants in combination with the inducible wild-type Tn10 showed dominance of the constitutive over the wild-type allele, suggesting that each mutant examined was operator constitutive (53). Tests on strains carrying two different wild-type plasmids have shown a distinction between inducible plasmids such as R57, R6, and R100 on the one hand and, on the other, RP1 and perhaps R46 and R199, which form a lower-level resistance group in which both repressor and operator are postulated to differ from those characteristic of the group that includes R100 (123).

We have exploited the transposability of Tn10 to compare the resistance levels both of wild-type Tn10 and of a constitutive allele (from pDU301; 53) when located in the chromosome and in a plasmid. Homozygotes in which a copy of the same allele of Tn10 is present in both locations simultaneously are no more resistant than are strains carrying the chromosomal allele alone (Howe and Chopra, unpublished data). This is surprising in view of the clear relation between gene dosage and resistance noted by Cabello et al. (see above and reference 20) in pSC101 and may reflect a difference in the products of pSC101 and R100 (see above).

There is evidence for a tetracycline gene dosage effect in S. aureus (128), where strains of S. aureus RN450 have been constructed containing a gene for tetracycline plus minocycline resistance and a gene for tetracycline resistance alone, the latter transduced from strain PS84. On challenge with tetracycline, the strain carrying both genes showed a higher resistance level than strains carrying either gene singly. S. aureus 649 Tet' Mir' carries 31 to 47 tetracycline resistance plasmids per cell (27, 93); on growth in the presence of 10 µg of tetracycline per ml, however, there was no evidence for increase in the tetracycline resistance plasmid copy number, nor were resistant derivatives of strain 649 Tet' Mir' isolated by incubation in the presence of the drug, in contrast to the finding of gene amplification in S. faecalis (see above).

ORIGIN OF TETRACYCLINE RESISTANCE GENES

The finding of some antibiotic-inactivating enzymes both in resistant bacteria and in antibiotic-producing streptomyces (12, 65, 155) prompts one to ask whether the tetracycline resistance genes discussed above might have originated from soil organisms. The ability of such genes from at least one group of naturally occurring plasmids to translocate from one replicon to another (Tn10; 54, 88) is consistent with this suggestion. Among those streptomycetes able to synthesize tetracycline or derivatives of it, the soil organism Streptomyces rimosus has been genetically analyzed in some detail (1, 66). This organism contains a fertility plasmid (SCP1; 67); the frequency with which the producer strain LST-118 gives rise to nonreverting oxytetracycline-sensitive variants is greatly increased by growth in the presence of acridines, suggesting that resistance is plasmid mediated (16). It is clear that tetracycline-producing streptomycetes must have some means of protecting themselves against their own product; with ribosomes from one strain of Streptomycyes aureofaciens that produces 2 mg of tetracycline per ml, protein synthesis is 50% inhibited in vitro by 50 µg of the drug per ml, whereas systems from low-level tetracycline producers are more sensitive, implicating a ribosomal resistance mechanism in this producer organism (42, 107).

The ability of a tetracycline resistance gene to be expressed is not restricted to the species in which the gene was isolated. A plasmid deter-
mining tetracycline resistance (pBC16; molecular weight, 2.8 x 10^6) has been isolated from Bacillus cereus GP7 (14); hybrid plasmids derived from pBC16, capable of replication in both Bacillus subtilis and E. coli, can confer resistance to tetracycline in both hosts, although other resistance genes present (for ampicillin, kanamycin, and chloramphenicol) are unable to be expressed in B. subtilis (89). One S. aureus plasmid coding for tetracycline resistance (pT127) has been introduced by transformation into B. subtilis, in which it continues to replicate and confer drug resistance (49). The promiscuous plasmid RP4 from Pseudomonas aeruginosa, which determines resistance to tetracycline and other antibiotics, is capable of conjugal transfer to a range of gram-negative bacteria (13, 39, 115). Such plasmids offer a wide variety of possibilities for spread of an ancestral gene among different bacteria, especially when considered together with the capacity of Tn10 to transpose. Further progress in understanding the origin and spread of resistance genes, however, will depend on whether tetracycline resistance in all organisms can be shown to result from an element related to Tn10 or whether (as possibly for pSC101) some completely different determinant is also found.

CONCLUSIONS

Franklin (60), writing in 1973 on antibiotic transport in bacteria, commented that "there are exceptional cases where an antibiotic sufficiently resembles a normal nutrient of the cell so as to be absorbed by the nutrient transport system." In fact, the majority of antimetabolites may prove fortuitously to use normal transport systems. For instance, it is already known that cycloserine uses the d-alanine transport system (156), phosphomycin uses the glycerol-3-phosphate system (133), arsenate uses phosphate systems (127), a variety of heavy metal cations use divalent cation transport systems (111, 112, 116, 157; A. A. Weiss, S. Silver, and T. G. Kinscherf, submitted for publication), and albomycin binds to the E. coli outer membrane TonA protein used in ferrichrome uptake (15). Aminoglycoside accumulation by E. coli provides a slightly different pattern of antimetabolite accumulation. By binding to ribosomes, some aminoglycosides are able to induce a normal polypeptide transport system which fortuitously promotes further aminoglycoside accumulation (83). In this case, however, the mechanism by which the aminoglycosides initially enter the cell is unknown (83). Passage of tetracycline across the bacterial cytoplasmic membrane may well prove to fit the general pattern of fortuitous transport; if the substrate it mimics can be identified, it may be possible to synthesize tetracyclines with improved properties of penetration by covalent linkage of antibiotic to the substrate.

High-level resistance to most of the agents listed above can result from chromosomal mutations that alter or lead to loss of the respective transport system or receptor. However, single-step chromosomal mutations which confer high levels of resistance to tetracycline do not occur, at least not in either E. coli (126) or S. aureus (92). The tetracycline transport system might, therefore, comprise part of a membrane complex essential for viability, which would explain the evolution of plasmid genes whose products specifically reduce the affinity of the transport system for tetracycline, yet do not disrupt membrane integrity. In contrast to this, some receptor proteins may have evolved as high-affinity binding components of transport systems for the uptake of substrates present in very low amounts in the environment (68, 75); dispensable structures of this kind could then be utilized as receptors by noxious agents such as bacteriophages and bacteriocins, and mutualistic loss of the receptors would confer bacteriophage or bacteriocin resistance without proving lethal for the cell.

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