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Metabolic Activities of Metronidazole-Sensitive and -Resistant Strains of *Helicobacter pylori*: Repression of Pyruvate Oxidoreductase and Expression of Isocitrate Lyase Activity Correlate with Resistance

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In this study, we compared metronidazole (Mtz)-sensitive and -resistant strains of *Helicobacter pylori* for metabolic differences that might correlate with drug resistance. Included in this study was an isogenic Mtz^r strain, HP1107, that was constructed by transforming genomic DNA from Mtz^r strain HP439 into Mtz^s strain HP500. Enzyme activities were also measured for Mtz^r strains grown in the presence or absence of 18 μg of metronidazole per ml (ca. one-half of the MIC). These studies confirmed the presence of the Embden-Meyerhof-Parnas, Entner-Doudoroff, and pentose pathways. *H. pylori* strains expressed enzymatic activities indicative of a complete and active Krebs cycle. All strains expressed pyruvate oxidoreductase (POR) and α-ketoglutarate oxidoreductase (KOR) as measured with the redox-active dye benzyl viologen (30 to 96 nmol/min/mg of protein for POR and 30 nmol/min/mg of protein for KOR). When grown in the presence of Mtz at ≥3.5 μg/ml, Mtz^r strains expressed no detectable POR or KOR activity. The apparent repression of POR and KOR activities by Mtz affected bacterial growth as manifest by extended lag periods and growth yield reductions of >30%. A dose-dependent relationship was demonstrated between the metronidazole concentration in the growth medium and the specific activity of POR measured in bacterial cell extracts. The observed repression was not due to inactivation of POR by Mtz. In addition to repression of POR and KOR activities, growth in the presence of Mtz also led to decreases in the activities of various Krebs cycle enzymes, including aconitase, isocitrate dehydrogenase, and succinate dehydrogenase. All of the Mtz^r strains examined expressed isocitrate lyase and malate synthase activities indicative of the glyoxylate bypass. No isocitrate lyase activity was detected in Mtz^s strain HP500. Isocitrate lyase activity was expressed by HP500 following transformation to Mtz resistance (Mtz^r strain HP1107) with DNA from an Mtz^r strain. The results of this study suggest that Mtz resistance may be a recessive trait, possibly involving inactivation of a regulatory gene, that results in constitutive expression of isocitrate lyase. Repression of POR and KOR activities in response to low levels of Mtz may be a general response of *H. pylori* strains to Mtz, but only resistant strains manage to survive via activation of compensatory metabolic pathways.

Microaerophilic bacteria exhibit a strictly respiratory form of metabolism and are generally restricted to environments of lowered oxygen tension (<21% O_2) (26). Survival in these nearly anaerobic environments may depend on metabolic features ordinarily found in anaerobic bacteria. For example, microaerophiles oxidize pyruvate via an anaerobic pyruvate:ferredoxin/flavodoxin oxidoreductase (POR) (7, 19, 38), rather than via an NAD-linked pyruvate dehydrogenase complex common to aerobes and facultative anaerobes. It is not known whether POR enables microaerophiles to survive periods of anaerobiosis by enabling broader utilization of alternative electron acceptors. However, microaerophiles, like anaerobes, are susceptible to the redox-active 5-nitroimidazole drugs (16, 31, 54). These drugs, when reduced by low-potential ferredoxins or flavodoxins in a four-electron transfer reaction, form short-lived, DNA-damaging hydroxylamine derivatives (22, 26, 29, 39, 54). In the presence of oxygen, oxidation of reduced metronidazole (Mtz) intermediates can generate biologically active superoxide anions (24, 39, 54).

In the case of *Helicobacter pylori*, a well-established cause of human gastritis and peptic ulcer disease (6, 32), nitroimidazole drugs are commonly included in the antimicrobial formulary employed for eradication of the infection (9, 57). However, resistance to Mtz is recognized as a major determinative factor in treatment failures (9, 11, 57). It is not known whether resistance occurs via spontaneous mutation or via acquisition of resistance genes through natural transformation. Both resistance mechanisms have been demonstrated in vitro (14, 58). Moreover, the mechanism of Mtz resistance is also unknown. Resistance might result from: (i) transport deficiency, (ii) drug modification or antiport, (iii) loss or modification of the biological target, (iv) increased activities of DNA repair enzymes, or possibly (v) enhanced oxygen radical scavenging capability. While Mtz is believed to enter cells via diffusion (39), recent studies indicate that uptake of Mtz by *H. pylori* requires an energized membrane (38). The uptake system was specific for related imidazole compounds and inhibited by KCN and uncoupling agents. However, differences in uptake between Mtz^s and Mtz^r strains did not correlate with drug resistance. Lacey et al. found that the viability of Mtz^r strains was decreased upon exposure to Mtz, even though less than 10% of the drug

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was utilized (27), suggesting that an adaptation period may be required for expression of resistance determinants. *Bacteroides* spp. expressing low-level Mtz resistance produce Mtz reductase (*nim* genes), which reduces the 5-nitro group to a nontoxic amino group (51). A similar reductase has not been reported in *H. pylori*, although there is evidence of decomposition of Mtz in bacterial extracts (38). In regard to loss of a biological target, studies with *Bacteroides* species demonstrate that a decrease or loss of POR activity correlates with high-level Mtz resistance (2, 40). Moreover, studies have shown that *Bacteroides fragilis* NCTC 11295 compensates for low POR activity by increasing the activity of lactate dehydrogenase (40). In the sexually transmitted protozoan *Trichomonas vaginalis*, Mtz resistance correlates with decreased levels of ferredoxin resulting from mutations in the promoter region of the structural gene (46). Hughes et al. (19) identified a four-subunit POR in *H. pylori* previously known to occur only in the hyperthermophilic arkaeons (25). Pyruvate-dependent reduction of flavodoxin, but not ferredoxin, was demonstrated in that study.

In the present study, we investigated the role of POR and α-ketoglutarate oxidoreductase (KOR) in the Mtz resistance of clinical isolates of *H. pylori*. Since so little is known regarding the metabolism of this microaerophilic human pathogen, we extended our studies to explore metabolic differences between isogenic Mtz^s and Mtz^r strains. For this study, we created isogenic Mtz^s and Mtz^r strains by transforming genomic DNA from an Mtz^r strain into an Mtz^s strain. The results presented herein establish that *H. pylori* is much more dependent on the Krebs cycle than was previously believed. Our studies also establish a correlation between Mtz resistance (both natural and transformed) and an ability to repress expression of the POR and KOR activities in the presence of low levels of Mtz coordinate with expression of compensatory metabolic activities, such as activation of the glyoxylate bypass.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Clinical isolates of *H. pylori* were obtained from the Victoria General Hospital, Halifax, Nova Scotia, Canada. The Mtz susceptibility of these isolates was determined by an E test (AB Biodisk, Sweden). All strains of *H. pylori* were maintained at −70°C in nutrient broth supplemented with 10% dimethyl sulfoxide. The bacteria were grown on brucella-based medium (Difco) supplemented with 2.5 to 5% pooled human serum. A biphasic system was used in which 20 ml of brucella agar formed a solid base in 75-cm^2 tissue culture flasks (Falcon) onto which 80 ml of brucella broth plus 2.5% human serum was layered. For some studies, various concentrations of Mtz were added (0.5 to 32 μg/ml). The flasks were initially incubated for 1 to 2 h in a microaerobic incubator (5% CO_2, 7% O_2, Forma) for gas equilibration and then incubated for 2 to 5 days on a rocking platform (Reliable Scientific, Memphis, Tenn.) placed in an aerobic incubator at 37°C.

Growth studies. The effect of increasing Mtz concentrations on the growth of *H. pylori* was determined in 250-ml side-armed flasks containing 100 ml of brucella broth supplemented with 5% human serum. The Mtz concentration ranged from 0 to 32 μg/ml. Optical density at 660 nm was measured in a Spectronic 20. The flasks were allowed to equilibrate in a microaerophilic incubator for 16 h prior to shaking on a gyratory shaker (New Brunswick) at 37°C.

Preparation of cell extracts. Bacteria were harvested at either 48 h (absence of Mtz) or 72 h (presence of Mtz) following microscopic examination to ensure a motile population devoid of coccoid bodies. Bacteria were pelleted by centrifugation (6,000 × *g*) for 5 min at 4°C, suspended in phosphate-buffered saline (4°C), and subjected to 15-s bursts from an ultrasonic probe (Branson Sonifier). Following five cycles of bursts interspersed with 1-min cooling periods in an ice bath, the crude extract was centrifuged at 10,000 × *g* for 30 min at 4°C to remove unbroken cells and debris. The supernatant was used immediately for enzymatic analyses. Some extracts were supplemented with 1 mM dithiothreitol during preparation to protect oxygen-sensitive enzymes.

Enzyme assays. All enzyme assays were carried out at 25°C in 1-ml volumes in a modified Cary-14 Spectrophotometer equipped with an OLIS data acquisition system (On Line Instrument Co., Bogart, Ga.) (23). All assays were performed in triplicate, and the means were computed from results obtained with replicate extracts. Enzymatic activities are reported as nanomoles per minute per milligram of protein. Protein determinations were done by using the Bradford procedure (Bio-Rad) with bovine serum albumin as the standard.

POR (EC 1.2.7.1) was assayed under anaerobic conditions with 75 mM potassium phosphate (pH 7.3), 10 mM sodium pyruvate, 5 mM benzyl viologen, 0.18 mM coenzyme A (CoA), and 5 μM thiamine PP_i. A few grains of sodium dithionite were added to render the cuvette anaerobic. The control cuvette contained no sodium pyruvate. Direct reduction of Mtz by POR was measured at 320 nm (5). Hydrogenase (EC 1.18.3.1) was assayed in a 1-ml cuvette containing 150 mM potassium phosphate buffer (pH 7.0) and 5 mM benzyl viologen and bubbled with hydrogen gas prior to assay (10). All benzyl viologen-based assays were done at 546 nm, and an extinction coefficient of 9.2 mM^{-1} cm^{-1} was used to determine specific activities. Formate dehydrogenase (EC 1.2.1.2) was also assayed with benzyl viologen as previously described (10). Malate dehydrogenase (EC 1.1.1.37) (60), isocitrate dehydrogenase (EC 1.1.1.41) (15), pyruvate dehydrogenase (EC 1.2.4.1), and α-ketoglutarate dehydrogenase (EC 1.2.4.1) (48) were assayed by monitoring the reduction (or oxidation) of NAD or NADP at 340 nm. Lactate dehydrogenase (EC 1.1.1.27) was assayed by monitoring the reduction of horse heart cytochrome *c* at 550 nm (10). Aconitate hydratase (aconitase) (EC 4.2.1.3) activity was assayed as described by Racker (47), and fumarate hydratase (fumarase) (EC 4.2.1.2) activity was determined by the method described by Massey (33). For these assays, cell extracts were prepared in buffer containing 1 mM dithiothreitol, as these enzymes were found to be particularly oxygen labile. Citrate synthase (EC 4.1.3.7) was measured by the method of Srere et al. (55) by monitoring the reaction of reduced CoA with 2-nitrobenzoic acid at 412 nm. Malate synthase (EC 4.1.3.2) was assayed with acetyl CoA and glyoxylate at 232 nm (49). Isocitrate lyase (EC 4.1.3.1) was measured by monitoring the reaction of phenylhydrazine with glyoxylate (49). The formation of the phenylhydrazine adduct was measured at 420 nm. An extract from *Azotobacter vinelandii* grown in an acetate minimal medium was used as a positive control for enzymes of the glyoxylate bypass. Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Veeger et al. (56) by monitoring the reduction of potassium ferricyanide at 455 nm.

Gluconeogenic enzymes, including pyruvate carboxylase (EC 6.4.1.1) (53), phosphoenolpyruvate (PEP) carboxykinase (EC 4.1.1.32) (4), and PEP carboxylase (EC 4.1.1.31) (45), were assayed by monitoring the oxidation of NADH in a coupled assay with excess malate dehydrogenase. Pyruvate kinase (EC 2.7.1.40) was assayed in a coupled reaction with excess lactate dehydrogenase (34). A coupled reaction was used to assay 6-phosphogluconate dehydratase (EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14), which contained an excess of lactate dehydrogenase (59). Hexokinase (EC 2.7.1.1) (21), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (43), and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) (44) were assayed by monitoring the reduction of NADP at 340 nm. Fructose 1,6-biphosphatase (EC 3.1.3.11) (52) and phosphoglucose isomerase (EC 5.3.1.9) (42) were assayed in a coupled reaction with glucose 6-phosphate dehydrogenase by monitoring the reduction of NADP. Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) was assayed by monitoring the reduction of NAD in a reaction containing aldolase and fructose 1,6-biphosphate as the substrate (1). Phosphofructokinase (EC 2.7.1.11) was assayed by a coupled reaction with triosephosphate isomerase, aldolase, and glycerol phosphate dehydrogenase by monitoring the oxidation of NADH in the presence of fructose 6-phosphate as the substrate (30). Aldolase (EC 4.1.2.13) was similarly assayed but with fructose 1,6-biphosphate in place of fructose 6-phosphate as the substrate (12).

Transformation of Mtz resistance determinants. Mtz^s strain HP500 was transformed with genomic DNA isolated from Mtz^r strain HP439. DNA was mixed with bacteria on a brucella agar plate in the absence of Mtz for 5 h under microaerobic conditions as described by Wang et al. (58). A loopful of bacteria was removed from the plate and streaked for isolation on brucella agar containing Mtz at 18 μg/ml. One of many Mtz^r colonies was picked and streaked onto brucella agar plates containing Mtz at 32 μg/ml. One colony was picked and designated HP1107. This strain was used in the enzymatic studies. To isolate spontaneous Mtz^r mutants of HP500, heavy suspensions (ca. 10^9 bacteria per ml) of bacteria were spread or spotted onto brucella agar plates containing a range of Mtz concentrations (6 to 18 μg/ml).

RESULTS

H. pylori strains were divided into Mtz^s and Mtz^r groups on the basis of E test results. The MICs for all of the resistant strains were >18 μg/ml, whereas strains for which the MICs were <6 μg/ml were considered sensitive. No MICs fell between these values. We first examined *H. pylori* isolates for POR activity, since studies with several anaerobic and microaerophilic bacteria had established that POR is involved in Mtz reduction (2, 8, 28, 31, 44). As seen in Table 1, cell extracts from Mtz^s strains generally exhibited higher POR specific activities (benzyl viologen reduction) than extracts from Mtz^r isolates grown in the absence of Mtz. However, when Mtz^r strains were grown in the presence of Mtz at 18 μg/ml, none of the strains contained detectable levels of POR. None of the strains examined in this study exhibited pyruvate:NAD dehy-

TABLE 1. POR activities of selected strains of *H. pylori*[a]

Strain	Mtz[b]	Mtz concn (μg/ml) in medium	POR sp act (nmol/min/mg of protein)
HP500	S	0	96
HP950	S	0	96
HP1134	S	0	180
HP1134R	R	20	0
HP934	S	0	37
HP439	R	0	30
HP439	R	28	0
HP439	R	18	0
HP1043	R	0	37
HP1043	R	18	0
HP1107	R	0	28
HP1107	R	18	0
HP1108	R	0	96
HP1108	R	18	0

[a] Extracts from *H. pylori* strains were assayed for POR activity with benzyl viologen as the electron acceptor.
[b] Mtz resistance (R), MIC of >32 μg/ml; Mtz sensitivity (S), MIC of <6 μg/ml.

drogenase activity. In this regard, *H. pylori* strains differ from *Campylobacter jejuni* and *C. coli* isolates, which appear to express both activities (10, 18). POR exhibited a requirement for thiamine PP_i and CoA. To determine whether POR is inactivated by Mtz, Mtz (0 to 100 μg/ml) was added to the benzyl viologen-based assay. Over this concentration range, Mtz, which has a much lower redox potential than benzyl viologen, exhibited no inhibitory effect on the reaction (Fig. 1). When Mtz was used as the electron acceptor in the POR assay for HP500 and HP439, only a very low rate of Mtz reduction was detected (<1 nmol/min/mg of protein). No further effort was made to optimize the Mtz-based POR assay or to evaluate various exogenous carriers (i.e., ferredoxin or flavodoxin). These results are consistent with numerous studies demonstrating that benzyl viologen is directly reduced by POR, whereas Mtz is indirectly reduced by low-redox carriers such as ferredoxin or flavodoxin (8, 28, 29). Therefore, the absence of POR activity in Mtz[r] strains grown in the presence of Mtz cannot be attributed to direct inhibition of POR by Mtz.

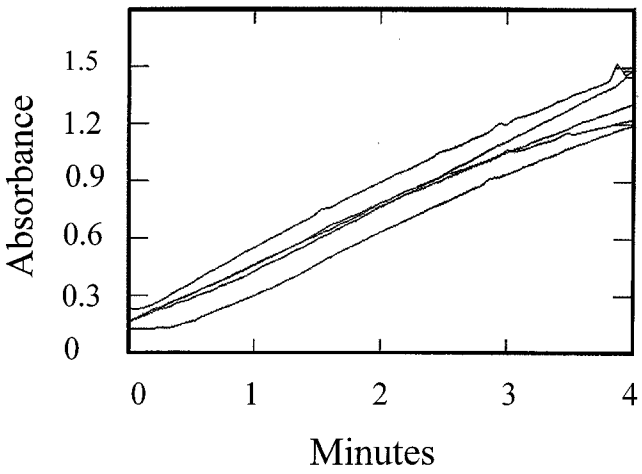

FIG. 1. Effect of Mtz concentration on POR enzymatic activity. Pyruvate-dependent reduction of benzyl viologen was monitored at 546 nm in crude extracts prepared from HP500. Mtz concentrations (bottom to top) were 0, 20, 40, 80, and 100 μg/ml. The tracings represent direct data compiled from individual runs. The slopes were all within 5% of the control.

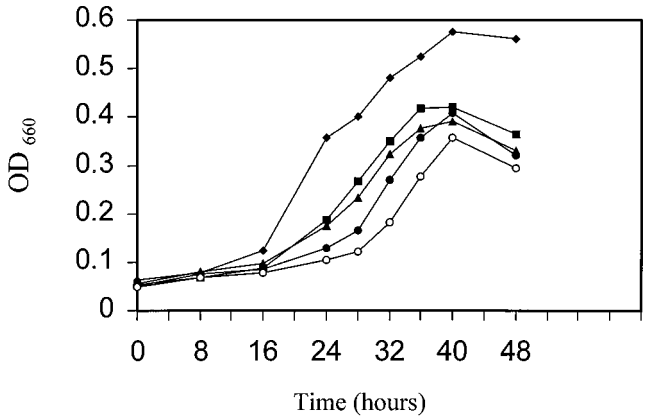

FIG. 2. Effect of Mtz on the growth of Mtz[r] *H. pylori*. The bacteria were grown under microaerobic conditions, as described in the text, in brucella broth containing Mtz at 0 (◆), 5 (■), 10 (▲), 18 (●), or 30 (○) μg/ml. OD_{660}, optical density at 660 nm.

Effect of Mtz concentration on growth. Mtz[r] strains grew more slowly in the biphasic system when Mtz (18 to 32 μg/ml) was present in the brucella medium. To investigate this apparent inhibitory effect, resistant strains were grown in brucella broth supplemented with various concentrations of Mtz. As seen in Fig. 2 for Mtz[r] strain HP439, both the lag phase and the maximum growth yield were dramatically affected in a dose-dependent manner by Mtz. Even in the presence of Mtz at 5 μg/ml, which is well below the MIC (>32 μg/ml), the lag phase was extended by nearly 10 h over that of the control. In exponential growth, the growth rates were not appreciably altered by the Mtz concentration. However, even at very low concentrations of Mtz, there was a 30 to 45% decrease in the maximum growth yield. The decreased yields were not due to oxygen limitation, since loosening the caps on the 250-ml sidearm flasks did not increase the growth yields (data not presented). The decreased growth yield was not strain specific, since low concentrations of Mtz also decreased the growth yields for Mtz[r] strains HP1043 and HP1107. The results of the growth studies suggested that Mtz either continues to exert a toxic effect on resistant strains or induces changes in their metabolic strategy that enfeebles them even in complex medium.

Dose-dependent effect of Mtz on POR activity. To determine whether decreased growth yields were attributable to an absence of POR activity, Mtz[r] strains were grown in the presence of various concentrations of Mtz and bacterial extracts were assayed for POR activity. As seen in Table 2, Mtz inhibited POR activity in a dose-dependent manner for Mtz[r] strain

TABLE 2. POR activity versus Mtz concentration[a]

Strain (phenotype)	Mtz concn (μg/ml)	POR sp act (nmol/min/mg of protein)
HP439 (Met[r])	0	50
	1.0	28
	3.5	0.3
	5.0	0
	18.0	0
HP500 (Met[s])	0	80
	1.0	16

[a] Bacteria were grown in biphasic brucella medium containing the indicated concentrations of Mtz. POR activity was measured as described in the text. The specific activities shown are averages of three determinations with <10% error.

TABLE 3. Activities of enzymes associated with glucose metabolism

No. in Fig. 3 and enzyme(s)	Mean sp act (nmol/min/mg of protein) (SEM)[a]				
	HP439		HP1107		HP500, no Mtz
	No Mtz	Mtz[b]	No Mtz	Mtz[b]	
1. Hexokinase	20.4 (0.1)	23.2 (3.1)	67.1 (0.3)	23.7 (0.1)	38.2 (1.7)
2. Phosphoglucose isomerase	21.8 (1.5)	7.4 (1.1)	78.8 (1.4)	38.5 (8.5)	NA[c]
3. Phosphofructokinase	1.4 (0.2)	6.5 (0.9)	NA	2.6 (0.7)	9.2 (0.7)
4. Fructose 1,6-biphosphatase	41.3 (5.9)	15.3 (0.7)	22.0 (1.6)	29.9 (0.8)	4.9 (0.1)
5. Aldolase	10.8 (1.7)	6.1 (0.9)	2.5 (0.6)	6.5 (2.7)	40.3 (1.0)
6. Glyceraldehyde 3-phosphate dehydrogenase	7.7 (0.8)	2.1 (0.6)	2.5 (0.2)	NA	11.2 (0.6)
7. Pyruvate kinase	2.6 (0.6)	2.5 (0.5)	1.0 (0.2)	2.2 (0.3)	13.4
8. Pyruvate:ferredoxin oxidoreductase	30.0 (1.0)	0	28.6 (2)	0	96.0 (5)
9. Glucose 6-phosphate dehydrogenase	20.0 (3.0)	12.0 (3.0)	117.3 (2.1)	29.5 (1.1)	119 (13.4)
10. 6-Phosphogluconate dehydrogenase	5.3 (0.6)	5.1 (0.4)	66.8 (6.1)	38.1 (6.1)	55.7 (0.6)
11. 6-Phosphogluconate dehydratase, KDPG aldolase	4.3 (1.2)	14.0 (3.0)	3.5 (0.2)	10.8 (0.4)	6.0 (0.6)
12. Lactate dehydrogenase	5.6 (1.3)	10.4 (1.1)	14.1	10	5.5 (2.2)
Hydrogenase[d]	40.7 (6)	52	22.3 (1.4)	14.4	78 (5.2)

[a] Each standard error of the mean was determined from two or more independent experiments performed in triplicate. Where none is listed, the determination represents the results of triplicate assays performed on a single extract. A zero indicates no detectable activity.
[b] The Mtz concentration was 18 μg/ml in biphasic brucella medium.
[c] NA, not assayed.
[d] Not depicted in Fig. 3.

HP439 (MIC, >32 μg/ml). As little as 1 μg of Mtz per ml reduced POR activity by over 50%, and 3.5 μg/ml nearly abolished POR activity. At an Mtz concentration of ≥5 μg/ml, POR activity was completely abolished. We also observed that the growth of strain HP439 was unaffected in the presence of Mtz at 1 μg/ml, but at 5 μg/ml, an inhibition similar to that reported in Fig. 2 for HP1043 was observed (data not presented). These results support a correlation between the activity of POR and growth of *H. pylori* strains. We next examined the effect of Mtz concentration on the growth of Mtz^s strain HP500. HP500 grew very slowly in biphasic brucella medium containing Mtz at 1 μg/ml (twice the MIC). POR activity measured in extracts from Mtz^s strain HP500 grown in this medium was similarly decreased from 80 to 16 nmol/min/mg of protein. At higher concentrations of Mtz, no growth of HP500 was observed. Moreover, no spontaneous Mtz^r mutants were isolated from the HP500 culture growing in medium containing Mtz at 1 μg/ml. While we could not exclude the possibility that low POR activities were a result of the poor viability of the culture, it is more likely that Mtz equally affected the expression of POR in both Mtz^s and Mtz^r strains. Since POR is at the crossroads of metabolism, it is likely that absence of POR activity leads to inhibition of growth. Therefore, Mtz^r strains probably express compensatory enzymatic activities that enable them to survive in the absence of POR activity.

Isolation of an isogenic Mtz^r strain of *H. pylori*. To effectively examine metabolic differences between Mtz^s and Mtz^r strains of *H. pylori*, we set out to construct isogenic Mtz^r and Mtz^s strains. Spontaneous Mtz^r mutants of Mtz^s strain HP500 were sought by direct plating onto brucella agar supplemented with various concentrations of Mtz. Despite numerous attempts, no Mtz^r mutants of HP500 were obtained. Failure to isolate Mtz^r mutants of HP500 might mean that more than one gene is involved in resistance. We next attempted to transform HP500 with genomic DNA from a Mtz^r strain. HP500 was readily transformed to resistance with genomic DNA from Mtz^r strain HP439 at frequencies similar to those reported by Wang et al. (i.e., 10^{-5}) (58). One of the transformed Mtz^r isolates (HP1107) was chosen for further study. HP1107 genomic DNA was again used to transform HP500 to create Mtz^r strain HP1108. Both strains grew in biphasic medium at an Mtz concentration of 32 μg/ml. Except where indicated otherwise, HP1108 results were identical to those presented herein for HP1107. The isogenic strains HP1107 and HP500 and Mtz^r strain HP439 were examined for compensatory metabolic activities that might correlate with resistance to Mtz.

Enzymes associated with glucose metabolism. Major enzymes associated with the Embden-Meyerhof-Parnas (EMP) pathway were detected in *H. pylori* strains (Table 3 shows specific activities, and Fig. 3 shows the metabolic scheme). Hexokinase, phosphoglucose isomerase, phosphofructrokinase, fructose 1,6-biphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase, and pyruvate kinase activities were present in all of the strains. We noted higher fructose biphosphatase activity than phosphofructokinase activity in Mtz^r strains, suggesting that in these strains gluconeogenesis may be favored over glycolysis. In Mtz^s strain HP500, higher phosphofructokinase than fructose 1,6-biphosphatase activity would favor the glycolic route. In addition, high fructose 1,6-aldolase and pyruvate kinase activities were also detected in strain HP500. Growth in the presence of Mtz did not substantially alter the activities of EMP enzymes. Unlike POR, none of the EMP enzymes assayed were absent in extracts of strains grown in the presence of Mtz. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities were found in all of the strains tested, confirming the presence of the pentose-phosphate pathway (35). HP500 and HP1107 exhibited substantially higher activities of these enzymes than did strain HP439.

Enzyme activity indicative of the Entner-Doudoroff pathway was also detected in all of the strains; however, in the presence of Mtz, the Mtz^r strains HP439 and HP1107 exhibited higher Entner-Doudoroff pathway activities (Table 2 and Figure 3). In contrast to Mtz^r strains of *B. fragilis* (40), there was no corresponding increase in lactate dehydrogenase activity in Mtz^r strains of *H. pylori* grown in the presence of Mtz. Lactate dehydrogenase was membrane bound in *H. pylori* strains and exhibited no activity with NAD as the electron acceptor. Lactate oxidation was coupled to electron transport and was measured by monitoring the reduction of horse heart cytochrome *c*. In this regard, the lactate dehydrogenase activity was similar to that reported for *C. jejuni* (17).

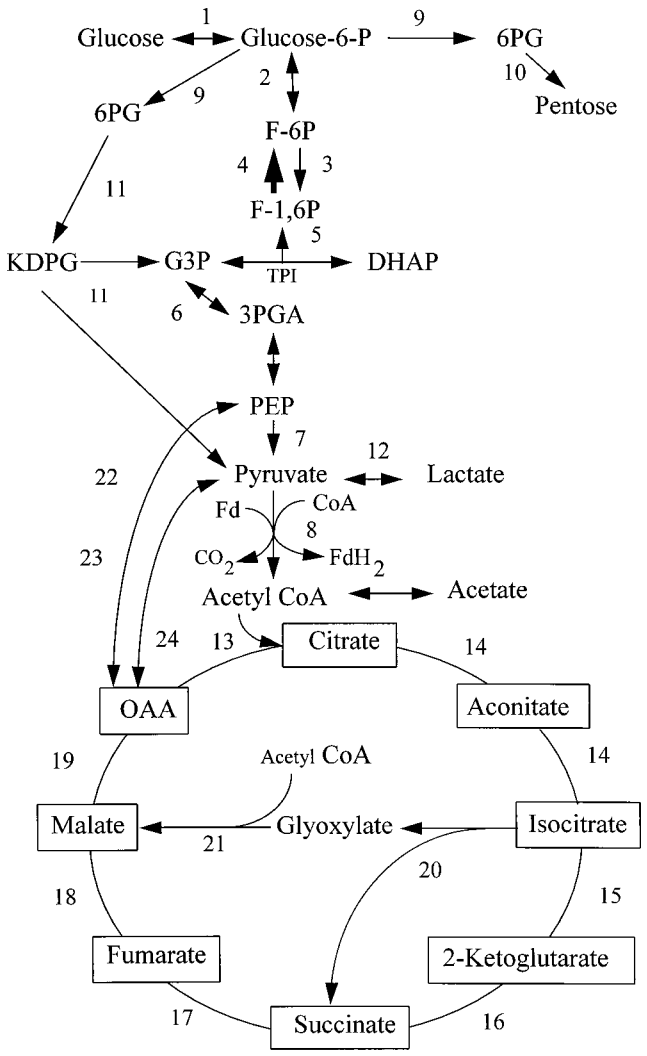

FIG. 3. Catabolic and anabolic activities detected in *H. pylori* strains grown in brucella medium. Thick arrows indicate major activities or direction. Fd, flavodoxin; TPI, triose phosphate isomerase; 6PG, 6-phosphogluconate; F-6P, fructose-6-phosphate; F-1,6-P, fructose-1,6-biphosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; 3PGA, 3-phosphoglycerate; OAA, oxaloacetate.

Krebs cycle enzymes. *H. pylori* strains contained a complete and active Krebs cycle (Table 4 and Fig. 3). Citrate synthase, the initial enzyme that condenses oxaloacetate with acetyl CoA, exhibited a high specific activity. The activity of citrate synthase was not appreciably affected by growth in the presence of Mtz. Isocitrate dehydrogenase exhibited a preference for NADP (95%) over NAD (5%), whereas malate dehydrogenase utilized NAD as an electron acceptor. Maximum fumarase and aconitase activities were detected in extracts of cells disrupted in the presence of reducing agents. KOR, like POR, could only be detected under anaerobic conditions with benzyl viologen as the electron acceptor. Generally, the KOR activities of the various strains were lower than the POR activities. No α-ketoglutarate dehydrogenase activity was detected with NAD or NADP (data not presented). An active succinate dehydrogenase was detected in all of the strains with potassium ferricyanide as the electron acceptor. We found variable fumarate reductase activity (data not shown) but were unable to demonstrate growth of HP500 under conditions requiring anaerobic respiration (data not shown). All of the strains examined contained a periplasmic hydrogenase which may couple the oxidation of hydrogen to the generation of ATP. We found no evidence of formate dehydrogenase in any of the *H. pylori* strains examined. This is somewhat unusual, considering that most related species of *Campylobacter* and *Wolinella* express high levels of this enzyme (10). With the exception of HP1134, none of the *H. pylori* isolates examined in this study exhibited measurable NADH oxidase activity (<0.1 nmol/min/mg of protein). HP1134 (Mtz^s) exhibited substantial NADH oxidase activity (30 nmol/min/mg of protein), in addition to an exceptionally active POR.

Growth of Mtz^r strains in the presence of Mtz led to decreases in the activities of several Krebs cycle enzymes. In this regard, both aconitase and isocitrate dehydrogenase levels were decreased three- to fourfold compared with activities determined for bacteria grown in the absence of Mtz. KOR, a POR-related enzyme complex, also exhibited no activity in extracts from Mtz^r strains grown in the presence of Mtz. Moreover, succinate dehydrogenase and fumarase activities were also diminished in strains grown in the presence of Mtz. Malate dehydrogenase activity was not appreciably affected by Mtz. All of the strains examined in this study exhibited malate synthase activity. This enzyme condenses glyoxylate with acetyl CoA to form malate. The specific activity of malate synthase was unaffected by the Mtz concentration. The first enzyme of this pathway (isocitrate lyase) was not detected in strain HP500. However, a low but consistent enzymatic activity was detected in Mtz^r strains regardless of whether Mtz was present in the medium (1 to 6.22 nmol/min/mg of protein). In comparison, the isocitrate lyase specific activity measured in *A. vinelandii* extracts was 56 nmol/min/mg of protein. *H. pylori* HP1107 expressed constitutive isocitrate lyase activity, suggesting that isocitrate lyase activity may cotransform with Mtz resistance. To test this possibility, genomic DNAs from Mtz^r strains HP439 and HP1107 were used to transform HP500 to Mtz resistance and the resulting Mtz^r strains had acquired isocitrate lyase activity. An examination of other Mtz^s strains for isocitrate lyase activity identified one Mtz^s strain (HP1134) that expressed isocitrate lyase activity. The strain expressed unusually high POR and NADH oxidase activities. When transformed to Mtz resistance with cosmid DNA originating from Mtz^r strain HP439, cell extracts from strain HP1134R expressed no POR activity when grown in medium containing Mtz at 18 μg/ml. Since Mtz resistance appears to be a recessive trait and one likely involving inactivation of a regulatory locus, the appearance of isocitrate lyase activity in HP1134 is likely the result of an independent mutation not involving the regulatory locus.

Other enzymes associated with pyruvate metabolism were examined (Table 4). Enzymes such as PEP carboxylase, PEP carboxykinase, and pyruvate carboxylase shuttle between three and four carbon acids and provide an alternate route for the entry of pyruvate and PEP into the Krebs cycle or for gluconeogenesis. Most of the activities of these enzymes were relatively low and may well be strain specific. However, we did note that PEP carboxykinase activity was present in strain HP500 and in strain HP1107 grown in the absence, but not in the presence, of Mtz. No activity of this enzyme was detected in extracts from strain HP439. In addition, PEP carboxylase activity was found only in *H. pylori* strains grown in the presence of Mtz. Pyruvate carboxylase was detected in HP439 (grown with or without Mtz) and in HP500 but not in strain HP1107 grown in the presence of Mtz. Because of the very low activities

TABLE 4. Krebs cycle and anaplerotic enzymes

No. in Fig. 3 and enzyme	Mean sp act (nmol/min/mg of protein) (SEM)[a]				
	HP439		HP1107		HP500, no Mtz
	No Mtz	Mtz[b]	No Mtz	Mtz[b]	
13. Citrate synthase	153.2 (2.9)	127.8 (5.8)	162.7 (7.4)	187.3 (36)	422.3 (53.1)
14. Aconitase	42.0 (4.0)	11.0 (1.1)	78.4 (4.6)	26.0 (4.0)	36.0 (4.0)
15. Isocitrate dehydratase	463.7 (21)	133.7 (25)	373.1 (3.5)	105.6 (10.1)	298.6 (15.3)
16. α-Ketoglutarate oxidoreductase	29.2 (3.5)	0	30.9 (4.2)	0	26.5
17. Succinate dehydrogenase	69.7 (2.3)	46.8 (2.6)	76.1 (7.9)	46.9 (6.8)	54 (14)
18. Fumarase	252 (29)	149 (5)	191 (3)	157 (7)	78.4 (3.1)
19. Malate dehydrogenase	3.8 (0.5)	2.2 (0.2)	6.08 (0.8)	5.0 (1.6)	9.3 (1.2)
20. Isocitrate lyase	1.26 (0.07)	1.1 (0.1)	2.62 (0.12)	6.22 (0.7)	0
21. Malate synthase	56.3 (8.1)	58.2 (5.5)	40.0 (1.0)	41.6 (1.0)	47.9 (3.6)
22. PEP carboxykinase	0	0	6.5 (1.7)	0	6.3 (0.3)
23. PEP carboxylase	7.9	7.8 (0.5)	NA[c]	6.0 (0.5)	4.93
24. Pyruvate carboxylase	5.5	1.3 (0.3)	3.8	0	3.8 (0.3)

[a] The standard error of the mean was determined from two or more independent experiments performed in triplicate. Where none is listed, the determination represents the results of triplicate assays performed on a single extract. A zero indicates no detectable activity.
[b] The Mtz concentration was 18 μg/ml in biphasic brucella medium.
[c] NA, not assayed.

of these enzymes obtained, we did not investigate these differences further.

DISCUSSION

We have investigated the basis for the Mtz resistance of selected clinical isolates of *H. pylori. Helicobacter* spp., like members of the genus *Campylobacter*, exhibit a strictly respiratory form of metabolism and oxidize amino acids and organic acids as carbon and energy sources (17, 28). Our study demonstrated that *H. pylori* possesses a complete and active Krebs cycle. In addition, all of the *H. pylori* strains examined in this study were found to contain POR and KOR activities, which in anaerobes are the major enzymes responsible for the reduction of nitroimidazole drugs (8, 29, 31). None of the *H. pylori* strains examined in this study exhibited NAD-linked pyruvate or α-ketoglutarate dehydrogenase activity, commonly found in aerobes and facultative anaerobes. Recently, the POR complex of *H. pylori* was purified and its four component enzymes were characterized (19). The POR complex exhibited pyruvate-dependent reduction of *H. pylori* flavodoxin but failed to reduce exogenous ferredoxin from *Clostridium pasteurianum*. Since ferredoxin is an integral component of the POR complex, it does not appear to function either as a soluble carrier of reducing equivalents or as a participant in the reduction of Mtz. A POR has also been described in the genus *Campylobacter* (7, 28). Studies performed with crude extracts from various species of *Campylobacter* indicate that Mtz reduction by POR was 10- to 100-fold lower than when assayed with viologen dyes (7). Similarly, only very weak pyruvate-dependent reduction of Mtz was detected in the present study with extracts of *H. pylori* in the absence of an added carrier. Generally, addition of an exogenous electron carrier enhances the rate of Mtz reduction, so our results do not necessarily exclude Mtz as an electron acceptor in vivo. Our studies also indicate that Mtz^r strains of *H. pylori* avoid the possible toxic consequences of reduction of Mtz by repression of POR and KOR activities by an unknown regulatory mechanism. We demonstrated that POR specific activity was Mtz concentration dependent, and as little as 3.5 μg/ml (well below the MIC of >32 μg/ml) led to nearly complete repression of detectable POR activity. Mtz^r mutants of *B. fragilis* also exhibit repression of POR activity when grown in medium containing Mtz (40). POR of *H. pylori* is not directly inhibited by Mtz, suggesting the possibility that POR is regulated at the level of transcription. The genes encoding the POR enzymes have not been cloned. One might speculate that DNA damage caused by reduced Mtz may lead to the induction of a POR-specific repressor that is activated by the SOS DNA repair system. The idea of a repressor, activated in response to low levels of Mtz, is supported by growth studies with Mtz^r strains. In this regard, we found that all of the Mtz^r clinical isolates grew substantially slower in the presence of Mtz at 18 or 32 μg/ml than in Mtz-free medium. Growth studies revealed that maximum growth yields were decreased by greater than 30% in the presence of as little Mtz as 5 μg/ml and that even at an Mtz concentration of 30 μg/ml, the maximum growth yield was only diminished another 5%. Mtz also dramatically affected the growth rate of Mtz^s strain HP500, and extracts from this strain also had decreased POR activities. However, HP500 failed to grow at Mtz concentrations that would lead to complete repression of POR and KOR in Mtz^r strains (ca. 3 μg/ml), suggesting the possibility that POR and KOR activities are essential for viability. Interestingly, the low concentrations of Mtz that lead to repression of POR and KOR activity are in the MIC ranges indicative of Mtz sensitivity. If, indeed, Mtz leads to repression of a major energy-yielding metabolic step, then Mtz resistance would be expected to involve the activation of some compensatory metabolic activity. For instance, studies with an Mtz^r mutant of *B. fragilis* NCTC 11295 indicated that increased activity of lactate dehydrogenase compensated for the loss of POR activity noted when the strain was grown in the presence of Mtz (20, 40). However, *H. pylori* strains expressed lactate dehydrogenase activity and there was no correlation between Mtz resistance and increased levels of this enzyme.

Our comparative metabolic studies identified isocitrate lyase as a possible compensatory enzyme expressed by Mtz^r strains HP439, HP1043, HP1107, and HP1108 but not by Mtz^s strain HP500 or HP950. However, the finding of constitutive isocitrate lyase activity in Mtz^s strain HP1134 raises the possibility that additional compensatory activities are required. Our recent studies suggest that Mtz resistance may be a recessive trait, possibly involving inactivation of a regulatory locus. The isocitrate lyase activity expressed by strain HP1134 may have arisen independently of inactivation of a regulatory locus. Thus, since this strain was rendered Mtz^r after transformation

with cosmid DNA from an Mtz^r strain, inactivation of this putative regulatory locus may have additional pleiotropic effects. Studies are in progress to identify the locus encoded by this DNA fragment and other genes that this locus might control.

The discovery of isocitrate lyase in some strains of *H. pylori* raises fundamental questions as to the origin of this activity and how it is regulated. In *Escherichia coli*, isocitrate lyase and malate synthase are products of the *aceBAK* operon, which is negatively regulated by IclR (13). In the presence of acetate, the operon becomes induced. If, indeed, a similar regulatory mechanism exists in *H. pylori*, one could envision mutations in an *iclR* homolog that would lead to constitutive expression of isocitrate lyase and negate a requirement for acetate as an inducer. Alternatively, the very low activity of isocitrate lyase raises the possibility that this activity arose from a mutation in an existing enzyme with a similar biological function. Perhaps such mutations can be used to explain both the increase in Mtz resistance in the general population and the basis for spontaneous mutants isolated in the laboratory (14). On the basis of our inability to isolate spontaneous Mtz^r mutants in our laboratory, we propose that resistance to Mtz may be attributable to multiple mutational events leading to a common phenotype. Thus, for some strains, one or more of these mutations may have already occurred. Such strains would exhibit a higher frequency of spontaneous resistance.

The possibility of additional compensatory metabolic changes led us to explore enzymes of glucose metabolism. Our enzyme-based studies confirm and extend nuclear magnetic resonance studies by others in establishing the presence of the EMP and Entner-Doudoroff pathways in *H. pylori* (3, 37). While glucose is minimally utilized by this organism (3, 36), our studies show that glucose metabolism is complex. For example, we found that the EMP pathway has the capacity to function in both gluconeogenic and glycolytic manners. In the presence of Mtz, Mtz^r strains appear to catabolize glucose more actively via the Entner-Doudoroff pathway. Clearly, more study is required to assess the contributions of these metabolic pathways to the over all metabolic strategy of *H. pylori*. One reason for examining the glucose catabolic pathways was to identify potential sources of acetyl CoA, since in the presence of Mtz, the major source (POR) would be inactive. While our search was not exhaustive, we found no major source of acetyl CoA. In this regard, Hughes et al. (19), found acetyl CoA carboxylase activity and malic enzyme activity in *H. pylori*. It is possible that other oxidative enzymes associated with pyruvate or acetate metabolism are present but were not detected with the methods employed in this study. These results are consistent with the hypothesis that POR activity is essential for optimal growth of *H. pylori*. Perhaps fatty acid oxidation or free acetate may serve as a source of acetyl CoA. Several chemically defined media have been described for the growth of *H. pylori* (41, 50), so it may be possible to adapt one of these formulations for further exploration of the nutritional defect created by Mtz and the compensatory activities associated with resistance.

Several earlier studies of *H. pylori* metabolism concluded that both glucose and pyruvate were oxidized to organic acids, suggesting that the Krebs cycle is either absent or incomplete in *H. pylori* (19, 36, 37). Our studies detected an active citrate synthase in *H. pylori*, suggesting that acetyl CoA generated by the oxidation of pyruvate would be rapidly catabolized via the Krebs cycle. In the absence of Mtz, an active isocitrate dehydrogenase was detected. In the presence of Mtz, isocitrate dehydrogenase was significantly decreased in activity, possibly in concert with the absence of both POR and KOR activities. Similarly, nearly all of the Krebs cycle enzymes were decreased in bacteria grown in the presence of Mtz. It is not clear whether other anaplerotic enzymes become activated in the presence of Mtz, but we were able to detect some of the carboxylating enzymes not detected in an earlier study (19). However, the activities were low and may be strain specific; nevertheless, PEP carboxylase, PEP carboxykinase, and pyruvate carboxylase activities were detected. These anaplerotic enzymes would be crucial to the operation of the Krebs cycle, particularly in the absence of POR and KOR activities, by providing a source of oxaloacetic acid needed for citrate synthesis.

Mtz is commonly used in the treatment of a variety of anaerobic infections and is one of the antimicrobial agents recommended for the treatment of *H. pylori* infection in humans. Resistance to this drug is the primary reason for failed eradication (9, 11, 27, 57). Mtz resistance also occurs in other organisms, including *Campylobacter* spp., *B. fragilis* (2), and *Trichomonas* spp. (46). It is highly likely that these organisms have similar resistance mechanisms, including regulation of POR and KOR. The observation that enzymes of the POR and KOR complex are repressed in response to low levels of Mtz needs to be further investigated, not only for the microaerophiles, but also for *Bacteroides* spp. Finally, the genetic variability noted among *H. pylori* strains will also be notably manifest in the enzymatic profiles of any particular strain. Thus, the strength of this study is that isogenic strains were compared, but a broader study of additional strains is required to confirm these observations. It is hoped that these preliminary findings will stimulate more research into the interesting metabolic strategy of these microaerophilic bacteria.

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REFERENCES

1. **Allison, W. S.** 1969. Glyceraldehyde 3-P dehydrogenase. Methods Enzymol. **13:**210–212.
2. **Britz, M. L., and R. G. Wilkinson.** 1979. Isolation and properties of metronidazole-resistant mutants of *Bacteroides fragilis*. Antimicrob. Agents Chemother. **16:**19–27.
3. **Chalk, P. A., A. D. Roberts, and W. M. Blows.** 1994. Metabolism of pyruvate and glucose by intact cells of *Helicobacter pylori* studies by ^{13}C NMR spectroscopy. Microbiology **140:**2085–2092.
4. **Chang, H. C., and M. D. Lane.** 1966. The enzymatic carboxylation of phosphoenolpyruvate. J. Biol. Chem. **241:**2414–2420.
5. **Chen, J. S., and D. K. Blanchard.** 1979. A simple hydrogenase-linked assay for ferredoxin and flavodoxin. Anal. Biochem. **93:**216–222.
6. **Cover, T. L., and M. J. Blaser.** 1992. *Helicobacter pylori* and gastroduodenal disease. Annu. Rev. Med. **43:**135–145.
7. **Daucher, J. A., and N. R. Krieg.** 1995. Pyruvate:ferredoxin oxidoreductase in *Campylobacter* species. Can. J. Microbiol. **41:**198–201.
8. **George, H. A., and R. M. Smibert.** 1982. Pyruvate oxidation by the Reiter strain of *Treponema phagedenis*. J. Bacteriol. **152:**1060–1065.
9. **Glupczynski, Y.** 1992. Results of a multicentre European survey in 1991 of metronidazole resistance in *Helicobacter pylori*. Eur. J. Clin. Microbiol. Infect. Dis. **11:**777–781.
10. **Goodman, T., and P. S. Hoffman.** 1983. Hydrogenase activity in catalase-positive strains of *Campylobacter* spp. J. Clin. Microbiol. **18:**825–829.
11. **Goodwin, C. S., B. J. Marshall, E. D. Blincow, D. H. Wilson, D. Blackbourn, and M. Phillips.** 1988. Prevention of nitroimidazole resistance in *Campylobacter pylori* by coadministration of colloidial bismuth subcitrate: clinical and in vitro studies. J. Clin. Pathol. **41:**207–210.
12. **Groves, W. E., J. Calder, and W. J. Rutter.** 1966. Fructose diphosphate aldolase. Methods Enzymol. **9:**486.
13. **Gui, L, A. Sunnarborg, B. Pan, and D. C. LaPorte.** 1996. Autoregulation of *iclR*, the gene encoding the repressor of the glyoxylate bypass operon. J. Bacteriol. **178:**321–324.
14. **Haas, C. E., D. E. Nix, and J. J. Schentag.** 1990. In vitro selection of resistant *Helicobacter pylori*. Antimicrob. Agents Chemother. **34:**1637–1641.

15. **Hathaway, J. A., and D. E. Atkinson.** 1963. The effect of adenylic acid on yeast nicotinamide adenine dinucleotide isocitrate dehydrogenase, a possible metabolic control mechanism. J. Biol. Chem. **238:**2875–2881.
16. **Hof, H., and V. Sticht-Groh.** 1984. Antibacterial effects of niridazole: its effect on microaerophilic campylobacter. Infection **12:**36–39.
17. **Hoffman, P. S., and T. G. Goodman.** 1982. Respiratory physiology and energy conservation efficiency of *Campylobacter jejuni*. J. Bacteriol. **150:**319–326.
18. **Hoffman, P. S., N. R. Krieg, and R. M. Smibert.** 1979. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. I. Physiological aspects of enhanced aerotolerance. Can. J. Microbiol. **25:**1–7.
19. **Hughes, N. J., P. A. Chalk, C. L. Clayton, and D. J. Kelly.** 1995. Identification of carboxylation enzymes and characterization of a novel four-subunit pyruvate:flavodoxin oxidoreductase from *Helicobacter pylori*. J. Bacteriol. **177:**3953–3959.
20. **Inoue, K., S. Narikawa, T. Suzuki, and M. Nakamura.** 1988. Relationship between metronidazole resistance and susceptibility factors in *Bacteroides fragilis* NCTC 11295. Chemotherapy (Tokyo) **36:**412–416.
21. **Kamel, M. Y., O. P. Allison, and R. L. Anderson.** 1966. Stereospecific D-glucokinase of *Aerobacter aerogenes*: purification and properties. J. Biol. Chem. **241:**690–694.
22. **Kedderis, G. L., L. S. Argenbright, and G. T. Miwa.** 1988. Mechanism of reductive activation of a 5-nitroimidazole by flavoproteins: model studies with dithionite. Arch. Biochem. Biophys. **262:**40–48.
23. **Keen, M. G., and P. S. Hoffman.** 1984. Metabolic pathways and nitrogen metabolism in *Legionella pneumophila*. Curr. Microbiol. **11:**81–88.
24. **Keyer, K., A. S. Gort, and J. A. Imlay.** 1995. Superoxide and the production of oxidative DNA damage. J. Bacteriol. **177:**6782–6790.
25. **Kletzin, A., and M. W. W. Adams.** 1996. Molecular and phylogenetic characterization of pyruvate and 2-ketoisovalerate ferredoxin oxidoreductases from *Pyrococcus furiosus* and pyruvate ferredoxin oxidoreductase from *Thermotoga maritima*. J. Bacteriol. **178:**248–257.
26. **Krieg, N. R., and P. S. Hoffman.** 1986. Microaerophily and oxygen toxicity. Annu. Rev. Microbiol. **40:**107–130.
27. **Lacey, S. L., S. F. Moss, and G. W. Taylor.** 1993. Metronidazole uptake by sensitive and resistant isolates of *Helicobacter pylori*. J. Antimicrob. Chemother. **32:**393–400.
28. **Lascelles, J., and K. M. Calder.** 1985. Participation of cytochromes in some oxidation-reduction systems in *Campylobacter fetus*. J. Bacteriol. **164:**401–409.
29. **Lindmark, D. G., and M. Muller.** 1976. Antitrichomonad action, mutagenicity, and reduction of metronidazole and other nitroimidazoles. Antimicrob. Agents Chemother. **10:**476–482.
30. **Ling, K., H. V. Paethau, F. Marcus, and H. A. Lardy.** 1966. Phosphofructokinase. Methods Enzymol. **9:**425–429.
31. **Lockerby, D. L., H. R. Rabin, and E. J. Laishley.** 1985. Role of the phosphoroclastic reaction of *Clostridium pasteurianum* in the reduction of metronidazole. Antimicrob. Agents Chemother. **27:**863–867.
32. **Marshall, B.** 1983. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet **i:**1273–1275.
33. **Massey, V.** 1955. Fumarase. Methods Enzymol. **1:**729–734.
34. **McQuate, J. T., and M. F. Utter.** 1959. Equilibrium and kinetic studies of the pyruvate kinase reaction. J. Biol. Chem. **234:**2151–2157.
35. **Mendz, G. L., and S. L. Hazell.** 1991. Evidence for a pentose-phosphate pathway in *Helicobacter pylori*. FEMS Microbiol. Lett. **84:**331–336.
36. **Mendz, G. L., S. L. Hazell, and B. P. Burns.** 1993. Glucose utilization and lactate production by *Helicobacter pylori*. J. Gen. Microbiol. **139:**3023–3028.
37. **Mendz, G. L., S. L. Hazell, and B. P. Burns.** 1994. The Entner-Doudoroff pathway in *Helicobacter pylori*. Arch. Biochem. Biophys. **312:**349–356.
38. **Moore, R. A., B. Beckthold, and L. E. Bryan.** 1995. Metronidazole uptake in *Helicobacter pylori*. Can. J. Microbiol. **41:**746–749.
39. **Muller, M.** 1983. Mode of action of metronidazole on anaerobic bacteria and protozoa. Surgery **93:**165–171.
40. **Narikawa, S., T. Suzuki, M. Yamamoto, and M. Nakamura.** 1991. Lactate dehydrogenase activity as a cause of metronidazole resistance in *Bacteroides fragilis* NCTC 11295. J. Antimicrob. Chemother. **28:**47–53.
41. **Nedenskov, P.** 1994. Nutritional requirements for growth of *Helicobacter pylori*. Appl. Environ. Microbiol. **60:**3450–3453.
42. **Noltmann, E. A.** 1964. Isolation of crystalline phosphoglucose isomerase from rabbit muscle. J. Biol. Chem. **239:**1545–1550.
43. **Noltmann, E. A., C. J. Gubber, and S. A. Kuby.** 1961. Glucose-6-phosphate dehydrogenase (Zwischenferment). I. Isolation of the crystalline enzyme from yeast. J. Biol. Chem. **236:**1225–1230.
44. **Pontremoli, S., and E. Grazi.** 1966. 6-Phosphogluconate dehydrogenase. Methods Enzymol. **9:**137–141.
45. **Quayle, J. R.** 1969. Phosphoenolpyruvate carboxylase. Methods Enzymol. **13:**292–296.
46. **Quon, D. V. K., C. E. D'Oliveira, and P. J. Johnson.** 1992. Reduced transcription of the ferredoxin gene in metronidazole-resistant *Trichomonas vaginalis*. Proc. Natl. Acad. Sci. USA **89:**4402–4406.
47. **Racker, E.** 1950. Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. Biochim. Biophys. Acta **4:**211–214.
48. **Reed, L. J., and B. B. Mukherjee.** 1969. α-Ketoglutarate dehydrogenase. Methods Enzymol. **13:**55–61.
49. **Reeves, H. C., R. Rabin, W. S. Wegener, and S. J. Ajl.** 1971. Assays of enzymes of the tricarboxylic and glyoxylate cycles. Methods Microbiol. **6A:**425–462.
50. **Reynolds, D. J., and C. W. Penn.** 1994. Characteristics of *Helicobacter pylori* growth in a defined medium and determination of its amino acid requirements. Microbiology **140:**2649–2656.
51. **Reysset, G.** 1996. Genetics of 5-nitroimidazole resistance in *Bacteroides* species. Anaerobe **2:**59–69.
52. **Rosen, O. M., S. M. Rosen, and B. L. Horecker.** 1965. Purification and properties of a specific fructose-1,6-diphosphatase from *Candida utilis*. Arch. Biochem. Biophys. **112:**411–420.
53. **Seubert, W., and H. Weicker.** 1969. Pyruvate carboxylase from *Pseudomonas*. Methods Enzymol. **13:**258–262.
54. **Smith, M. A., and D. I. Edwards.** 1995. Redox potential and oxygen concentration as factors in the susceptibility of *Helicobacter pylori* to nitroheterocyclic drugs. J. Antimicrob. Chemother. **35:**751–764.
55. **Srere, P. A., H. Brazil, and L. Gonen.** 1963. The citrate condensing enzyme of pigeon breast muscle and moth flight muscle. Acta Chem. Scand. **17**(Suppl.)**:**S129–S134.
56. **Veeger, C., D. V. DerVartanian, and W. P. Zeylemaker.** 1970. Succinate dehydrogenase. Methods Enzymol. **13:**81–90.
57. **Veldhuyzen van Zanten, S. J. O., and P. M. Sherman.** 1994. Indications for treatment of *Helicobacter pylori* infection: a systematic overview. Can. Med. Assoc. J. **150:**189–198.
58. **Wang, Y., P. Roos, and D. E. Taylor.** 1993. Transformation of *Helicobacter pylori* by chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker. J. Gen. Microbiol. **139:**2485–2493.
59. **Wood, W. A.** 1971. Assay of enzymes representative of metabolic pathways. Methods Microbiol. **6A:**411–424.
60. **Yoshida, A.** 1965. Enzymic properties of malate dehydrogenase of Bacillus subtilis. J. Biol. Chem. **240:**1118–1124.