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The *fdxA* Ferredoxin Gene Can Down-Regulate *frxA* Nitroreductase Gene Expression and Is Essential in Many Strains of *Helicobacter pylori*

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Very few examples of metabolic regulation are known in the gastric pathogen *Helicobacter pylori*. An unanticipated case was suggested, however, upon finding two types of metronidazole (Mtz)-susceptible strains: type I, in which *frxA* (which encodes a nitroreductase that contributes to Mtz susceptibility) is quiescent, and type II, in which *frxA* is well expressed. Here we report that inactivation of the *fdxA* ferredoxin gene (*hp277*) in type I strains resulted in high-level *frxA* expression (in effect, making them type II). However, *fdxA* null derivatives were obtained from only 6 of 32 type I strains tested that were readily transformed with an *frxA*::*aphA* marker. This suggested that *fdxA* is often essential. This essentiality was overcome in 4 of 20 strains by inactivating *frxA*, which suggested both that *frxA* overexpression is potentially deleterious and also that *fdxA* has additional, often vital roles. With type II strains, in contrast, *fdxA* null derivatives were obtained in 20 of 23 cases tested. Thus, *fdxA* is dispensable in most strains that normally exhibit (and tolerate) strong *frxA* expression. We propose that restraint of *frxA* expression helps maintain balanced metabolic networks in most type I strains, that other homeostatic mechanisms predominate in type II strains, and that these complex results constitute a phenotypic manifestation of *H. pylori*'s great genetic diversity.

Helicobacter pylori, the gram-negative pathogen implicated in peptic ulcer disease and gastric cancer, chronically infects more than half of all people worldwide and is one of the most genetically diverse bacterial species (for reviews see references 14, 19,and 38). Independent clinical isolates typically differ by 3% or more in DNA sequences of representative housekeeping genes (2, 20) and by 5% or more in gene content (4, 5, 46). Any two independent clinical isolates are usually distinguishable from one another by DNA fingerprinting (3) or by sequencing a few housekeeping genes (2, 20).

Inspection of the two fully sequenced *H. pylori* genomes had revealed homologs of only a few of the regulatory genes known from other bacterial species. This had suggested that *H. pylori* might actually be relatively inflexible in a conventional regulatory sense; that is, it is hard wired for its special gastric niche (5, 16, 52). It seemed, however, that *H. pylori* would achieve phenotypic flexibility and diversity through mutation (56), interstrain and interspecies gene exchange and recombination (30, 51, 52), and frameshift mutations in repetitive sequences, the hallmark of highly mutable contingency genes (47, 52). More-recent studies have demonstrated considerable regulation of gene expression in response to growth phase and environmental parameters, such as acidity and concentrations or availability of iron, nickel, and other metals, and have identified more than a dozen genes with regulatory activity (15). Another case of metabolic regulation was suggested by our studies of susceptibility to the clinically important anti-*H. pylori* drug metronidazole (Mtz) (27, 28, 50). Susceptibility results from the action of one or two related nitroreductases that each mediate conversion of Mtz from harmless prodrug to hydroxylamine, a bactericidal and mutagenic agent; RdxA, which is abundant in essentially all Mtz^s clinical isolates; and FrxA, which is present at only very low levels in most isolates (designated type I strains) but at higher levels in others (type II strains) (27, 28). RdxA and FrxA differ in substrate specificity (49), but their normal substrates, products, and roles (e.g., whether purely metabolic or protective against reactive nitrogen and oxygen metabolites that are produced in the host response to infection; see reference 40) are not known.

The two types of Mtz^s strains can be distinguished provisionally in a forward mutation assay. Typically, Mtz^r colonies are found at frequencies of about 10^{-4} in cultures of type I strains and are found at frequencies of $\leq 10^{-8}$ in cultures of type II strains. This reflects the need to inactivate just one gene (*rdxA*) rather than two genes (both *rdxA* and *frxA*) to achieve resistance (27, 28). Although *frxA* inactivation does not affect Mtz susceptibility when *rdxA* is functional, its inactivation in type I strains that are already mutated in *rdxA* usually increases resistance by about twofold (from 16 to 32 μg/ml). This illustrates that *frxA* is expressed, but only weakly, in type I strains. In accordance with this fact, *frxA* transcripts were detected by reverse transcriptase PCR (RT-PCR) in both type I and type II strains, but Northern blot analysis showed that they were abundant only in type II strains (27). In principle, the observed patterns of *frxA* expression might reflect differences in a regulatory site or in a *trans*-acting regulatory factor.

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Here we identify a ferredoxin gene (*fdxA*; *hp277* in the genome sequence) as a negative regulator of *frxA* gene expression and show that it is essential for many type I strains and that part of this essentiality can involve restraint of nitroreductase gene expression.

MATERIALS AND METHODS

***H. pylori* culture conditions.** *H. pylori* strains were grown on brain-heart infusion agar (BHI; Difco) supplemented with 7% horse blood, 0.4% Isovitalex, and the antibiotics amphotericin B (8 μg/ml) and trimethoprim (5 μg/ml) (this solution is referred to hereafter as BHI agar) (27). Mtz was added to this medium when needed at a concentration appropriate for the experiment, as detailed below. The plates were incubated at 37°C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Transformation (electroporation) was carried out as follows: exponentially growing *H. pylori* cells were harvested from BHI agar (approximately 10^8 to 10^9 cells), washed twice in 10% glycerol, and suspended in a final volume of 80 μl of 10% glycerol at 4°C. Five micrograms of purified plasmid DNA or PCR fragment was added to the cells for 1 min on ice. The suspension of cells and DNA was transferred to a prechilled 0.2mm-gap electroporation cuvette and was subjected to single-pulse electroporation with an initial voltage of 2.5 kV in a Bio-Rad Gene Pulser, spread on BHI agar, and incubated for 24 h at 37°C. The cells were then transferred to BHI agar containing chloramphenicol (Cam) (15 μg/ml), kanamycin (Kan) (20 μg/ml), or Mtz (8 μg/ml), as appropriate, and were incubated for 3 to 10 days, as needed, to select for transformants.

Determination of Mtz susceptibility and resistance. *H. pylori* cells growing exponentially on Mtz-free BHI agar were suspended in phosphate-buffered saline (PBS) buffer, a series of 10-fold dilutions of these cell suspensions was prepared, and 10 μl of each dilution was spotted on freshly prepared BHI agar containing various concentrations of Mtz (0, 0.2, 0.5, 1.5, 3, 8, 16, 32, 64, and 128 μg/ml) (essentially as described in references 27 and 28). The susceptibilities of strains to Mtz are described here in terms of MIC, defined operationally as the lowest of the Mtz concentrations listed above that reduces the efficiency of colony formation by at least 10-fold. When Mtz-resistant mutants were rare ($<10^{-6}$) and accurate estimates of these frequencies were needed, culture aliquots were spread directly on the surface of an entire plate of Mtz-containing BHI agar. We used this culture dilution protocol here because it is more sensitive and reliable than traditional standard agar dilution or Etest methods for studying Mtz susceptibility (MIC) in *H. pylori*, as detailed in reference 27.

***H. pylori* strains.** The *H. pylori* strains used here were Mtz^s clinical isolates from diverse parts of the world. Most have been studied previously in other contexts (27, 28, 31, 39). The type I (*frxA* quiescent) and type II (*frxA* expressed) Mtz^s *H. pylori* strains (as defined in references 27 and 28) that were used here and their origins are as follows. Type I strains included 26695 (from the United Kingdom) (52); TN2, GS3, HPK5, CPY6261, CPY6271, and CPY6311 (Japan); HUPB48, HUPB57, HUPB63, HUPB71, HUPB72, and HUPB77 (Spain); Lit11, Lit13, Lit50, Lit55, and Lit76 (Lithuania); Ind27, Ind66, Ind121, Ind92, Che5, and Che13 (India); HK192 (Hong Kong); and PeCan9a (Peru). Type II strains included SS1 (Australia); X47 (United States, mouse adapted, and ultimately from a domestic cat; also known as X47-2AL [18]); 88-3887 (United Kingdom, 26695-related) (29 and Fig. 1); 98QM3 (from domestic cat; D. Dailidiene, K. W. Simpson, and D. E. Berg, unpublished data); 2600, 2667, and 2714 (Texas) (32); Lit5-34, Lit28-1, Lit43, Lit66-1, Lit75-1, Lit102, Lit113, Lit119, Lit120, and Lit122 (Lithuania); Alas219, Alas381, and Alas10103 (Alaska); Ind31 (India); R10 (South Africa); and HK152 (Hong Kong).

Five of these strains merit special comment. Strain 26695 (52) is nonmotile due to a frameshift mutation in the *fliP* flagellar assembly gene and had come from an initially mixed culture that also contained closely related motile cells, represented by strain 88-3887 (29). The relatedness of 26695 and 88-3887 is illustrated in randomly amplified polymorphic DNA (RAPD) fingerprint data (Fig. 1; no bands differed among some 25 scored in tests with four arbitrary primers). It was important for the present study that mutational tests suggested that 88-3887 expressed *frxA* (type II Mtz^s phenotype), whereas 26695 did not (type I). Most critically, inactivation of strain 88-3887's *rdxA* gene (transformation with an *rdxA*::*cat* allele and selection for Cam^r, as described in references 21, 27, and 28) left it vulnerable to Mtz: it formed colonies 10^3-fold and 10^6-fold less efficiently on medium with 8 and 16 μg of Mtz per ml, respectively, than on medium with no Mtz or only 3 μg of Mtz per ml (MIC = 8 μg/ml). In contrast, *rdxA* inactivation in strain 26695 allowed this strain to form colonies with 100% efficiency on medium with up to 16 μg of Mtz per ml (MIC = 32 μg/ml). Inactivation of both *rdxA* and *frxA* resulted in a MIC of 64 μg/ml for both strains.

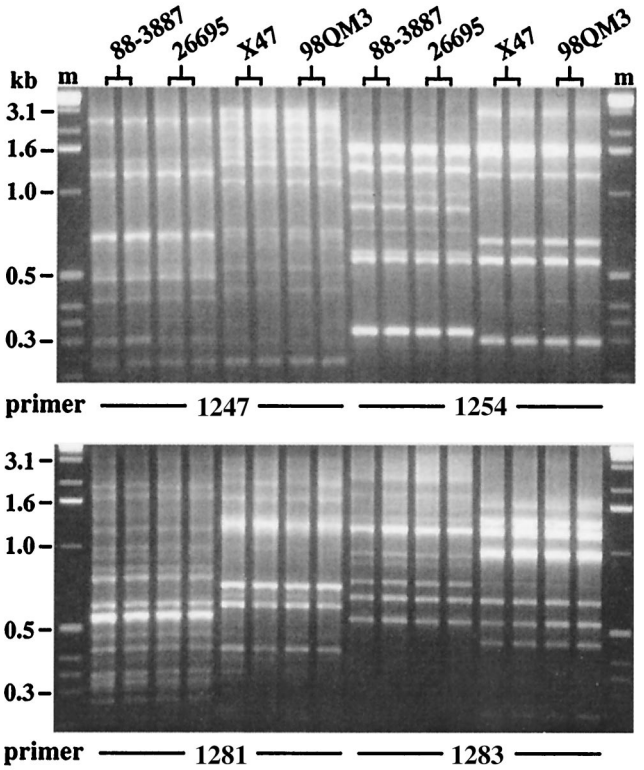

FIG. 1. RAPD analysis of relatedness. RAPD tests were carried out on the related strain pairs 88-3887 and 26695 and also on X47 and 98QM3, as discussed in the text, by using primers 1247 (left eight lanes), 1254 (top right eight lanes), 1281 (bottom left), and 1283 (bottom right) (3). The first and second lanes in each set contain products of duplicate RAPD tests, carried out with 5 and 20 ng of template DNA, to ensure that any differences seen are reproducible (or to learn when they are not). Lanes labeled m contain 1-kb marker size standards from Gibco-BRL.

These data showed that the residual Mtz susceptibility of *rdxA*-deficient 88-3887 involves its *frxA* gene, not some other function. We also note that *frxA* expression seemed weaker in 88-3887 than in several other type II strains in that *rdxA* inactivation in this strain did confer low-level Mtz resistance; it allowed colony formation with 100% efficiency on medium with 3 μg of Mtz per ml instead of just 1 or 1.5 μg per ml, as had been seen with SS1 and several other type II strains (27, 28). Also noteworthy are the type II strains X47 and 98QM3 because of differences in ease of *fdxA* inactivation (see below). Strain X47 derives from an *H. pylori* strain that had been isolated from a domestic cat and that was then adapted to mice by several sequential passages (18). 98QM3 was isolated from a member of the same cat colony some 5 years later by D. Dailidene, K. W. Simpson, and D. E. Berg and seemed closely related or identical to X47 in RAPD fingerprint (no bands differed among more than 25 scored in tests with four arbitrary primers; as in Fig. 1). Fifth, HUPB57 was considered type I because, even though *rdxA* inactivation resulted in an MIC of 64 μg/ml (higher than that seen for most strains after *rdxA* inactivation), inactivation of *frxA* as well as *rdxA* resulted in a higher level of resistance (MIC = 128 μg/ml).

DNA methods. *H. pylori* genomic DNAs were isolated from confluent cultures grown on BHI agar by using a Qiamp Tissue kit (Qiagen Corporation, Chatsworth, Calif.) or a standard cetyltrimethylammonium bromide phenol method (4). RAPD fingerprint analysis was carried out essentially as described previously (3) in 25-μl reaction mixtures containing either 5 or 20 ng of genomic DNA (to assess reproducibility of patterns), 5 mM $MgCl_2$, 20 pM concentrations of a given primer, 0.25 mM concentrations of each deoxynucleoside triphosphate, and 1 U of Biolase thermostable DNA polymerase (Midwest Scientific) in a solution containing 10 mM Tris-HCl (pH 8.3) and 50 mM KCl under the following cycling conditions: 45 cycles of 94°C, 1 min; 36°C, 1 min; and 72°C, 2 min. Gene-specific PCR was carried out in 20-μl volumes containing 1 to 10 ng of genomic DNA, 10 pmol of each primer, 1 U of Biolase, and 0.25 mmol of each deoxynucleoside

TABLE 1. Primers used[a]

Primer	Sequence	Position of 5′ end
RAPD		
1254	5′-CCGCAGCCAA	
1281	5′-AACGCGCAAC	
1283	5′-GCGATCCCCA	
1290	5′-GTGGATGCGA	
Gene-specific		
fdxA-F1	5′-CGCTTGTTCAAGGCTCTGATG	250 bp upstream of *hp277* (*fdxA*)
fdxA-R1	5′-CGCTACAAACTCCAGCCGATT	300 bp downstream of *hp277*
fdxA-F2	5′-GCCTCGTTGCGTGAGCGTAT	144th nt of *hp277* (*fdxA*)
fdxA-R2	5′-CGCACGCAATGCATTCATCA	18th nt of *hp277*
frxRT-F	5′-GGACAGAGAACAAGTGGTTGCTT	3rd nt of *hp642* (*frxA*)
frxRT-R	5′-GCGAACCTAGAATTAGTGTCAT	319th nt of *hp642*
rdxART-F	5′-GCATGCTGTGGTTGAATCTCAC	367th nt of *hp954* (*rdxA*)
rdxART-R	5′-CGAGCGCCATTCTTGCAAGATGT	42nd nt of *hp954*
ureB-F	5′-CGTCCGGCAATAGCTGCCATAGT	781st nt of *hp072* (*ureB*)
ureB-R	5′-GTAGGTCCTGCTACTGAAGCCTTA	340th nt of *hp072*

[a] The genes are listed according to their numerical designations in the genome sequence database of strain 26695 (52). nt, nucleotide.

triphosphate in standard PCR buffer. Gene-specific PCR entailed 2 min of preincubation at 94°C followed by 30 cycles of 94°C, 40 s; 58°C, 40 s; and 72°C for 1 min per kilobase pair, plus a final elongation step of 72°C for 10 min. The genetic structures of transformants were checked by PCR to verify that they had resulted from allelic replacement by using primers *fdxA*-F and *fdxA*-R, *rdxA*-F and *rdxA*-R, or *frxA*-F1 and *frxA*-R1, as appropriate (see Table 1 for primer sequences).

Mutant alleles used in strain construction. The *rdxA*::*cat*, *rdxA*Δ111, and *frxA*::*aphA* alleles used to generate *rdxA* and *frxA* null mutant strains by DNA transformation and selection for transformants by resistance to chloramphenicol, metronidazole, and kanamycin, respectively, have been described previously (27, 28). An *fdxA* null allele was generated as follows: (i) PCR was used to amplify an 828-bp *fdxA*-containing DNA fragment from strain 26695 with primers *fdxA*-F1 and *fdxA*-R1 (Table 1); (ii) this fragment was cloned into a pBluescript plasmid vector (Stratagene); (iii) the resultant clone was linearized by PCR with primers *fdxA*-F2 and *fdxA*-R2 to delete 126 bp of *fdxA*; (iv) ligation of the linearized clone DNA was performed with a minimal *cat* cassette (44); and (v) plasmids containing *cat* cassette inserts were selected and PCR was used to identify one in which *cat* and *fdxA* are in the same orientation. This *fdxA*::*cat* DNA was used to generate *fdxA* null *H. pylori* strains by DNA transformation. It is important to note that the stem-loop structure that is just downstream of the open reading frame in many *cat* cassettes has been removed here. This cassette is considered nonpolar on distal gene expression, because its insertion between DNA segments encoding the β and β′ domains of the large β-β′ RNA polymerase subunit (normally fused in *H. pylori*) does not impair growth (44). An *fdxA*::*aphA* insertion allele that is probably polar on distal gene expression, because *fdxA* and *aphA* are in opposite orientations, was generated similarly by using the *aphA* cassette from the *frxA*::*aphA* allele.

Measurement of survival in stationary phase. Concentrated suspensions of *H. pylori* cells that had been growing exponentially as overnight cultures on BHI agar medium were prepared in PBS buffer (about 2×10^9 cells per ml), and 20 μl (~4×10^7 cells) was spread uniformly on the surface of fresh BHI agar (150-mm-diameter petri plate). The viability of this initial inoculum on each day, beginning at day three, was determined by suspending aliquots of confluent bacterial growth from these plates in PBS, measuring the optical density, and determining viable counts by quantitative culture (CFU per optical density unit).

RT-PCR analysis of mRNA levels. Exponentially growing *H. pylori* strains were spread on BHI medium alone or with Mtz (0.2 μg/ml for SS1; 1.5 μg/ml for 26695). Following 2 days of incubation, bacterial cells were collected and total RNA was prepared by using a Qiagen RNeasy kit, as recommended by the manufacturer (Qiagen Corp). After elution from the RNeasy column, the RNA was treated with RNase-free DNaseI, extracted twice with phenol:chloroform, and extracted once with chloroform-isoamyl alcohol. It was then precipitated with ammonium acetate (final concentration of 2.5 M) and 2.5 volumes of ice-cold ethanol, washed in 75% ethanol, and resuspended in RNase-free water. The integrity of the 16S and 23S rRNA was checked on a 1% agarose gel. Genomic DNA contamination was checked by PCR with *Taq* DNA polymerase without RT. RT-PCR was carried out by using the One-Step RT-PCR kit (Gibco-BRL) and primers *frxRT*-F and *frxRT*-R (for *frxA* mRNA), *rdxRT*-F and *rdxRT*-R (for *rdxA* mRNA), and *ureB*-F and *ureB*-R (for *ureB* mRNA). RT-PCR was carried out in a volume of 50 μl in a Perkin-Elmer GeneAmp PCR system 2400 thermal cycler with the following conditions: 50°C for 20 min; 94°C for 2 min; and then 35 cycles of 94°C for 15 s, 58°C for 30 s, 72°C for 40 s, with a final incubation at 72°C for 10 min.

RESULTS

The *fdxA* gene (*hp277* in the strain 26695 genome sequence [52]) encodes a [Fe_4 S_4]-type ferredoxin (5, 52), a carrier of reducing equivalents, that may variously accept, donate, shift, and/or store electrons in key steps in central metabolism (8). The possibility that this protein's redox potential might be low enough to reduce Mtz and thereby convert it from prodrug to bactericidal agent motivated our interest in testing whether *fdxA* inactivation affected Mtz susceptibility. Others (32) had also sought to study *fdxA* but were not able to obtain *fdxA* null mutant transformants in their laboratory strain (2600). They therefore concluded that *fdxA* is essential for viability (32). The *cat* resistance gene cassette they had inserted into *fdxA* was probably polar on distal gene expression (44); however, we suspected that one or more of the genes downstream of and probably cotranscribed with *fdxA*, which were annotated as guanosine pentaphosphate pyrophosphatase (*hp278*), lipopolysaccharide heptosyltransferase (*hp279*), and lipid A acyltransferase (*hp280*) (5, 52), might be essential. Accordingly, we decided to reexamine *fdxA*'s importance for *H. pylori*.

A nonpolar *fdxA* null allele was generated in cloned DNA by replacing much of the *fdxA* sequence with a chloramphenicol resistance determinant (*cat*) that lacks transcription pause sites (44), and the resulting *fdxA*::*cat* DNA was used to transform the type I reference strain 26695. Hundreds of Cam^r transformant colonies were obtained, as is typical with other DNAs and this strain, although the colonies were slow growing (4 days, instead of 3, were needed to detect them by eye). PCR with *fdxA*-specific primers confirmed that these Cam^r transformants had resulted from replacement of a wild-type allele with the *fdxA*::*cat* allele (data not shown). Equivalent slow-growing *fdxA* null transformants were also obtained with the type II

TABLE 2. Efficiency of colony formation on Mtz-containing medium[a]

Strain	Mtz^r frequency
26695 wild type (type I)	$\sim10^{-4}$
26695 *rdxA*	~1.0
26695 *frxA*	$\sim10^{-4}$
26695 *fdxA*	$<10^{-8}$
26695 *fdxA rdxA*	$\sim10^{-4}$
26695 *fdxA frxA*	$\sim10^{-4}$
26695 *fdxA rdxA frxA*	~1.0
SS1 wild type (type II)	$<10^{-8}$

[a] Efficiency of colony formation on BHI agar containing 8 μg of Mtz per ml. When this efficiency is less than 1.0 (which implies that the strain is fully resistant to Mtz), this frequency generally corresponds to frequencies of new Mtz^r mutants, generated and selected by the mutagenic and bactericidal properties of Mtz once activated.

mouse-adapted strain SS1. In contrast, attempts to generate *fdxA*-deficient derivatives of these strains with a different null allele, *fdxA*::*aphA*, which is probably polar on distal gene expression because *fdxA* and *aphA* are in opposite orientations, were unsuccessful (no transformant colonies were scored as ≤0.01% of normal yield), presumably because of polarity on distal gene expression. We conclude that *fdxA* is not essential for viability in strains 26695 or SS1, although it probably contributes to the vigor of their growth.

Three sets of results indicated that *fdxA* inactivation increased nitroreductase gene expression in strain 26695. First, Mtz^r mutants were found in cultures of *fdxA* null derivatives of strain 26695 at frequencies of $\leq10^{-8}$ (Table 2). This contrasts with a frequency of about 10^{-4}, which is characteristic of 26695 wild type, in which just one gene (*rdxA*) needs to be inactivated to achieve Mtz resistance (27, 28, 50). Second, 26695 derivatives with null alleles of *rdxA* and *fdxA* (*frxA* functional) or of *frxA* and *fdxA* (*rdxA* functional) each remained Mtz^s but gave rise to Mtz^r mutants at frequencies of about 10^{-4} rather than $\leq10^{-8}$. A triple mutant, containing null alleles of both *rdxA* and *frxA* as well as of *fdxA* (*rdxA*Δ111, *frxA*::*aphA*, *fdxA*::*cat*), was Mtz^r (Table 2). Thus, inactivation of *frxA* as well as *rdxA* was needed to render the *fdxA* null derivative of 26695 resistant to Mtz. Third, RT-PCR indicated that *fdxA* inactivation increased the *frxA* mRNA level about fivefold relative to that of a *ureB* internal standard (Fig. 2). The *rdxA* transcript level also seemed to be increased about twofold in the *fdxA*-null derivative, which suggested that *fdxA* might help regulate both nitroreductase genes. In contrast, *fdxA* inactivation in SS1, which normally expresses *frxA* at a high level, did not affect *frxA* or *rdxA* mRNA levels (Fig. 2). Further tests revealed similar mRNA levels in cultures grown with sublethal levels of Mtz (see the legend of Fig. 2). Collectively, these outcomes support the view that the nitroreductase gene expression level inferred from Mtz susceptibility patterns reflects bacterial genotype (*fdxA* status) per se but not induction of gene expression by Mtz or the cellular damage that it causes.

It is noteworthy that the *rdxA frxA* double mutant derivative of 26695 was fully resistant to 32 μg of Mtz per ml (100% efficiency of colony formation), whereas the isogenic *rdxA frxA fdxA* triple mutant exhibited full resistance only up to 16 μg of Mtz per ml in each of three independent trials (Fig. 3). The quantitative differences in efficiency of colony formation by double and triple mutants on plates with the critical 32 μg of Mtz per ml were seen when cells of each strain were spotted on the same plate. The greater susceptibility of the triple mutant might reflect either increases in other enzymes that also activate Mtz (28) or a nonspecific effect of the *fdxA* mutant's less vigorous growth.

***fdxA* is essential in most but not all type I Mtz^s strains.** The generality of findings with strains 26695 was tested by using 31 additional transformable strains that had been classified as type I by forward mutation tests (see Materials and Methods). Expected yields of transformants (generally at least hundreds of Cam^r colonies) were obtained with only two of them (Ind121 and HUPB57) (Table 3). Mutational tests showed that they each resembled 26695 in that *fdxA* inactivation in them caused a decrease in the Mtz^r mutant frequency from approximately 10^{-4} to $<10^{-8}$ and a need to inactivate both *frxA* and *rdxA*, rather than only *rdxA*, to achieve Mtz resistance. Thus, *fdxA* inactivation seemed to have turned on *frxA* expression in these two type I strains.

Just a few Cam^r transformants were also obtained from 3 of the other 29 type I isolates (Lit055, CPY6271, and HUPB48), yields that were, in each case, less than 1% of those obtained with *frxA*::*aphA* DNA used as an internal control in the same transformation mixes. PCR tests indicated that some of these rare Cam^r transformants still retained the wild-type *fdxA* allele, suggesting that the *fdxA*::*cat* DNA had been inserted at another locus, perhaps by an illegitimate (mutation-like) recombination event (data not shown). However, at least one exceptional Cam^r transformant of each lineage was found by PCR to contain *fdxA*::*cat* in place of the resident wild-type *fdxA* gene, and these transformants were studied further. Inactivation of *fdxA* in these three strains had also, in each case, caused reduction in frequencies of Mtz^r mutants in young cultures from $\sim10^{-4}$ to $\leq10^{-8}$. The Mtz^r mutant frequencies were increased

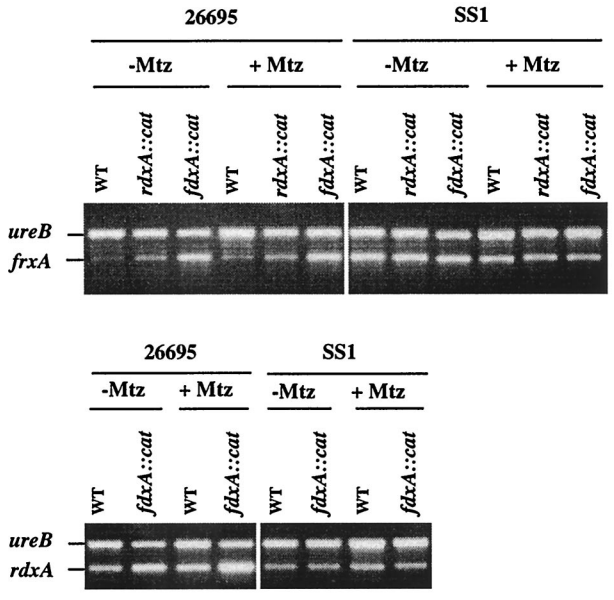

FIG. 2. RT-PCR analysis of mRNA levels. *H. pylori* cells were grown, RNA was extracted, and RT-PCR was carried out as detailed in Materials and Methods. Threshold deleterious levels of Mtz were included in BHI agar where indicated (0.2 μg/ml for strain SS1; 1.5 μg/ml for strain 26695). WT, wild type.

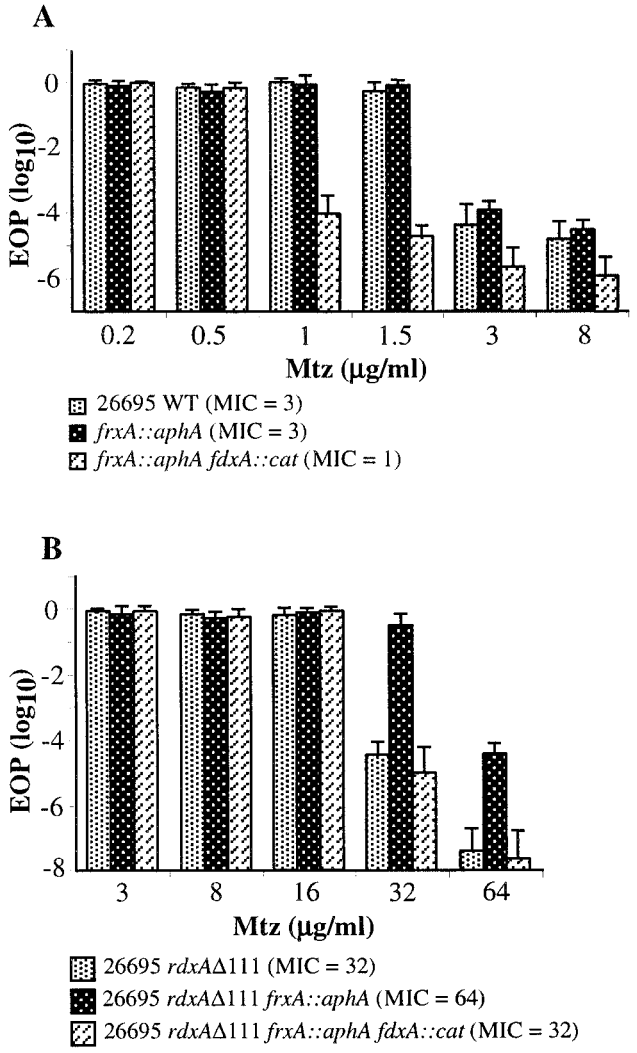

FIG. 3. Profiles of susceptibility to Mtz of strain 26695 wild type (WT) and isogenic mutant derivatives of it. Each test was carried out at least three times.

again (to $\sim 10^{-4}$) by further derivatives in which *frxA* had been inactivated. This indicated that *fdxA* also helped down-regulate *frxA* expression in these three exceptional type I strains.

No Cam^r transformants of any of the other 26 type I strains were obtained in repeated transformations with *fdxA*::*cat* DNA. In each case, the use of *frxA*::*aphA* DNA as a parallel control or as an internal control in the same transformation mix resulted in 100 or more Kan^r transformants (Table 3), and PCR tests showed that each strain did contain an *fdxA* gene. Thus, *fdxA* seemed to be essential in most type I strains. It also seemed that this essentiality could be relieved by suppressor mutations in at least some strains (Lit055, CPY6271, and HUPB48). The ease of generating *fdxA* null derivatives of strains 26695, Ind121, and HUPB57 suggested that possibly equivalent suppressors already preexist at low frequency in natural populations.

***fdxA* is dispensable in most type II Mtz^s strains.** Replacement of resident *fdxA* alleles with the *fdxA*::*cat* null allele was achieved readily in 20 of 23 type II strains tested (Table 3), although in each case *fdxA* null transformant colonies grew less rapidly than did colonies of their *fdxA*-proficient parents. Among the type II strains in which *fdxA* was readily inactivated were (i) strain 2600, in which *fdxA*-null derivatives had first been sought but without success (32), probably because of transcription polarity (noted above); (ii) 88-3887, a 26695-related strain that, remarkably, is type II in its Mtz-susceptible phenotype (26695 is type I); and 98QM3, but not X47, which is closely related to 98QM3 (see Fig. 1 and Materials and Methods).

No Cam^r transformant colonies were obtained from 3 of 23 type II isolates in repeated trials with *fdxA*::*cat* DNA (nonpolar null allele) (X47, a North American isolate, HK152 from Hong Kong, and R10 from South Africa), despite obtaining many Kan^r transformants with *frxA*::*aphA* control DNA. Each of these three strains was retested and was confirmed as type II; their Mtz^s phenotypes were changed from stable ($\leq 10^{-8}$ Mtz^r) to metastable ($\sim 10^{-4}$ Mtz^r) by inactivation of either *rdxA* or *frxA*, and they became Mtz^r if both *rdxA* and *frxA* were inactivated. Among these *fdxA*-requiring strains was X47, which is remarkable because *fdxA* null derivatives of the closely related strain 98QM3 were readily obtained. This indicates that small differences in background genotype may determine whether *fdxA* is essential or not.

Premature death in stationary phase. *H. pylori* cells began dying soon after reaching stationary phase. Although death in

TABLE 3. Efficiency of recovery of *fdxA*::*cat* (null) transformants depends on bacterial genotype

Frequency of recovery of *fdxA* null transformants	Fraction of strains	Examples
Type I strains		
Readily obtained[a]	3 of 32	26695, Ind121, HUPB57
Obtained with difficulty[b]	3 of 32	Lit055, CPY6271, HUPB48
Obtained only after *frxA* inactivation	4 of 20	Ind27, Ind66, Chen13, Lit050
Not obtained after *frxA* inactivation[c]	16 of 20	Various[d]
Obtained only after *frxA* and *rdxA* inactivation	1 of 2	HK192[e]
Type II strains		
Readily obtained	20 of 23	SS1, 98QM3, 88-3887 and others[f]
Not obtained	3 of 23	X47, HK152, R10

[a] Readily obtained indicates that the yield of Cam^r *fdxA* null transformants was similar to that of Kan^r *frxA* null transformants generated with *frxA*::*aphA* DNAs in the same transformation mix.

[b] Obtained with difficulty indicates that the yield of Cam^r *fdxA* null transformants was <1% that of Kan^r *frxA* null transformants generated in the same transformation mix. In these cases, some Cam^r transformants did not contain allelic replacements of the wild-type *fdxA* allele by the null mutant allele and thus may have resulted from illegitimate recombination or some other mutation event.

[c] Not obtained also indicates that the yield of Cam^r *fdxA* null transformants was <1% that of Kan^r *frxA* null transformants generated in the same transformation mix. We do not know if the not obtained class differs from the obtained with difficulty class.

[d] These strains were TN2, GS3, HPK5, CPY6261, and CPY6311 (Japan); HUPB63, HUPB71, HUPB72, and HUPB77 (Spain); A-11, A-13, and O76 (Lithuania); Ind92 and Chen5 (India); HK192 (HongKong); and PeCan9a (Peru).

[e] Incorporation of *frxA*::*aphA* and *rdxA*Δ111 null mutations into HK192 allowed recovery of slow-growing *fdxA* null transformants in high yield, whereas incorporation of these mutations into PeCan9a did not allow *fdxA* null transformants.

[f] These additional strains were Lit5, Lit28-1, Lit43, Lit66-1, Lit75-1, Lit102, Lit113, Lit119, Lit120, Lit122, Alas219, Alas381, Alas10103, 2600, 2667, 2714, and Ind31.

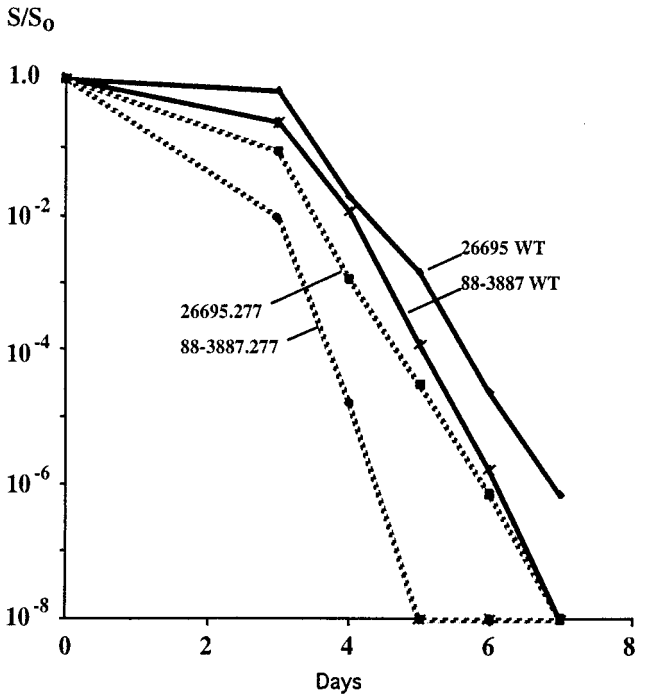

FIG. 4. Kinetics of death in stationary phase. Young exponentially growing cells were spread on BHI agar and were incubated. Aliquots were withdrawn daily, and efficiencies of colony formation, relative to culture optical density, were determined. WT, wild type.

stationary phase is not understood, we suspected that it might be accelerated by disturbance of metabolic networks. Accordingly, the survivals of the wild-type strains 26695 and 88-3887 were compared with those of their isogenic *fdxA* derivatives (Fig. 4). In each case, *fdxA* null derivatives tended to die more rapidly than their wild-type parents in stationary phase, but the kinetics of death seemed strain specific. By the time that the viabilities of wild-type 88-3887 and 26695 had declined to about 1% of the initial level, the viabilities of their *fdxA* null derivatives had declined another ~400-fold and ~20-fold, respectively (Fig. 4). This was seen in each of two independent trials, each with an independently constructed *fdxA* null transformant. The *fdxA* null derivative of the unrelated SS1 strain also died prematurely in stationary phase, with a severity similar to that of strain 26695 but not 88-3887 (data not shown). Given that 88-3887 and 26695 are closely related, it would seem that effects of *fdxA* on fitness can also be strongly affected by small differences in background genotype.

Lethality of *fdxA* inactivation can involve *frxA*. One explanation for *fdxA*'s essentiality in many type I strains and dispensability in most type II strains supposes that high FrxA nitroreductase levels are deleterious in strains of particular genotypes. This idea is based on indications that inactivation of *fdxA* caused increased *frxA* expression in at least some type I strains, that *fdxA* seemed to be essential in most type I strains, and that *fdxA* was dispensable in most type II strains. To test this idea, we made *frxA* null (*frxA*::*aphA*) transformants of 20 representative *fdxA*-requiring type I strains and transformed *frxA* null derivatives of each lineage with *fdxA*::*cat* DNA. Cam^r transformant colonies were obtained at normal frequency in four lineages (Ind27, Ind66, Chen13, and Lit050) (Table 3). In each case, the colonies obtained were much smaller and slower growing than those made by the parental (*frxA*-deficient but *fdxA*-functional) strain. PCR tests of two single-colony isolates and of pools of 20 to 50 transformants from each lineage demonstrated the expected allelic replacement (original *fdxA* allele by *fdxA*::*cat* allele) in each case. This suggests that *fdxA*'s essentiality in some type I strains reflects the ability of its gene product to down-regulate *frxA* gene expression. However, the inability to obtain *fdxA* null transformants of *frxA* null derivatives of 16 of these 20 selected type I strains and the slow growth of *fdxA* null transformants, when obtained, indicated that *fdxA* must have additional role(s).

DISCUSSION

***fdxA*, an often essential regulatory gene.** The possibility of metabolic regulation of nitroreductase gene expression in *H. pylori* emerged first with the finding of two types of Mtz^s clinical isolates: type I, in which *frxA* is relatively quiescent; and type II, in which *frxA* is highly expressed (27, 28). Here we report that (i) *fdxA* (*hp277*, ferredoxin gene) helped down-regulate *frxA* expression in some type I *H. pylori* strains; (ii) the *fdxA* gene was essential for viability in many of them; (iii) this essentiality reflected a need to restrain *frxA* expression, at least in some cases; and (iv) *fdxA* was dispensable in most type II strains (which naturally express *frxA*). The complexity of these results—the inability to predict with certainty how any one strain will behave based on findings with other strains—provides a new phenotype-level illustration of *H. pylori*'s great genetic diversity and may give insight into how this gastric pathogen evolves and interacts with its human hosts during long-term chronic infection.

The involvement of *fdxA* in down-regulating *frxA* expression was most evident in six type I strains that tolerated *fdxA* inactivation; in each case, this caused a need to mutate *frxA* (along with *rdxA*) to achieve Mtz resistance. The repeated failure to obtain *fdxA* null transformants of most other type I strains, however, indicated that FdxA was often essential. Although a failure to obtain *fdxA* null transformants of strain 2600 had also been interpreted as indicating *fdxA* essentiality (32), that particular result can now be ascribed to polarity on distal gene expression, because *fdxA* null derivatives of strain 2600 were readily generated here by using a nonpolar *fdxA*::*cat* allele.

The requirement for *fdxA* was overcome in 4 of 20 strains by inactivating their *frxA* genes. This suggested that FdxA protein also regulated *frxA* expression in these strains and that keeping *frxA* quiescent was an adaptive trait for them. Indications of additional roles for *fdxA* included our inability to obtain *fdxA* null transformants in most type I strains, even after making them *frxA* deficient; the slow growth of *fdxA* null transformants (although some of this might also be ascribed to residual polarity of the *cat* cassette used to inactivate *fdxA*); and their premature death in stationary phase. This additional role(s) may include carriage of reducing equivalents for multiple metabolic reactions and possibly also regulating expression of additional genes (8, 9).

In light of *fdxA*'s essentiality in most type I strains, it was striking that *fdxA* null derivatives were obtained in most type II strains, the class that normally exhibits (and tolerates) strong *frxA* expression. This might reflect either (i) the presence in

them of genes with equivalent or compensatory functions (e.g., additional ferredoxins) and their absence from most type I strains or (ii) the presence in them of naturally occurring suppressor mutations, perhaps equivalent to the suppressors invoked above to explain the rare *fdxA* null transformants of three type I strains (Lit055, CPY6271, and HUPB48). An illustration that rather small differences in background genotype might determine whether *fdxA* is essential or not was provided by studies of two closely related strains, X47 (*fdxA* requiring) and 98QM3 (*fdxA* independent) (Fig. 1).

Consequences of increasing *frxA* expression and *fdxA* inactivation. Although the normal role of FrxA nitroreductase is not known (e.g., whether it is strictly metabolic or protective against reactive metabolites produced in the host response to infection), we suggest that the detrimental effect of excess *frxA* expression in *H. pylori* strains of certain genotypes stems from changes in metabolite pools. In one model, excess FrxA might cause a potentially injurious metabolite to accumulate to toxic levels, analogous to that seen with 2-ketobutyrate, sugar phosphates, and 3′-phosphoadenoside 5′-phosphosulfate (PAPS) in certain mutant strains of enteric bacteria (33, 34, 41). In an alternative model, excess FrxA might cause depletion of a critical intermediate or end product, analogous to starvation variously for succinyl-coenzyme A, caused by excess glutamate dehydrogenase and a resultant siphoning of most alpha ketoglutarate into glutamate synthesis (26); or for several serine-derived metabolites, caused by excess serine deaminase and a resultant siphoning of most serine into pyruvate synthesis (6). In our experiments, the tolerance of high nitroreductase levels in most type II strains and a few type I strains might stem from differences in levels of other metabolic enzymes that, in a toxicity model, consume the metabolite, interfere with its synthesis, or produce an antidote or that, in an intermediate depletion model, increase flux through undersupplied pathways or activate alternative modes of synthesis of the essential end product. Such flexibility, the compensation of deleterious effects of one metabolic alteration by changes in other metabolic functions, is a familiar theme in traditional biochemical genetics (see, for example, references 6, 10, 11, and 26).

How FdxA might act. Two models for FdxA-mediated downregulation of *frxA* expression seem attractive. One invokes a ferredoxin-mediated effect on a metabolite that itself is regulatory; e.g., ferredoxin-dependent synthesis of a corepressor or consumption of an inducer. A second model envisions direct action of FdxA itself and is suggested by studies of the FdI ferredoxin of *Azotobacter vinelandii*. This ferredoxin interacts with a pyruvate dehydrogenase subunit and enables it to bind the *fpr* promoter and block *fpr* gene transcription (45). Other useful precedents include various larger iron-sulfur proteins, such as SoxR, aconitase-iron regulatory protein, and IscR, which bind specific RNA or DNA sequences or participate in protein-protein interactions in reactions that also depend critically on oxidation states and/or iron binding to their iron-sulfur centers and that thus can respond sensitively to environmental and intracellular cues (9, 48).

Evolutionary inferences. The complexity among *H. pylori* strains of patterns of *fdxA* essentiality and *frxA* regulation illustrates, at the phenotypic level, *H. pylori*'s extraordinary genetic diversity. Much of this diversity may reflect accumulation of numerous genetic differences, many of which may have quantitative effects on metabolite flux in one or more biochemical pathways. The following possible sources of this diversity have been much discussed: general mutation (56), frameshifts in repetitive sequences in contingency genes (47), and recombination within and between strains (24, 29, 51, 52). We suggest that the present level of diversity also reflects several additional features: (i) *H. pylori*'s mode of transmission, which is preferentially intrafamilial and occurs efficiently in early childhood (7, 13, 23, 36); (ii) the tendency of infections to persist for decades; and (iii) the rarity of new infections in adulthood (1, 37, 55). These three features create a highly fragmented bacterial population and diminish competition among strains from unrelated persons and selection for any one or few potentially most-fit genotypes (selective sweeps [22]). These features would promote genetic drift even if all people were identical physiologically. Given human diversity in traits that may be important to individual *H. pylori* strains (17, 24, 35, 43), we also imagine that at least subtly different phenotypes may be selected in different infected people. These features of *H. pylori* and of human populations create, in effect, rugged evolutionary landscapes (12, 53, 57). The chance of ingestion, especially in infancy, as much as any near-ideal match between bacterial genotype and particular host physiology may dictate which *H. pylori* strain becomes established in any new human host. This feature should often result in selection for adaptive changes that make each infecting strain better suited for its present host. The selection for adaptive changes may continue for years, in part because gastric physiology changes with age and in response to chronic infection. Adaptation will often involve many small steps and operate along different trajectories in different strains and infected people (12, 43, 54, 57)—Jacob's concept of evolution by tinkering (25). The resultant constellations of quantitative trait determinants should, in turn, affect the chance that a given strain will productively infect a particular human host and the chance that persistent infection will lead to overt disease.

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