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Emergence of Tetracycline Resistance in *Helicobacter pylori*: Multiple Mutational Changes in 16S Ribosomal DNA and Other Genetic Loci

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Tetracycline is useful in combination therapies against the gastric pathogen *Helicobacter pylori*. We found 6 tetracycline-resistant (Tet^r) strains among 159 clinical isolates (from El Salvador, Lithuania, and India) and obtained the following four results: (i) 5 of 6 Tet^r isolates contained one or two nucleotide substitutions in one part of the primary tetracycline binding site in 16S rRNA ($AGA_{965\text{-}967}$ [*Escherichia coli* coordinates] changed to gGA, AGc, guA, or gGc [lowercase letters are used to represent the base changes]), whereas the sixth (isolate Ind75) retained $AGA_{965\text{-}967}$; (ii) PCR products containing mutant 16S ribosomal DNA (rDNA) alleles transformed recipient strains to Tet^r phenotypes, but transformants containing alleles with single substitutions (gGA and AGc) were less resistant than their Tet^r parents; (iii) each of 10 Tet^r mutants of reference strain 26695 (in which mutations were induced with metronidazole, a mutagenic anti-*H. pylori* agent) contained the normal $AGA_{965\text{-}967}$ sequence; and (iv) transformant derivatives of Ind75 and of one of the Tet^r 26695 mutants that had acquired mutant rDNA alleles were resistant to tetracycline at levels higher than those to which either parent strain was resistant. Thus, tetracycline resistance in *H. pylori* results from an accumulation of changes that may affect tetracycline-ribosome affinity and/or other functions (perhaps porins or efflux pumps). We suggest that the rarity of tetracycline resistance among clinical isolates reflects this need for multiple mutations and perhaps also the deleterious effects of such mutations on fitness. Formally equivalent mutations with small but additive effects are postulated to contribute importantly to traits such as host specificity and virulence and to *H. pylori*'s great genetic diversity.

Helicobacter pylori is a genetically diverse gram-negative bacterial species that chronically infects more than half of all people worldwide and that can provoke development of peptic ulcer disease and contribute to the risk of gastric cancer (for reviews, see references 10 and 47). Several combination therapies have been developed to eradicate this pathogen and cure or prevent associated diseases. First-line therapies usually involve a proton pump inhibitor plus clarithromycin and either metronidazole or amoxicillin. They tend to fail in more than 10% of clinical trials, however, often because of bacterial resistance to metronidazole, bacterial resistance to clarithromycin, and/or patient noncompliance. This led to the recommendation that a need for additional treatment be anticipated whenever *H. pylori* eradication is attempted and that tetracycline generally be used in such second-line (rescue) therapies (15, 27). In practice in developing countries, however, first-line therapies often also involve tetracycline because metronidazole-resistant strains tend to be common and clarithromycin is too expensive for most people (29; R. H. Gilman and A. Chowdhury, personal communication).

Tetracycline is bacteriostatic, not bactericidal, against most susceptible bacterial pathogens, *H. pylori* included (45). It binds tightly to a pocket in 16S rRNA, where it interferes sterically with binding of aminoacyl-tRNA to the ribosome A site and thereby blocks protein synthesis and bacterial growth. The tetracycline binding pocket has been defined with atomic resolution in the *Thermus thermophilus* ribosome and consists of two domains in 16S rRNA: helix 34 and the loop next to helix 31 (6, 32). Resistance to tetracycline is common in many bacterial species. In *Propionibacterium acnes* this resistance is ascribed to a mutation affecting one specific position in the tetracycline binding site (position G1058 in rRNA helix 34) in a remarkable 85% of tetracycline-resistant (Tet^r) clinical isolates (34, 35). In most other species, however, resistance tends to be conferred by auxiliary genes carried on plasmids, transposons, or other mobile DNA elements and typically involves proteins that variously (i) promote tetracycline efflux, (ii) bind to and change the 30S ribosomal subunit conformation and thereby interfere with tetracycline-ribosome binding, or (iii) cause enzymatic inactivation of the drug (9). Cases of resistance due to changes in chromosomally encoded porins or efflux pumps are also known (23, 25, 30).

Tetracycline resistance is uncommon among *H. pylori* clinical isolates, at least in the United States and Europe. For example, no Tet^r strain was found among more than 6,000 clinical isolates from the United States tested in D. Y. Graham's laboratory (cited elsewhere [31] as unpublished data) or among any of nearly 1,900 strains from Portugal (8), The Netherlands (12), Sweden (19), Germany (40), or Australia (16). Just 6 Tet^r strains were found among 607 isolates tested from

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Italy (38), Saudi Arabia (2), Bulgaria (5), and Spain (11). However, some 5 to 7% of strains from Japan and Korea (24) and a remarkable 59% of strains from Shanghai, People's Republic of China (48), were reported to be Tet^r. The last two reports, if confirmed, would indicate that tetracycline resistance is far more common in East Asia than in the West and would be in accord with indications that East Asian and Western *H. pylori* gene pools differ markedly from one another (22, 49). One case of very high level tetracycline resistance in *H. pylori* has been examined closely: Trieber and Taylor (42) recently found that resistance in a pair of closely related clinical isolates from the same patient (28) (in effect, one strain) was due to a multisite mutation, a replacement of AGA by TTC in the loop next to helix 31, one side of the tetracycline binding pocket (16S rRNA positions 965 to 967) (42).

In the experiments described here we studied 6 *H. pylori* strains that are resistant to low levels of tetracycline and that were found among 159 independent clinical isolates from El Salvador, India, and Lithuania. The initial search for resistance in this strain collection was motivated by a consideration that tetracycline is available without prescription in each of these three countries; that it is used extensively against cholera and other diarrheal diseases in India and El Salvador; and that it had also been much used in Lithuania, often to avoid complications from other infections (even common colds, although such tetracycline use decreased as other antimicrobial agents became more available after independence in 1990). These six Tet^r strains were studied to understand the mechanisms that underlie the resistance phenotype and why tetracycline resistance is so uncommon, at least in Western *H. pylori* populations. Three findings contributed importantly to our study design. First, resistance of *H. pylori* to the macrolide clarithromycin results from any of several specific single nucleotide substitutions in 23S rRNA; mutant ribosomal DNA (rDNA) alleles are easily moved to new strains by transformation and selection for resistance. Most such clarithromycin-resistant (Cla^r) transformants contain only the mutant allele (which encodes resistance), even though *H. pylori* contains two copies of each rRNA gene (43, 44, 46). Thus, a recessive rDNA allele can be acquired, rendered homozygous, and expressed relatively efficiently in *H. pylori*. Second, resistance to metronidazole, which is common in many *H. pylori* populations, usually results from inactivation of chromosomal genes for nitroreductases (*rdxA* and, in some cases, *frxA*) that mediate conversion of metronidazole from a prodrug to a bactericidal agent (20, 21). Thus, unlike drug resistance in many pathogens, resistance in *H. pylori* usually involves only mutant alleles of normal chromosomal genes, not mobile DNA element-borne auxiliary resistance determinants. Third, tetracycline resistance in *P. acnes* is often due to a mutation in the tetracycline binding pocket in 16S rRNA (34, 35), as noted above.

The six Tet^r clinical isolates studied here were each less resistant than the Tet^r strain that Trieber and Taylor (42) characterized. Five of the six contained one or two nucleotide changes in the loop next to helix 31, which forms part of the tetracycline binding pocket, whereas the sixth did not. Further tests showed that tetracycline resistance in *H. pylori* can be complex and multifactorial and can involve various mutant alleles of 16S rRNA genes or non-rDNA determinants, or both. Most individual mutations have only weak effects, but these effects are additive: high-level, probably clinically significant, resistance can emerge from combinations of multiple mutations with small but cumulative individual effects.

MATERIALS AND METHODS

Bacterial strains and culture methods. The *H. pylori* reference strains used in the present study were HPK5, which is highly transformable (39), and 26695, whose genome has been sequenced entirely (41). The 81 Lithuanian, 38 Salvadorean, and 40 Indian clinical isolates screened for tetracycline resistance were from adults with gastric complaints who warranted endoscopy and were from patients of two of the authors (L.K. and M.A.P.) and Abhijit Chowdhury (29), respectively. Genomic DNA from highly resistant Australian *H. pylori* strain Aus108 (28), in which resistance is due to a change from AGA to TTC at 16S rDNA positions 965 to 967 (42), was kindly provided by C. Trieber and D. Taylor. All patients whose *H. pylori* strains were cultured had provided written informed consent under protocols approved by local institutional human studies committees (Kaunas University of Medicine, Kaunas, Lithuania; Hospital Rosales, San Salvador, El Salvador; and the Institute of Post Graduate Medical Education and Research, Calcutta, India).

H. pylori isolates were cultured on brain heart infusion agar (Difco) supplemented with 7% horse blood, 0.4% IsoVitaleX, and the antibiotics amphotericin B (8 μg/ml), trimethoprim (5 μg/ml), and vancomycin (6 μg/ml) (referred to here as BHI agar) and were incubated at 37°C under microaerobic conditions (10% CO_2, 5% O_2) as described previously (20). Metronidazole susceptibility and resistance were estimated by spotting aliquots of appropriately diluted bacterial cultures on BHI agar with 8 μg of metronidazole per ml and scoring the efficiency of colony formation on metronidazole-containing agar relative to that on metronidazole-free agar, as described previously (20).

To score tetracycline susceptibility or resistance, cells growing exponentially on tetracycline-free BHI agar were suspended in phosphate-buffered saline buffer, and about 10^6 cells were spotted on medium with 2 μg of tetracycline per ml (tet2 medium), and on tetracycline-free medium as a control. An isolate was considered resistant if any bacterial growth was observed after 1 week of incubation. The level of resistance of Tet^r isolates was also examined more closely by an efficiency of colony formation (efficiency of plating) test, in which a series of 10-fold dilutions of cell suspensions was prepared; and 10 μl of each dilution was spotted on freshly prepared BHI agar containing various concentrations of tetracycline (e.g., 0, 0.5, 1.0, 2.0, and 4 μg/ml). When accurate estimates of very low frequencies of colony formation ($<10^{-6}$) were needed, culture aliquots were spread directly on the surface of an entire plate of tetracycline-containing BHI agar rather than spotted in a small area.

Levels of resistance were more difficult to quantify with tetracycline than with metronidazole. For example, small increases in near-threshold tetracycline concentrations tended to cause progressive decreases in the fraction of cells able to form colonies and also in colony size (culminating in invisibility without the use of a magnifying glass, independent of the time of incubation). This contrasted with the abrupt changes in colony-forming efficiency, but little change in colony size, with changes in concentration that were seen in similar analyses of metronidazole susceptibility (20). As a consequence, estimates of a strain's colony-forming efficiency at a given tetracycline concentration (in effect, resistance level) varied slightly from trial to trial. However, the same rank order of resistance levels for different clinical isolates or transformants was observed in repeated parallel tests. Variations in test results may reflect the fact that tetracycline is more bacteriostatic than bactericidal and the fact that it is somewhat unstable during incubation; any effects of subtle differences in bacterial physiologic states on the effectiveness of tetracycline inhibition would also contribute to variability. Given these observations, for parsimony in assembling the results, a strain was considered to still be rather resistant to any level of tetracycline that allowed colony formation with at least 10% the efficiency of that seen with the next lower tetracycline level; and it was considered susceptible to the next higher tetracycline concentration used if that concentration caused at least a 1,000-fold decrease in the efficiency of colony formation. It is noteworthy that for several isolates tested, cells from minute colonies recovered from media with partially inhibitory concentrations of tetracycline had susceptibility profiles that matched those of the parental cell line, which had not been grown with tetracycline. These cells seemed not to be spontaneous resistant mutants, which is quite different from the pattern seen with cells recovered from media with partially inhibitory concentrations of metronidazole (20, 21).

PCR and DNA sequencing. DNAs were prepared from confluent BHI agar plate cultures derived from single colonies or from pools of colonies, as appropriate, with QIAamp Tissue DNA extraction kits (Qiagen Corporation, Chats-

worth, Calif.). To detect changes in 16S rRNA sequences associated with tetracycline resistance, nearly full-length (1.4-kb) segments of 16S rRNA genes were amplified by PCR with primers 16S-F (5′-CGGTTACCTTGTTACGACTTC AC) and 16S-R (5′-TATGGAGAGTTTGATCCTGGCTC). The PCR was carried out in 50-μl volumes containing 10 ng of genomic DNA, 10 pmol of each primer, 1 U of Biolase (*Taq* DNA polymerase equivalent) from Midwest Scientific (St. Louis, Mo.), and 0.25 mmol of each deoxynucleoside triphosphate in standard PCR buffer. The reaction mixtures were preincubated for 2 min at 94°C and were then subjected to 30 cycles of 94°C for 40 s, 52°C for 40 s, and 72°C for 2 min, with a final elongation step of 72°C for 10 min. PCR fragments were purified for sequencing with a QIAquik PCR purification kit (Qiagen Corporation). PCR-amplified 16S rDNAs were sequenced by using primers 16S-R and 16S-F and also primer 16S-F2 (5′-TCAAGCCTAGGTAAGGTTCTTCG). Sequencing reactions were carried out with a Big Dye Terminator cycle sequencing kit (PE Applied Biosystems, Foster City, Calif.), and products were run on Applied Biosystems automated sequencers in the Washington University Molecular Microbiology core facility.

Nucleotide sequence accession numbers. The 16S rDNA sequences determined in the present study have been deposited in GenBank under accession numbers AF535194 to AF535200 (for strains Sal05, SalI0, Lit69, Lit76, Lit81, HPK5, and Ind75, respectively).

RESULTS

Tet^r clinical isolates and mutant 16S rRNA genes. Preliminary tests showed that individual cells from diluted cultures of several reference *H. pylori* strains, including strains 26695 and HPK5 (39, 41), did not form colonies on freshly prepared medium with 2 μg of tetracycline per ml (tet2 medium) under our conditions but did form colonies on media with lower tetracycline concentrations (0.5 or 1 μg/ml) about as efficiently as they did on tetracycline-free medium. Cells from colonies of several strains that had grown on tet0.5 or tet1 medium remained unable to grow on tet2 medium, which indicates that they were not mutants. A set of 38 clinical isolates from El Salvador, 81 isolates from Lithuania, and 40 isolates from India (159 isolates in total) was then screened for growth on tet2 medium. Five strains (two Salvadorean and three Lithuanian strains) were judged to be resistant. They formed colonies with nearly 100% efficiency on tet2 medium, whereas most other clinical isolates screened could not grow on this medium (no colonies per 10^6 cells plated). One Indian strain (strain Ind75) formed minute colonies efficiently on tet1.5 medium, unlike any of the other 39 Indian strains screened, and thus was also designated resistant. For each of these six strains, however, the speed of colony development was reduced on tet1.5 or tet2 medium (colonies could be seen without magnification at ~5 days on tet2 medium, whereas they could be seen without magnification at ~3 days on tetracycline-free medium). The three Lithuanian and two Salvadorean Tet^r clinical isolates also made minute colonies on tet4 medium, but generally with efficiencies of only 1% or less, and they became visible only after 6 days of incubation. Three of these six Tet^r strains (strains Lit69, Lit76, and Lit81) were susceptible to metronidazole, which contrasts with the reported metronidazole resistance of all Tet^r strains in two East Asian *H. pylori* collections (24, 48).

Given the many Tet^r *P. acnes* isolates in which resistance was due to substitution at 16S rRNA position G1058 (helix 34), which forms part of the tetracycline binding pocket (34, 35), we amplified by PCR and sequenced 16S rRNA genes from each of these six new Tet^r *H. pylori* strains. The sequences of five of them differed from that of the canonical wild-type strain in the loop adjacent to helix 31 (positions 965 to 967) (Fig. 1), a segment that cooperates with helix 34 to form the primary tetracycline binding site. Two strains were doubly mutant ($AGA_{965\text{-}967}$ changed to gtA and gGc [lowercase letters are used to represent the base changes]); three others contained just single nucleotide substitutions at this same site (AGA changed to gGA or AGc); and the sixth (strain Ind75) contained the canonical AGA sequence at this site. In contrast, each of 33 *H. pylori* 16S rRNA entries found in a database search in June 2002 (excluding three entries for the Tet^r strains [with ttc in place of AGA] of Trieber and Taylor [42]) contained AGA at this site. Several other differences in the rDNA sequences of Tet^r isolates and tetracycline-susceptible (Tet^s) reference strains were also found, but none was consistently found among all Tet^r isolates (Fig. 1). It is striking, however, that none of the Tet^r *H. pylori* clinical isolates contained sequence changes at or near G1058, the residue that is so important in tetracycline resistance in *P. acnes*, nor were changes found in other, lower-affinity tetracycline binding sites (32).

			1111111
Position	3778	**999**	0001122
in *E. coli*	99996488	**666**	2353699
coordinates	23560123	**567**	6386723
Reference Tet^s Strains			
26695	GTCTGGAC	**AGA**	GCGTCAC
J99	A-TG...C	**AGA**	ATGCCGC
HPK5	C	**AGA**	GCGTCAT
Tet^r Clinical Isolates and Transformants			
Sal05(clin)	.......G	**g..**	AT.CTG.
HPK5-Sal05	.	**g..**	AT.C...
Sal10(clin)	A-TG...G	**g..**	AT.CTG.
HPK5-Sal10	.	**g..**	AT.CT.T
Lit69(clin)	A.....	**..c**	...C...
HPK5-Lit69	.	**..c**	...C...
Lit76(clin)	.....	**gt.**	...C...
HPK5-Lit76	.	**gt.**	...C...
Lit81(clin)	A....AC.	**g.c**	.....
HPK5-Lit81	.	**g.c**	...C..T
Ind75(clin)	.	...	.....
ind75-Lit76	.	**gt.**	...C...
Aus108/9	A...A...	**ttc**	...C...

FIG. 1. Diagram of polymorphic sites in 16S rDNAs of Tet^s reference strains of *H. pylori*, Tet^r clinical isolates, and Tet^r transformant derivatives of HPK5 (Tet^s) and Ind75 (low-level Tet^r) generated as detailed in the text and in GenBank accession numbers AF535194 to AF535200. The positions in the rRNA sequence (adapted to *E. coli* 16S rRNA coordinates; GenBank accession number AE000474) are listed vertically. For example, the 1st column of sequence corresponds to position 92; the 5th column corresponds to position 360; and the 9th, 10th, and 11th columns (boldfaced) correspond to positions 965, 966, and 967, the sites of mutations inferred to contribute to tetracycline resistance (lowercased), as discussed in the text. Periods indicate base substitutions relative to the 16S rDNA sequence. Dashes indicate absent nucleotides (1-bp deletions). Empty spaces indicate sequences not determined. clin, clinical isolate. HPK5 and Ind75 used as recipients for transformation using rDNAs from Tet^r clinical isolates are italicized. Highly resistant strain Aus108/9, examined by others (42), is included for comparison. Not shown here is position 1024, which is 1024T in HPK5 but 1024C in most *H. pylori* strains, including all Tet^r clinical isolates identified in our studies.

16S rRNA sequence changes underlie much but not all of

the tetracycline resistance in clinical isolates. To further test if nucleotide substitutions in 16S rRNA contributed to resistance, PCR-amplified 16S rDNAs from each of the six Tet^r isolates were used to transform Tet^s strain HPK5, and resistant transformants were selected on tet2 medium. Single discrete colonies were obtained at frequencies of about 10^{-4} in 4 days after transformation with DNAs containing double substitutions from positions 965 to 967 (strains Lit76 and Lit81); much smaller, barely visible colonies were obtained only at 6 days after transformation with DNAs from the three strains with single substitutions (strains Lit69, Sal05, and SalI0); and no colonies were obtained by using the PCR products from the 16S rDNA from strain Ind75 (which contains AGA at positions 965 to 967) or any of three Tet^s control strains. The expected rDNA alleles with changes at positions 965 to 967 were found by sequencing in each of the 10 Tet^r transformants tested (two from each of the five donor DNAs; Fig. 1). Nearby sequences that distinguished the Tet^r clinical isolates from the HPK5 recipient were also present in many transformants; these may reflect normal genetic linkage, not mechanistic involvement in resistance.

Although *H. pylori* contains two copies of the 16S rRNA gene, inspection of sequence tracings showed that 9 of the 10 transformants were homozygous (contained only the mutant allele), indicating that the emergence of transformants with a resistant phenotype typically involves acquisition of the mutant sequence by both 16S rRNA gene copies, presumably one after the other. One transformant generated with Lit081 DNA was heterozygous, which, although not understood (susceptibility was expected to be dominant), is reminiscent of the finding of occasional heterozygous transformants generated with 23S rDNA alleles that confer clarithromycin resistance (18, 44). In summary, these results show that changes in the 16S rRNA sequence can confer resistance to tetracycline in *H. pylori*. The weaker Tet^r phenotype of transformants containing rDNA alleles with single substitutions relative to the Tet^r phenotype of their parental clinical isolates and the inability of rDNA sequences from Tet^r isolate Ind75 to confer resistance on the Tet^s recipient strain both imply that non-rRNA determinants also contribute to tetracycline resistance in *H. pylori*.

In follow-up experiments, weakly Tet^r clinical isolate Ind75 (grown on tet1.5 medium) was transformed with PCR-amplified 16S rDNA from Tet^r strains Lit76 and Aus108, and colonies that grew well on tet3 medium were selected. Several representative transformants made with Lit76 DNA (AGA to gtA) grew, albeit weakly, on tet8 but not on tet16 medium; a transformant made with a PCR product from Aus108 (AGA to ttc) grew on tet32 but not on tet64 in the same experiment (Table 1). The 16S rDNAs of representative transformants were sequenced and found to contain the expected $gtA_{965\text{-}967}$ and $ttc_{965\text{-}967}$ alleles. Because these transformants were more resistant than either parent, we infer that the naturally occurring 16S rDNA of Lit76 or Aus108 and the non-rRNA determinants of Ind75 contribute additively to resistance phenotypes.

Tet^r mutants generated in culture. To further assess potential contributions of non-rDNA versus rDNA gene mutations to tetracycline resistance, we selected and characterized Tet^r mutant derivatives of reference strain 26695. No ($<10^{-8}$) spontaneous Tet^r mutants were found in preliminary experiments. We therefore mutagenized cells of strains 26695 by growing them on medium containing low concentrations (2 or 3 μg per ml) of metronidazole, a condition in which many cells are killed by this anti-*H. pylori* drug and in which survivors contain a markedly increased frequency of mutations (36, 37). Some 10^{-6} to 10^{-5} of cells harvested from metronidazole-containing medium formed colonies on tet2 medium, whereas $<10^{-8}$ cells harvested from metronidazole-free medium formed colonies. These estimates of colony-forming efficiency were imprecise, however, because colony sizes ranged from barely perceptible to near normal (diameter, ≥1 mm) even after 7 days of incubation. Six to 7 days of incubation was also needed for these Tet^r colonies to appear on the initial tet2 selection plates, which is about 2 days longer than the time needed for the emergence of Tet^r transformants. However, no colonies or background growth was detected after parallel plating of 10^8 cells from a nonmutagenized control culture of the same strain on tet2 medium. Further tests with 16 Tet^r mutants selected on tet2 plates indicated that their resistant phenotype was retained after subculture on tetracycline-free medium. Two of eight and five of eight mutants from cultures pregrown with 2 and 3 μg of metronidazole per ml, respectively, formed colonies on tet6 medium, albeit less rapidly and some 100- to 1,000-fold less efficiently than on tet2 medium; the other nine mutants grew well on tet2, only weakly on tet4 medium, and not at all on tet6 medium. Thus, intrinsic levels of tetracycline resistance varied among the new Tet^r mutants.

The sequence of the 0.7-kb segment near the 3′ end of 16S rRNA (containing the helix 31 loop and the helix 34 components of the primary tetracycline binding pocket) was determined as described above for 10 of the metronidazole-induced Tet^r mutants (5 that grew on tet6 medium and 5 that were less resistant, that is, that grew on tet2 medium and to some extent on tet4 medium). None of these Tet^r mutants contained changes in or near the primary tetracycline binding site or in any of the potential secondary binding sites (6, 32) in this 3′ half of the 16S rDNA sequence. The sequences of much of the other (5′) half of the 16S rRNA gene (which contains one arm of one of the secondary tetracycline binding sites [32]) were also determined from two of the most resistant mutants. Again, no sequence changes likely to affect tetracycline binding were found (GenBank accession numbers AF535194 to AF535198

TABLE 1. Level of tetracycline resistance[a] is determined by a combination of background genotype and 16S rDNA allele

Recipient strain	Tetracycline concn (μg/ml) allowing growth of strain[b]:		
	WT ($AGA_{965\text{–}967}$)	Lit76 ($gtA_{965\text{–}967}$)	Aus108 ($ttc_{965\text{–}967}$)
Ind75	1.5	8	32
WT 26695	0.5	8	16
Tet^r 26695 (non-rDNA)	6	16	64

[a] Tetracycline resistance levels were difficult to define with precision, because increases in tetracycline levels resulted in progressive decreases in colony size and efficiency of colony formation, as detailed in Materials and Methods.

[b] The tetracycline concentration that still allowed pretty good growth. The levels tested by efficiency-of-plating assays were 0 0.5, 1.5 or 2, 4, 6, 8, 12, 16, 32, 64 and 128 μg of tetracycline per ml. The resistance levels given here are the highest levels that cause no more than a 10-fold decrease in efficiency of colony formation relative to that caused by the next highest level of tetracycline, as detailed in Materials and Methods. WT, wild type.

and AF535200). This implies that each of these 10 laboratory-generated Tet^r mutants had arisen as a result of changes in other (non-rDNA) genes.

One mutant that grew on tet4 medium, albeit very weakly, was transformed with PCR products containing the Lit76 (AGA to gtA) and Aus108 (AGA to ttc) alleles, and transformants that grew well on tet4 medium were selected and purified by restreaking them on fresh tet4 medium before analyses of their rDNA alleles. The gtA and ttc alleles each inactivate a *Bst*BI site (positions 959 to 965; TTCGAA to TTCGAg or TTCGAt due to the changes to AGA at positions 965 to 967). Restriction analysis of PCR-amplified 16S rDNAs from several representative transformants showed that the mutant alleles had indeed been acquired and that the transformants were homozygous in each case (data not shown). Representative transformants generated with Lit76 DNA grew weakly on tet32 medium but not on tet64 medium, whereas those generated with Aus108 DNA were slightly more resistant in that they grew on tet64 medium but not on tet128 medium (Table 1). The corresponding transformants of the ancestral 26695 wild-type strain grew only on tet12 medium but not on tet16 medium (strain Lit076) and on tet32 medium but not on tet64 medium (strain Aus108) (Table 1). Because these transformants were also more resistant than either parent, we infer once again that higher-level tetracycline resistance can be achieved through the additive effects of resistance mutations in 16S rDNA plus additional mutations at one or more unknown non-rDNA loci.

DISCUSSION

We have studied the mechanisms of tetracycline resistance, a trait with a potentially high degree of clinical significance that is exhibited by a small minority of *H. pylori* clinical isolates. In five of the six Tet^r isolates studied, resistance could be ascribed at least in part to one or two substitutions in a three-nucleotide loop region that forms part of tetracycline's primary binding site in the ribosome, positions 965 to 967 in 16S rRNA. Studies of isogenic strains generated by the transformation of mutant rDNA alleles into a uniform genetic background showed that single nucleotide substitutions conferred only weak resistance; that double substitutions conferred a somewhat higher level of resistance; and that the triple substitution, which others (42) had first analyzed, conferred high-level resistance, as expected. Thus, the strength of resistance seems proportionate to the severity of change (deformation) of tetracycline's primary ribosome binding site. None of the Tet^r clinical isolates had mutations in the other (helix 34) arm of this binding site, nor had specific mutagenesis by others (42) of the position in that arm that underlies most resistance in *P. acnes* (position G1058) yielded Tet^r *H. pylori*. The different outcomes in *H. pylori* and *P. acnes* may reflect subtle differences in ribosome structure. For example, given the importance of tetracycline's primary binding site for aminoacyl-tRNA binding to the ribosome (6, 32), perhaps no change in the helix 34 arm of *H. pylori* can significantly diminish tetracycline binding yet still allow ribosomes to act effectively in protein synthesis; or, given multivalent tetracycline binding, perhaps no single change in this arm alters tetracycline binding sufficiently to confer detectable resistance.

That other (non-rRNA) genes can also contribute to tetracycline resistance was indicated by findings that (i) the level of resistance conferred by rDNA alleles with single substitutions in the critical trinucleotide from positions 965 to 967 after transformation of strain 26695 was weaker than the resistance of parental Tet^r clinical isolates, (ii) one weakly resistant clinical isolate had no mutation in its 16S rRNA tetracycline binding site, and (iii) the rDNA sequences of laboratory-generated Tet^r mutant derivatives of strain 26695 were also unchanged from those of their Tet^s parent. In addition, introduction of mutant rDNA alleles into either the clinical isolate or a 26695 mutant with non-rRNA-based resistance resulted in higher-level tetracycline resistance than the level of resistance of either parent. The two types of resistance determinants (non-rRNA- and rRNA binding site-based resistance) are inferred to contribute to resistance by complementary mechanisms. Possible contributors to non-rRNA-based resistance include (i) various porin genes (1, 4, 30, 50); (ii) the putative *tetA* efflux gene, HP1165 (42), homologs of which are present in several cryptic *H. pylori* plasmids (13, 33); and (iii) several other putative efflux genes (*arcB* family HP0607, HP0969, and HP1329), which are unrelated to HP1165 (*tetA*) (42). Regardless of the exact nature of these determinants, their apparently low abundance in *H. pylori* clinical isolates would be explained if they decrease fitness and thus tend to be contraselected during human infection. More generally, we suggest that tetracycline resistance may be uncommon in *H. pylori* for three reasons: (i) the rarity of mobile DNA elements carrying auxiliary resistance genes; (ii) a need for multiple very specific nucleotide sequence changes in rDNA and/or other genes to achieve high resistance; and (iii) the effects of resistance mutations, whether in rDNAs or elsewhere, on bacterial fitness.

Our results illustrate that accumulations of genetic determinants with small individual effects can, in the aggregate, significantly affect *H. pylori* phenotypes. They suggest that mutant *H. pylori* strains with even slight tetracycline resistance are favored whenever inhibitory concentrations of tetracycline are encountered. This may occur repeatedly during typical multiyear chronic infections, especially in the many countries in which tetracycline use is not regulated by prescription. Because tetracycline is primarily bacteriostatic, resistance need not be very strong to be advantageous: any mutant whose growth is less inhibited by tetracycline than its parent's should tend to outgrow the parent whenever this drug is present, quite independent of the dose or the duration of treatment. Even slight inhibition of such mildly resistant mutants during subsequent exposures to tetracycline will foster the stepwise emergence of derivatives with higher-level resistance. This could be due to a series of additional mutations and/or to interstrain transfer of DNA with already formed resistance determinants. That resistance is nevertheless uncommon in current *H. pylori* populations suggests an additional mitigating force. In particular, as noted above, many resistance determinants may be deleterious when no tetracycline is present, although such effects are often overcome by yet additional compensatory mutations (3). Eventually, this series of adaptive mutations may culminate in strains that are sufficiently resistant to render tetracycline-based rescue therapy ineffective and that yet retain their virulence. This was probably the case with the triple mutant strain (strain Aus108 [AGA965ttc]) (42) that was isolated from an

elderly peptic ulcer patient after several failed tetracycline-based courses of anti-*H. pylori* therapy (28).

More generally, we suggest that quantitative genetic determinants, formally equivalent to those studied here, contribute importantly to bacterium-human host interactions and the great genetic diversity of *H. pylori* strains worldwide. We propose that the human gastric mucosa be considered a rugged evolutionary landscape (7), diverse and challenging to each strain that infects it. Any differences among us in traits that could be important to individual *H. pylori* strains (13) and any changes in gastric physiology caused by our host response to chronic infection (26) should each select for adjustments in bacterial phenotype. Many such adjustments will be quantitative and, due to an accumulation of genetic changes with small but additive individual effects, will result in an evolutionary tinkering equivalent to that leading to the emergence of tetracycline resistance.

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