Biological Effect of Metronidazole Resistance in *Helicobacter pylori*

Gin Hyug Lee[1], Sun-Mi Lee, Won-Hee Nam, Eun Sil Kim, Jin Ho Kim[1], Ho Young Kang[2] and Jin-Yong Jeong*

Asan Institute for Life Sciences, Asan Medical Center, University of Ulsan College of Medicine, Seoul 138-736, Korea, [1]Division of Gastroenterology, Department of Internal Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul 138-736, Korea, [2]Department of Microbiology, Pusan National University, Pusan 609-735, Korea

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Resistance to metronidazole, a key component of therapies against *Helicobacter pylori*, is common in clinical isolates. Resistance generally requires inactivation of *rdxA* (HP0954), and sometimes also *frxA* (HP0642), two related nitroreductase genes. Here we studied the effect of resistance to metronidazole on fitness of the gastric pathogen *H. pylori*. The effect of metronidazole resistance for *H. pylori* in culture was assessed first by looking at colonies formed by freshly constructed mutant derivatives of *H. pylori* strain 26695. Mutations resulting in metronidazole resistance caused premature death of *H. pylori* in stationary phase, but had no significant effect on early exponential growth. The effect of nitroreductase deficiencies on fitness in vivo was tested by infecting C57BL/6 mice with 1:1 mixtures of SS1 wild type and its isogenic metronidazole resistant derivatives. Inactivation of *rdxA* caused an inability to colonize mice in SS1 *H. pylori* strain. Derivatives of a metronidazole resistant strain that survived better in stationary phase, although remaining metronidazole resistant, could again colonize mice. In conclusion, metronidazole resistance diminishes *H. pylori*'s fitness, but their costs can be suppressed by additional mutation.

Key words – *Helicobacter pylori*, Metronidazole resistance, *rdxA*, *frxA*, nitroreductase genes.

The development and widespread use of many potent new antibiotics in human medicine and agriculture during the last half century has led to very frequent resistance to these agents among pathogens, and thus marked decreases in their effectiveness in treating infectious disease [1,2]. It is believed or hoped, however, that such acquired resistances diminish the vigor of bacterial growth (fitness) in antibiotic-free environments, such that resistant strains would decrease in abundance as less important uses of antibiotics are curtailed. Several studies with enteric bacteria have shown that laboratory induced resistance mutations or genes cloned from naturally occurring R factor plasmids or transposons are indeed deleterious, although compensating mutations that restore normal fitness were easily selected [3,4].

Here we study the cost of metronidazole resistance (Mtz^R) to *Helicobacter pylori*, a gastric pathogen that colonizes more than half of all people worldwide, and that is the major cause of peptic ulcer disease and an early risk factor for gastric cancer. Metronidazole (Mtz) is a key component of one of the important combination therapies used against this pathogen, but resistance is widespread, ranging from 10-20% of strains in US populations to more than 50% in many developing countries [6,12,13]. Most people with Mtz^R *H. pylori* have never been treated for their *H. pylori* infections; rather, the Mtz^R *H. pylori* they carry are may reflect frequent Mtz usage against various parasitic and anaerobic infections, typically in doses that may induce and select for resistance in *H. pylori*, but that are not sufficient to eradicate sensitive strains [11].

Tests of *H. pylori* strains from many parts of the world have shown that clinically significant Mtz^R depends on inactivation of a normal chromosomal gene, *rdxA* (HP0954), which encodes an oxygen insensitive NADPH nitroreductase [8]. In metronidazole susceptible (Mtz^S) cells, this enzyme mediates conversion of Mtz from harmless prodrug to bacteriocidal agent, although its normal role for *H. pylori* (e.g., nutrition or detoxification) is not known. A homolog of *rdxA*, the *frxA* gene (HP0642) also has the potential to cause Mtz^S, but in most strains it is rather little transcribed, and its inactivation increases the level of Mtz that resistant strains will tolerate, but has little if any effect on the intrinsic ability of *rdxA*+ cells to tolerate very low levels

*Corresponding author
Tel : +82-2-3010-4105, Fax : +82-2-3010-4182
E-mail : jyjeong@amc.seoul.kr

of Mtz [7,8,9,14]. Although *rdxA* and *frxA* can not be essential for viability, it has been tempting to imagine that Mtz^R, the result of they contribute to bacterial vigor, at least under certain circumstances.

Here we report studies in culture and in animal model that show that nitroreductase gene mutations that confer Mtz^R contribute significantly to bacterial fitness.

Material and Methods

Bacterial strains and culture conditions

The *H. pylori* strains 26695 and SS1 used here were obtained from Douglas Berg. These strains were grown on brain-heart infusion agar (BHI; Difco) supplemented with 7% horse blood, 0.4% Isovitalex and the antibiotics amphotericin B (8 ug/ml), trimethoprim (5 ug/ml), vancomycin (6 ug/ml) (referred to here as agar medium), and with Mtz when needed at a concentration appropriate for the experiment. The plates were incubated at 37℃ under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). *H. pylori* transformation (electroporation) was carried out as described [15].

E. coli DH5α was grown on Luria-Bertani (LB) medium. The small multicopy plasmid vector pBluescript SK- (here designated pBS) was used as a cloning vector, and cells carrying it were selected on medium with 50 ug/ml ampicillin.

Determination of Mtz sensitivity and resistance

Frozen bacterial cultures were streaked onto Mtz-free agar medium and incubated for three days, and cells from these plates were spread on fresh Mtz-free medium and incubated for one day. The resulting young exponentially growing cells were suspended in PBS buffer; a series of 10-fold dilutions of these suspensions was then prepared, and 10 ul of each dilution was spotted on freshly prepared agar medium containing appropriate concentration of Mtz. (variously, 0, 0.2, 0.5, 1.5, 3, 8,16, 32, or 64 ug/ml, as appropriate).

Construction of Mtz^R mutant strains

A 111 bp in-frame deletion in *rdxA* (*rdxA*△111) was engineered as follows. A 1343 bp PCR product was generated by amplification of 26695 genomic DNA with oligonucleotide primers specific for genes that flank *rdxA* (primers rdxA-F1 "5′-CGGACTCATGGAATTGCTCCAT", downstream of *rdxA*, and rdxA-R1 "5′-GGCAAATCATAGGCATTATGGTG", upstream of *rdxA*), and cloned into the *Eco*RV site of pBS. A second PCR was carried out on a pBS-*rdxA* clone with outward facing primers containing *Xba*I sites near their 5′ ends (rdxA-F2 "5′-GTCATCTAGACCTGGCGATTTCAGCGATTTCT" and rdxA-R2 "5′-GTCATCTAGAAGCGCTTCAGCGTTAATGGTGGT"). *Xba*I digestion of the linear PCR product, ligation, recovery in DH5a, transformation into *H. pylori* with selection for Mtz^R (3 or 8 ug/ml).

Mono culture

Wild-type Mtz^S and Mtz^R cells were incubated in BHI agar plates under microaerobic condition separately. All experiments were performed in the absence of Mtz. Bacterial cells were taken at each time point and suspended with PBS buffer. Same amounts of cells (OD_{600}=1) were spotted on BHI plates by serial 10-fold dilution for survival bacterial titer.

Mixed culture

First, frozen bacterial strains were streaked onto BHI blood agar plates (supplements with 8 ug/ml Mtz in the case of Mtz^R strain) and incubated three days. Colonies were inoculated into fresh agar without supplemental antibiotic for any of the strains. These cultures were incubated for 48 hours prior to the actual start of the competition experiment. Wide type Mtz^S and Mtz^R mutant were collected from the plate and resuspended with PBS buffer. Same amounts of cells were mixed [input ratio of 1:1, Mtz^R mutant to Mtz^S wild type]. The mixture was grown on solid medium, and aliquots were removed daily to assess the ratio of the two types. PCR using the mixed cell population was performed with primers that flank the site of deletion in *rdxA*.

Mouse colonization

Mouse colonization assays were performed essentially as described previously [16]. Briefly, *H. pylori* wild type and mutant were started from freezer stocks and were grown on BHI agar plates with 7% horse blood separately. After 48 hours, the cells were harvested in PBS buffer, the concentration was adjusted according to the OD at 600nm. The animals were orogastrically inoculated twice (day 0 and 2) with 200 ul of an *H. pylori* cell suspension of 1×10^9 CFU/ml in PBS through a feeding needle. After 4 weeks, the mice were sacrificed and the stomachs were removed

and extracted DNA with Qiagin Tissue Kit according to manufacture's protocol. To assess the ratio of the two types, PCR was performed with primers that flank the site of deletion in *rdxA*.

Results

Construction of defined *rdxA* (Mtz^R) mutant strains

To test whether the loss of *rdxA* function, which is responsible for a Mtz^R phenotype, affects bacterial growth we generated a 111 bp in-frame deletion in *rdxA* in 26695, an *H. pylori* strain whose genome was fully sequenced [17], and also in a strain SS1. This entailed generating a deletion by PCR in a cloned *rdxA* gene, and then DNA transformation and selection for Mtz^R (see Fig. 3; Materials and Methods). Transformants resistant to metronidazole (8 ug/ml) were obtained at normal frequencies (~10^{-4} per CFU), and their haploid mutant structure was verified by PCR. These Mtz^R mutant transformants also grew well on medium with 16 ug/ml, but not 32 ug/ml of Mtz. This phenotype will be designated 16R.

In vitro fitness of Mtz^R strains

The fitness of isogenic Mtz^R (16R) and Mtz^S parental (WT) cells was monitored in several ways. First, the changes in optical density with time during exponential growth were monitored to assess fitness in liquid culture. Fig. 1 shows that the Mtz^R mutant grew as well as Mtz^S (wild type) parent. The effect of Mtz^R on fitness was monitored further by scoring viability in stationary as well as exponential growth phases. Fig. 2A shows that the Mtz^R (16R) derivative of 26695 dies prematurely in stationary

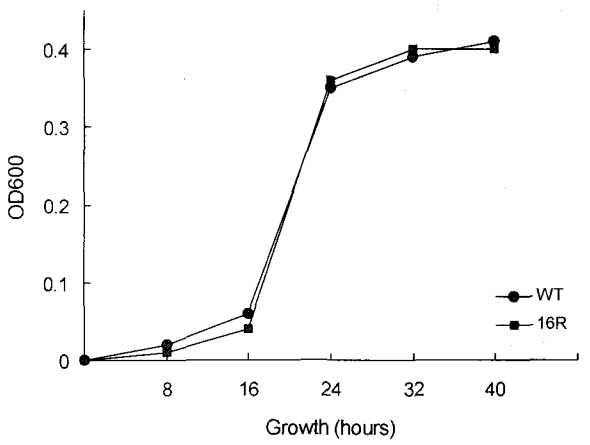

Fig. 1. Exponential growth analysis of Mtz^R and Mtz^S *H. pylori* strains.

phase, relative to its wild type parent. In contrast, the Mtz^R mutant transformant was just as viable as its Mtz^S parent during the first two days of incubation (exponential growth).

Two other 26695 derivatives with different Mtz^R phenotypes were also tested: (i) a derivative containing base substitution mutation of *rdxA* that resulted in mild Mtz^R phenotypes (resistant to 3 ug/ml, but not 8 ug/ml; designated 3R), and (ii) an uncharacterized spontaneous mutant derivative of *rdxA*-deletion strain (16R) that is resistant to a much higher level of Mtz (>64 rather than just 16 ug/ml; designated 64R); this hyper-resistance mutation occurs at an as yet unmapped locus, outside the *rdxA* gene. Fig. 2A shows that the fitness of the 3R derivatives of 26695 are intermediate between those of wild type (Mtz^S) and the 16R mutant, and that the hyper-resistant strain (64R; containing *rdxA* deletion plus a resistance enhancing mutation) is

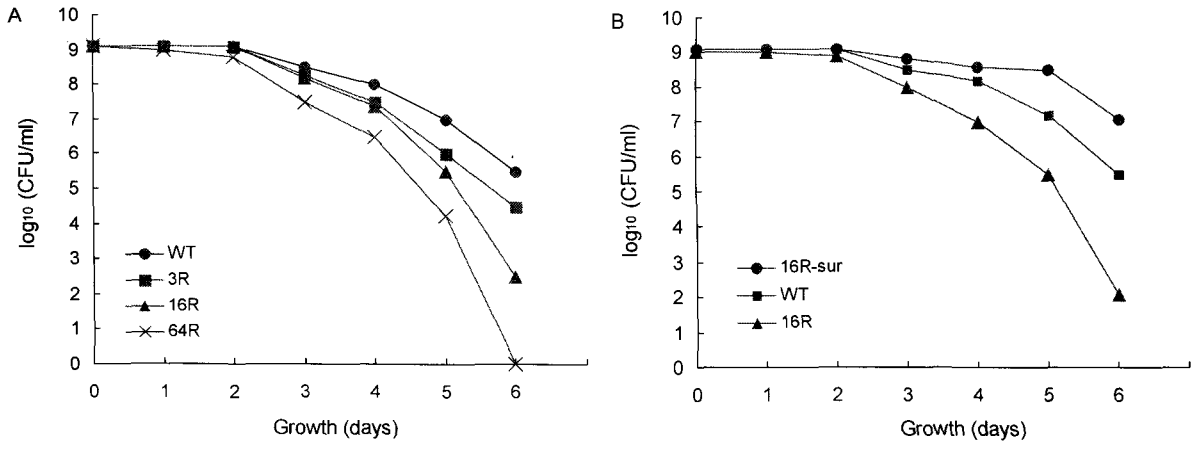

Fig. 2. Growth of *H. pylori* strains. (A) effect of various Mtz^R *H. pylori* mutants, (B) effect of sur mutant.

even less fit than the 16R mutant strain.

Mutation that compensates for decreased fitness caused by Mtz^R

A spontaneous derivative of the Mtz^R strain that survives well in stationary phase was found by screening survivors of the 16R (26695) strain after six days of growth as in Fig. 2A. Three of six such survivors tested exhibited markedly improved survival in stationary phase (designated "sur" mutants). Further tests with one representative "sur" mutant showed that it actually survived better than wild type in stationary phase (Fig. 2B), although it retained its 16R (32S) phenotype.

The possibility of complementing the premature death of Mtz^R cells in stationary phase by co-culture with isogenic Mtz^S(wild type) was tested by (i) growth well into stationary phase, (ii) subculture at various times on fresh plates, (iii) re-growth and further subculture. Aliquots from these mixed cultures were taken each day, and the frequencies of Mtz^R and Mtz^S types were estimated by PCR. Fig. 3A shows results of representative competition experiments [input ratio of 1:1, Mtz^R mutant to Mtz^S wild type]. The constant ratio of the two diagnostic fragments throughout the growth period shows that the Mtz^R mutant (lower band) and Mtz^S parent (upper band) grow equally well in exponential phase. In contrast, the subculturing after 6 days growth led to preferential outgrowth of the wild type (Fig. 3A). This result shows again Mtz^R *H. pylori* cells die prematurely in stationary phase, and that their deficiency cannot be compensated by co-growth with wild type *H. pylori*. Spontaneous mutant derivatives of Mtz^R strains (designated "sur" mutants) that survive well in stationary phase were found by screening survivors after six days of growth. These sur strains actually survived better than wild type in stationary phase (Fig. 2B and 3B).

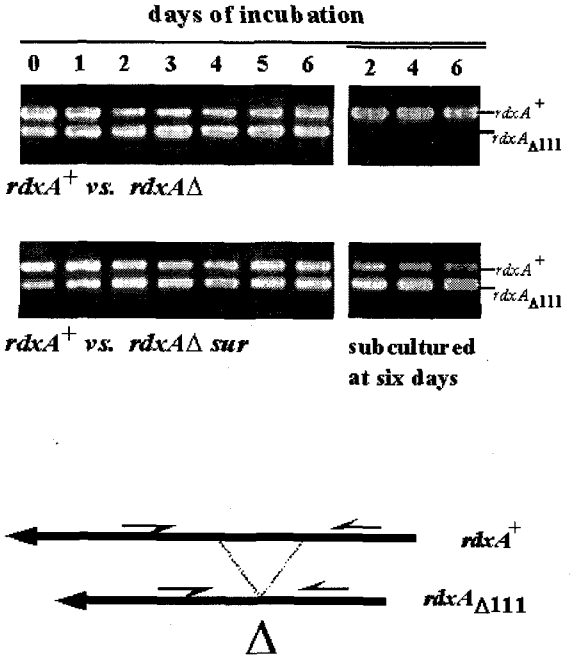

Fig. 3. Fitness of Mtz^R mutant in mixed culture with wild-type Mtz^S strain. Bacterial cell were grown into stationary phase and then subculture at various times on fresh plates and re-growth and re-subculture. Aliquots from these mixed cultures were taken each day, and the frequencies of wild type and Mtz^R mutant were estimated by PCR.

Fitness of Mtz^R *H. pylori* strains during infection of the experimental animal

Introduction of an *rdxA* in-frame deletion into the mouse colonizing strain SS1 also resulted in a premature stationary phase death phenotype, and did not affect exponential growth or viability of young cultures, as expected based on analyses with strain 26695; compensatory mutations that markedly enhanced stationary phase survival were also easily obtained (data equivalent to those in Fig. 2 and 3).

The effect of inactivation of *rdxA* on mouse colonization was tested by infecting C57BL/6 mice with a 1:1 mixture of the Mtz^R mutant and a isogenic wild type strain. Mixed infections by SS1-16R and its SS1 wild type parent resulted in colonization only by wild type, not by SS1-16R (Fig. 4A). In contrast, mixed infection involving a sur mutant

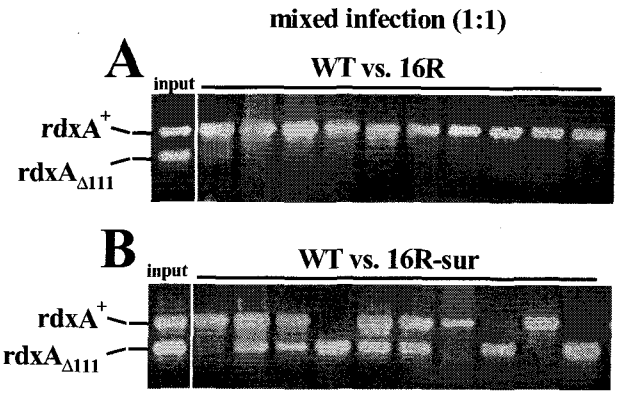

Fig. 4. Fitness of Mtz^R strains during infection of C57BL/6 mice. *H. pylori* wild type and mutants were mixed and inoculated of C57BL/6 mice. After 4 weeks, infected bacterial cells were recovered from mouse stomach and bacterial DNA were prepared and tested by PCR.

derivative of SS1-16R and its wild type parent resulted in establishment of each strain in an apparently balanced mixed infection (Fig. 4B)

Discussion

The present experiments have shown (i) that Mtz^R (*rdxA*) mutant strains of *H. pylori* die prematurely in stationary phase cultures, relative to isogenic Mtz^S wild type strains; (ii) that the severity of this premature death depends on the level of Mtz^R; and (iii) that the premature death of Mtz^R strains can be compensated, and indeed overcompensated, by mutation another as yet uncharacterized gene. We propose that this decreased fitness of Mtz^R *H. pylori* in culture could be due to accumulation in stationary phase of metabolites that are toxic, but that in Mtz^S cells are detoxified by their more complete complement of nitroreductases.

In terms of the human condition, many of the putatively Mtz^R *H. pylori* infections in socieities in which Mtz^R is commonplace (e.g., Latin America, Eastern Europe) are actually mixed, and often contain many Mtz^S *H. pylori* and fewer Mtz^R *H. pylori* cells. The two strain types in these mixed infections are often very closely related, differing only by point mutations in the *rdxA* gene [7,10]. This abundance of mixed infections was attributed to frequent use of Mtz for diverse other infections in these societies, typically at doses or in drug combinations that may be sufficient to induce and select for Mtz^R, but usually not sufficient to eradicate this organism. The demonstration here that Mtz^R cells are less fit than Mtz^S cells in Mtz-free culture supports this interpretation.

Another indication of possibly decreased fitness of Mtz^R in vivo comes from a report of Mtz^R *H. pylori* being relatively more common in benign infections and Mtz^S *H. pylori* being more common in infections associated with overt disease [5]. This could be explained if Mtz^R strains grew less vigorously in vivo, achieved lower titers in the gastric mucosa, and thereby caused less damage to host tissue directly (e.g., via toxins) or indirectly (e.g., by exciting inflammatory responses). It could also be explained if Mtz^R strains were generally less resistant than Mtz^S strains to bacteriocidal substances produced during inflammation, which is generally more severe in the more virulent infections.

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References

1. Andersson, D. I. and B. R. Levin. 1999. The biological cost of antibiotic resistance. Curr Opin Microbiol. **2(5)**:489-93.
2. Bjorkman, J. and D. I. Andersson. 2000. The cost of antibiotic resistance from a bacterial perspective. Drug Resist Updat. **3(4)**:237-245.
3. Bjorkman, J., D. Hughes. and D. I. Andersson. 1998. Virulence of antibiotic-resistant Salmonella typhimurium. Proc Natl Acad Sci U S A. **95(7)**:3949-53.
4. Bjorkman, J., I. Nagaev, O. G. Berg, D. Hughes and D. I. Andersson. 2000. Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. Science. 2000 **287(5457)**:1479-82.
5. Ching, C. K., K. P. Leung, R. W. H. Yung, S. K. Lam, B. C. Y. Wong, K. C. La, and C. L. Lai. 1996. Prevalence os metronidazole resistant *Helicobacter pylori* strains among Chinase peptic ulcer disease patients and normal controls in Hong Kong. Gut **38**:675-67.
6. Glupczynski, Y. 1998. Antimicrobial resistance in *Helicobacter pylori*: a global overview. Acta. Gastroenterol. Belg. **61**:357-36.
7. Goodwin, A., D. Kersulyte, G. Sisson, S. J. O. Veldhuyzen van Zanten, D. E. Berg, and P. S. Hoffman. 1998. Metronidazole resistance in *Helicobacter pylori* is due to null mutations in a gene (*rdxA*) that encodes an oxygen-insensitive NADPH nitroreductase. Mol. Microbiol. **28**:383-393.
8. Jeong, J. Y., A. K. Mukhopadhyay, D. Dailidiene, Y. Wang, B. Velapatio, R. H. Gilman, A. J. Parkinson, G. B Nair, B. C. Y. Wong, S. K. Lam, R. Mistry, I. Segal, Y. Yuan, H. Gao, T. Alarcon, M. L. Brea, Y. Ito, D. Kersulyte, H.-K. Lee, Y. Gong, A. Goodwin, P. S. Hoffman, and D. E. Berg. 2000. Sequential inactivation of *rdxA* (HP0954) and *frxA* (HP0642) nitroreductase genes cause moderate and high-level metronidazole resistance in *Helicobacter pylori*. J. Bacteriol. **182**:5082-5090.
9. Jeong, J. Y., A. K. Mukhopadhyay, J. K. Akada, D. Dailidiene, P. S. Hoffman and D. E. Berg. 2001. Roles of FrxA and RdxA nitroreductases of *Helicobacter pylori* in susceptibility and resistance to metronidazole. J. Bact. **183**:5155-5162.
10. Kersulyte, D., A. K. Mukhopadhyay, B. Velapati-o, W. W. Su, Z. J. Pan, C. Garcia, V. Hernandez, Y. Valdez, R. S. Mistry, R. H. Gilman, Y. Yuan, H. Gao, T. Alarcon, M. Lopez Brea, G. B. Nair, A. Chowdhury, S. Datta, M. Shirai, T. Nakazawa, R. Ally, I. Segal, B. C.Y. Wong, S. K. Lam, F. Olfat, T. Boren, L. Engstrand, O. Torres, R. Schneider, J. E. Thomas, S. Czinn, and D. E. Berg. 2000. Differences in Genotypes of *Helicobacter pylori* from Different Human Populations. J. Bacteriol. **182**:3210-3218.

11. Megraud, F., N. Lehn, T. Lind, E. Bayerdorffer, C. O'Morain, R. Spiller, P. Unge, S. Y. van Zanten, M. Wrangstadh, and C. F. Burman. 1999. Antimicrobial susceptibility testing of *Helicobacter pylori*in a large multicenter trial: the MACH 2 study. Antimicrob. Agents Chemother. **43**:2747-275.
12. Miyaji, H., T. Azuma, S. Ito, H. Suto, Y. Ito, Y. Yamazaki, F. Sato, M. Hirai, M. Kuriyama, T. Kato and Y. Kohli. 1997. Susceptibility of *Helicobacter pylori* isolates to metronidazole, clarithromycin and amoxycillin in vitro and in clinical treatment in Japan. Aliment. Pharmacol. Ther. **11**:1131-1136.
13. Mukhopadhyay, A. K., D. Kersulyte, J. Y. Jeong, S. Datta, Y. Ito, A. Chowdhury, S. Chowdhury, A. Santra, S. K. Bhattacharya, T. Azuma, G. B. Nair, and D. E. Berg. 2000. Distinctiveness of genotypes of *Helicobacter pylori* in Calcutta India. J. Bacteriol. **182**:3219-3227.
14. Mukhopadhyay A. K, J. Y. Jeong, D. Dailidiene, P. S. Hoffman and D. E. Berg. 2003. The *fdxA* ferredoxin gene can down-regulate *frxA* nitroreductase gene expression and is essential in many strains of *Helicobacter pylori*. J Bacteriol. **185(9)**:2927-2935.
15. Raudonikiene. A., N. Zakharova, W. W. Su, J. Y. Jeong, L. Bryden, P. S. Hoffman, D. E. Berg, and K. Severinov. 1999. *Helicobacter pylori*with separate beta- and beta'-subunits of RNA polymerase is viable and can colonize conventional mice. Mol. Microbiol. **32**:131-13.
16. Seyler, R. W., J. W. Olson and R. J. Maier. 2001. Superoxide dismutase-deficient mutants of Helicobacter pylori are hypersensitive to oxidative stress and defective in host colonization. Infect Immun. **69(6)**:4034-40.
17. Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. Ketchum, H. Klenk, S. Gill, B. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. Khalak, A. Glodek, K. McKenney, L. Fitzegerald, N. Lee, M. Adams, E. Hickey, D. Berg, J. Gocayne, T. Utterback, J. Peterson, J. Kelley, M. Cotton, J. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. Hayes, M. Borodovsky, P. Karp, H. Smith, C. Fraser, and J. Venter. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature. **388**:539-547.

초록 : *Helicobacter pylori*에서 metronidazole 내성이 미치는 생물학적 영향

이진혁[1] · 이선미 · 남원희 · 김은실 · 김진호[1] · 강호영[2] · 정진용

(울산대학교 의과대학 아산생명과학연구소, [1]울산대학교 의과대학 서울아산병원 소화기내과, [2]부산대학교 생명과학부)

메트로니다졸은 인체 위장질환의 원인균인 헬리코박터 파일로리를 박멸하기위해 처방하는 주요 약제이다. 그러나 인체로부터 분리한 헬리코박터 파일로리 균주는 메트로니다졸에 내성을 가지는 경우가 일반적이며, 이러한 내성원인은 이 균주의 염색체 상에 존재하는 두 종류의 nitroreductase 유전자인 *rdxA*와 *frxA* 유전자가 비활성화 됨에 따라 유발된다. 본 연구에서는 헬리코박터 파일로리 균에서 *rdxA* 유전자에 변이를 도입하여 메트로니다졸에 내성을 가지는 균주를 구축하여, 메트로니다졸 내성이 균주에 미치는 생물학적 영향을 관찰하고자 하였다. In vitro상에서 메트로니다졸 내성균주는 대조균과 비교하여 exponential phase에서는 거의 차이 없이 증식하였으나 stationary phase에서는 빠르게 생육활성을 잃는 것을 관찰할 수 있었다. 또한 생쥐를 이용한 동물 실험에서 메트로니다졸 내성균주는 생쥐의 위장 내에서 서식하는 능력을 상실함을 알 수 있었다. 그러나 이러한 생육활성을 회복시켜주는 compensatory mutation을 가진 균주를 쉽게 얻을 수 있었으며 이 균주는 생쥐에 감염시키면 위장 내에서 서식하는 능력을 회복함을 알 수 있었다.