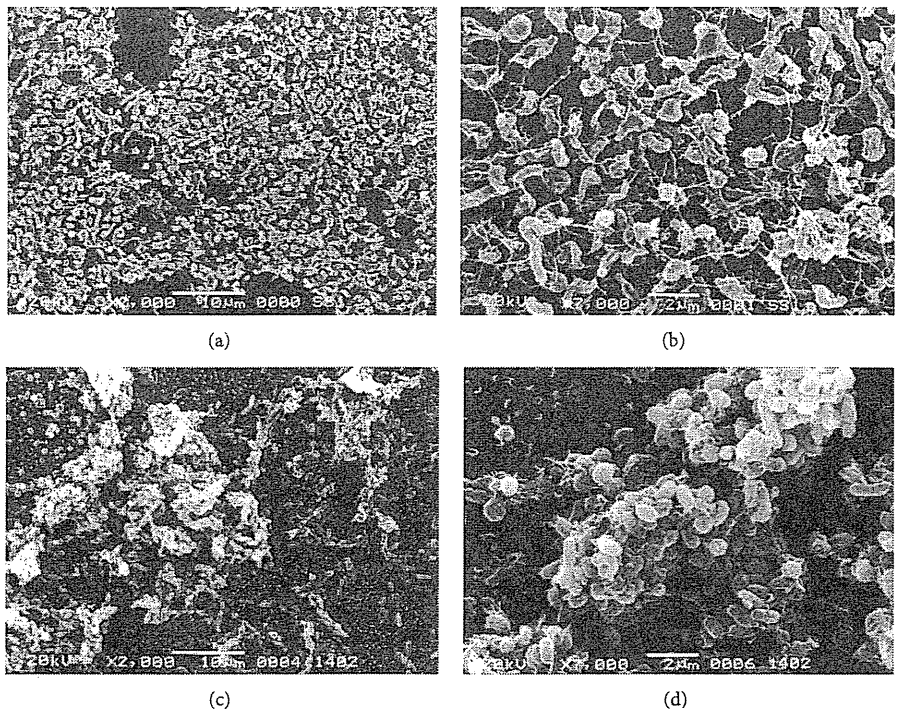

FIGURE 2: SEM images of *H. pylori* strains SS1 ((a) and (b)) and TK1402 ((c) and (d)) biofilms. The 3-day biofilm of each strain on cover glass was investigated using SEM. Photographs were taken at low (×2000; (a) and (c)) or high (×7000; (b) and (d)) magnification. Scale bar (2 μm) is shown at the bottom of each electron micrograph.

by scanning electron microscopy (SEM) (Figure 2) [34]. In the SS1 biofilms, the bacteria attached to glass surfaces in thin layers, and the biofilms consisted mainly of bleb-like or amorphous structures (Figures 2(a) and 2(b)). On the other hand, the TK1402 biofilms were composed primarily of cells with bacillary morphology which were clearly outlined (Figures 2(c) and 2(d)). We also analyzed the biofilm cells of the other strains using SEM. However, the majority of these biofilm cells consisted of autolysed cells, suggesting that the strong biofilm forming ability of TK402 may have resulted from an active metabolic state for a relatively long time without exhibiting morphological changes or autolysis. In addition, the biofilms of TK1402 strain showed the presence of many outer membrane vesicles (OMVs) on the glass surfaces as well as on the bacterial cell surfaces. These structures were not detected in the biofilms of the other strains. OMVs were more closely observed in the thin-sectioned biofilms using transmission electron microscopy (TEM) and the OMVs were located at the substratum-bacterium interface and in the extracellular spaces. In addition, biofilm formation by strain TK1402 was strongly correlated with the production of OMV. These results suggested that the OMV produced by strain TK1402 may serve as an EPS matrix for these biofilms. OMV production is a physiologically normal function of gram-negative bacteria [35, 36]. In *Pseudomonas aeruginosa*, OMVs have multifunctional biological roles including microbial interaction and host infection as well as maintenance of the structure of biofilm [37, 38]. In *Porphyromonas gingivalis*, OMVs promote attachment, aggregation, and biofilm formation and the functions of OMVs in biofilms have been discussed [39, 40]. Similar to most gram-negative bacteria, *H. pylori* released OMV into the extracellular space [41, 42]. Major protein and phospholipid components associated with the OMVs were identified [43]. We analyzed the protein profile of the OMV produced by strain TK1402 to determine which components of the OMV contribute to biofilm formation in *H. pylori*. The results indicated that a specific approximately 22 kDa protein might be involved in the biofilm forming ability of this strain [44]. Additional research is now in progress to determine what factors are directly involved in biofilm formation by strain TK1402.

Concerning the *H. pylori* biofilm matrix, Grande et al. demonstrated that extracellular DNA is a component of EPS structures and is important in stabilizing biofilm structures [45]. Yang et al. indicated that mannose-related proteoglycans (proteomannans) are one component of the EPS structures and proteomannans are also involved in the process of *H. pylori* biofilm formation [46]. They also reported that the neutrophil-activating protein A (NapA) is upregulated in biofilm cells compared to planktonic cells, and biofilm formation with a *napA* deficient mutant exhibited a different phenotypic biofilm. Recently, Grande et al. demonstrated that biofilms developed by multiple *H. pylori* strains are more complex than those associated with single strains and such conditions might promote genetic exchange favoring the generation of more virulent strains [47].

4. Quorum Sensing in *H. pylori*

The *luxS* gene is the only known quorum-sensing gene present in the sequenced *H. pylori* genome. Several reports indicated that *H. pylori* produces extracellular signaling molecules related to AI-2, and production of AI-2 is dependent on *luxS* function [48–50]. These reports have indicated that the production of AI-2 by *luxS* is growth-phase dependent, with maximal production occurring in the mid-exponential phase of growth. Several reports indicated that LuxS has an alternative role in regulation of motility by modulating flagellar transcription and flagellar biosynthesis [51, 52]. Our previous study also demonstrated that strain TK1402 *luxS* deficient mutant exhibited significantly lower motility than that of parental strain [53]. In addition, the *luxS* mutant exhibited a reduced infection rate relative to the wild-type parent strain TK1402 in a Mongolian gerbil model. Cole et al. reported the relations of *luxS* quorum sensing and biofilm formation in *H. pylori* [15]. They demonstrated that the *luxS* mutants of clinically isolated strains, SD3 and SD4, were approximately twofold more better at forming a biofilm than the parental strains. On the other hand, Doherty et al. indicated that LuxS fulfills primarily a metabolic role in the activated methyl cycle, which generates the *S*-adenosylmethionine required by methyltransferases and recycles the product via methionine as well as cell-to-cell signaling [54]. Further investigations are expected to elucidate the function of LuxS.

5. *H. pylori* Biofilm Formation in the Environment

The principal mode of transmission proposed for *H. pylori* is person to person contact via the faecal-oral, oral-oral, or gastro-oral routes [55–58]. However, especially in developing countries, the patterns of *H. pylori* transmission suggest a universal source for exposure rather than person to person transmission [59]. Thus, the drinking water supply was highlighted as an important source of *H. pylori* infection and, indeed, *H. pylori* was only detected with special procedures in water distribution systems [60, 61]. In addition, the role of water sources and associated biofilms acting as environmental transmitters of *H. pylori* has been suggested by the detection of *H. pylori* DNA by molecular methods, such as PCR, in sewage, well water, pond and river water, river water, and shallow ground water in developed countries as well as in developing countries [61–66]. These data suggested that *H. pylori* exists in water distribution systems and that the organism may survive in biofilms in these systems. However, in fact, it does not appear that *H. pylori* forms biofilms at locations which are relatively stressful conditions such as less than optimal temperatures and nutrient limitation. In oligotrophic water systems, the bacterial genera *Pedomicrobium*, *Hyphomicrobium*, *Gallionella*, and *Caulobacter* were regularly found [67]. It is likely that these bacteria form biofilms in drinking water distribution systems and are then contaminated with *H. pylori* from sewage, well water, pond and river water, river water, and shallow ground water and are embedded in such bacterial biofilm structures. Indeed, *H. pylori* has never been cultured from drinking water distribution systems using standard cultivation techniques [68, 69]. These reports indicated that it is impossible to distinguish between alive and dead cells of *H. pylori* in such systems. Recently, it was reported with several new methods such as in situ fluorescent hybridization (FISH) [20, 70] to detect viable *H. pylori* in various water sources. Continuous critical investigation is necessary as it remains unclear to what extent there is a health risk from this source.

6. *H. pylori* Biofilm Formation on Human Gastric Mucosa

The first photographic documentation of the existence of *H. pylori* biofilms on human gastric mucosa was reported by Carron et al. using endoscopically directed biopsies and scanning electron microscopy [17]. Mature biofilms were present and attached to the cell surface of *H. pylori*-positive specimens. Their group subsequently reported that, among patients with peptic ulcer disease who were tested urease positive for *H. pylori*, the average rate of total cell surfaces covered by biofilms was 97.3%, as opposed to 1.64% for urease-negative patients [18]. Cellini et al. reported that a prevalent S-shape *H. pylori* morphotype which coexisted with coccid aggregated bacteria embedded in an abundant matrix was demonstrated by SEM analysis with biopsies from patients harboring culturable bacteria [19]. On the other hand, samples from patients shown as *H. pylori*-positive only through the molecular methods showed clustered coccid bacteria arranged in a microbial biofilm. Cammarota et al. reported that, among the patients who had a history of at least four *H. pylori* eradication failures, SEM analysis of gastric biopsies showed that *H. pylori* formed biofilms on the gastric mucosa in all of the patients and that the biofilm disappeared in all of them when the microorganism was eradicated [71].

7. Effects of *H. pylori* Biofilms on Susceptibility to Antimicrobial Agents

Eradication of *H. pylori* is important not only for the treatment of gastric/duodenal ulcer, but also for the treatment and prevention of *H. pylori*-associated diseases such as gastric cancer, as well as for inhibiting the spread of this microorganism. For the eradication of *H. pylori*, a combination therapy using an antiacid agent (proton pump inhibitor (PPI) or H_2 blocker) and two anti-*H. pylori* agents (amoxicillin and either clarithromycin (CAM) or metronidazole) has been recommended [72–74]. Fluoroquinolones have also been selected as anti-*H. pylori* agents. In Japan, a combination of a proton pump inhibitor, amoxicillin, and CAM is commonly used in first-line eradication therapy [72]. However, CAM resistance is an increasing problem for the first-line therapy of *H. pylori* infection, since the major cause of eradication failure is thought to be the existence of CAM resistant *H. pylori* [72, 74–77]. CAM resistant *H. pylori* are extremely common and the frequency of CAM resistant clinical isolates ranges from approximately 10 to 30% [74, 78]. Point mutations in the domain V loop of the 23S rRNA gene (commonly an

Table 1: Generation of CAM resistance mutations in biofilm and planktonic cells. The 2-day and 3-day biofilms and planktonic cells were exposed to the indicated concentrations of CAM (biofilms were exposed to one-eighth, one-quarter, or one-half of the MBC of CAM at concentrations of 0.125, 0.25, and 0.5 μg/mL, concentrations which are equivalent to 8x, 16x, and 32x MIC and planktonic cultures were also exposed to one-quarter or one-half of the MBC of CAM at concentrations of 0.063 and 0.125 μg/mL, concentrations which are equivalent to 4x and 8x MIC) for 24 h under microaerobic conditions at 37°C with shaking. After incubation, cells were recovered in fresh *Brucella* supplemented with 7% FCS agar, and the generation of CAM resistant mutants was assessed in media supplemented with 1.0 μg/mL CAM. When no CAM resistant cells were detected, exposure to CAM was repeated up to 5 times. The table indicates the accumulation ratio of the generated CAM resistance in biofilms (number of samples was 12 or 13) or in planktonic cultures (number of samples was 12).

Samples	Passage time				
CAM concentrations	1st	2nd	3rd	4th	5th
2-day biofilm					
CAM 0.5 μg/mL	0/12 (0%)	0/12 (0%)	1/12 (8%)	2/12 (17%)	4/12 (33%)
CAM 0.25 μg/mL	1/12 (8%)	4/12 (33%)	6/12 (50%)	8/12 (67%)	9/12 (75%)
CAM 0.125 μg/mL	0/12 (0%)	1/12 (8%)	2/12 (17%)	3/12 (25%)	4/12 (33%)
2-day planktonic					
CAM 0.125 μg/mL	0/12 (0%)	0/12 (0%)	1/12 (8%)	4/12 (33%)	4/12 (33%)
CAM 0.063 μg/mL	0/12 (0%)	0/12 (0%)	3/12 (25%)	3/12 (25%)	3/12 (25%)
3-day biofilm					
CAM 0.5 μg/mL	1/12 (8%)	3/12 (25%)	4/12 (33%)	6/12 (50%)	6/12 (50%)
CAM 0.25 μg/mL	1/13 (8%)	5/13 (38%)	11/13 (85%)	11/13 (85%)	11/13 (85%)
CAM 0.125 μg/mL	1/13 (8%)	2/13 (15)	3/13 (23%)	5/13 (38%)	6/13 (46%)
3-day planktonic					
CAM 0.125 μg/mL	0/12 (0%)	1/12 (8%)	1/12 (8%)	1/12 (8%)	3/12 (25%)
CAM 0.063 μg/mL	1/12 (8%)	1/12 (8%)	1/12 (8%)	1/12 (8%)	3/12 (25%)

adenine-to-guanine transition at position 2142 or 2143) have been reported as the basis for resistance [72, 74–79].

In other bacterial biofilms, biofilm grown cells express properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents [26, 80–83]. Based on these reports, the biofilm cells can become 10–1000 times more resistant to the effects of antimicrobial agents. Multiple mechanisms of biofilm resistance to antimicrobial compounds were suggested: (i) failure of the antimicrobial compounds to penetrate the biofilm, (ii) slow growth of the biofilm cells owing to nutrient limitation, and (iii) activation of the general stress response [26, 84–88]. However, the effect of *H. pylori* biofilm formation on antibiotics susceptibility is not well documented. Thus, we investigated the effects of CAM on *H. pylori* biofilms [89]. Biofilm formation in *H. pylori* increased the resistance to CAM at minimum inhibitory concentration (MIC) levels by up to 4-fold in 2-day biofilms (intermediated biofilms) and to 16-fold in 3-day biofilms (mature biofilms) as well as minimum bactericidal concentration (MBC) levels by up to 4-fold compared to planktonic cells. Participation of the efflux pumps of the resistance-nodulation-cell division (RND) family was involved in the development of CAM resistance in *H. pylori* biofilm and failure of CAM penetration into the biofilm interior due to the presence of the extracellular matrix was also demonstrated. In addition, we demonstrated that *H. pylori* biofilm formation can affect the generation of CAM resistance mutations (Table 1). CAM resistant cells were detected more frequently in biofilms after treatment with CAM. Our results indicated that the relatively high concentration, especially one-quarter of MBC (0.25 μg/mL, which are concentrations equivalent to 16x MIC), of CAM may facilitate the generation of CAM resistance mutations in *H. pylori* biofilms.

8. Therapy for Preventing *H. pylori* Biofilm Infection

Antibiotic resistance in *H. pylori* can therefore be acquired by the selection of spontaneous mutation events that occur due to the magnitude and duration of antibiotic use on the human gastric mucosa. Nakamura et al. reported that CAM concentrations in gastric juices, mucosa, or serum after administration of 500 mg of the drug for 7 days were 550.6, 64.6, and 2.5 μg/mL at 2 hours after administration and 43.4, 36.2, and 2.2 μg/mL at 6 hours, respectively [90]. These concentrations might be sufficient to reduce the levels of *H. pylori in vivo* so that this microorganism formed biofilms. However, to reach such high concentrations of CAM on the gastric mucosa for extended periods, the drug needs to be taken with sufficient dosage. In addition, in cases with inadequate compliance with eradication therapy, the concentration of CAM does not reach high levels in the gastric mucosa. Further, macrolides including CAM are frequently used in the treatment of various infectious diseases in pediatric, respiratory, and otorhinolaryngology settings. In these cases, biofilm formation by *H. pylori* may contribute to the acquisition of CAM resistance.

Novel approaches to prevent biofilm formation and to treat infections by biofilm-forming bacteria are currently under development [91, 92]. Recently, a clinical trial for effective strategies targeting *H. pylori* biofilm infections through the use of molecules such as *N*-acetylcysteine (NAC) was

reported [71, 93]. NAC is a mucolytic and a thiol-containing antioxidant agent and is considered a nonantibiotic drug that has antibacterial properties. In 1977, Parry and Neu found that NAC had the ability to inhibit the growth of both gram-positive and gram-negative bacteria, including *Staphylococcus aureus*, *P. aeruginosa*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* [94]. The antibacterial effect of NAC may be due to competitively inhibiting amino acid (cysteine) utilization or by virtue of possessing a sulfhydryl group it may react with bacterial cell proteins. Moreover, previous studies demonstrated decreased biofilm formation by a variety of bacteria in the presence of NAC [95–98], leading to an inhibition of bacterial adherence, a reduction in the production of the extracellular polysaccharide matrix promoting the disruption of mature biofilms, and a reduction in sessile cell viability [95–98]. Relative to *H. pylori* biofilms, NAC is effective in both inhibiting *H. pylori* biofilm formation and disrupting developed biofilms *in vitro* [71]. In addition, NAC treatment preceding the initiation of antibiotic eradication therapy is able to provide eradication of resistant *H. pylori* infections. Large scale studies regarding the effectiveness of NAC *in vivo* for reducing *H. pylori* biofilms are still required.

9. Conclusions

Pathogenic bacteria including *H. pylori* within biofilms can escape from both host immune responses and the effects of antimicrobial agents. Consequently, chronic infections by biofilm forming bacteria become troublesome and difficult to treat. Some of the previous studies have shown that *H. pylori* forms biofilm on human gastric mucosa. Nevertheless, assessment of *H. pylori* strain susceptibility to antibiotics *in vitro* has traditionally been evaluated using planktonic cells, so that MICs are not reliable predictors of the antibiotic effects in the human stomach. The assessment of the ability to form biofilms in *H. pylori* could play an important role in preventing and controlling the generation of antibiotic resistance. It is expected that enhancing our knowledge of *H. pylori* biofilm formation will lead to new treatment therapies for preventing *H. pylori* infections. However, it is recognized that our understanding of *H. pylori* biofilm formation is still in its infancy. Further studies of the mechanism of *H. pylori* biofilm formation need to be performed. In addition, investigation into novel *H. pylori* eradication strategies for the human gastric mucosa using biofilm-dissolving compounds, quorum sensing inhibitors, or conventional antibiotics may provide advantages in resolving *H. pylori* infections.

Conflict of Interests

The authors have declared that no competing interests exist.

Acknowledgments

This work was supported in part by JSPS KAKENHI no. 24593166 and a grant for Strategic Research Base Development Program for Private Universities from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (MEXT), 2010–2014 (S1001024). This work was also partially supported by Labour Sciences Research Grants for Research on global health issues from the Ministry of Health, Labor and Welfare, Japan.

References

[1] B. J. Marshall and J. R. Warren, "Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration," *The Lancet*, vol. 1, no. 8390, pp. 1311–1315, 1984.

[2] B. E. Dunn, H. Cohen, and M. J. Blaser, "*Helicobacter pylori*," *Clinical Microbiology Reviews*, vol. 10, no. 4, pp. 720–741, 1997.

[3] M. J. Blaser, "*Helicobacter pylori*: its role in disease," *Clinical Infectious Diseases*, vol. 15, no. 3, pp. 386–393, 1992.

[4] D. Y. Graham, "*Campylobacter pylori* and peptic ulcer disease," *Gastroenterology*, vol. 96, no. 2, supplement 2, pp. 615–625, 1989.

[5] J. Parsonnet, G. D. Friedman, D. P. Vandersteen et al., "*Helicobacter pylori* infection and the risk of gastric carcinoma," *The New England Journal of Medicine*, vol. 325, no. 16, pp. 1127–1131, 1991.

[6] A. C. Wotherspoon, C. Doglioni, T. C. Diss et al., "Regression of primary low-grade-B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*," *The Lancet*, vol. 342, no. 8871, pp. 575–577, 1993.

[7] International Agency for Research on Cancer and World Health Organization, "Schistosomes, liver flukes, and *Helicobacter pylori*," in *Monographs on the Evaluation of Carcinogenic Risks to Humans*, vol. 61, pp. 218–220, 1994.

[8] J. J. E. Bijlsma, C. M. Vandenbroucke-Grauls, S. H. Phadnis, and J. G. Kusters, "Identification of virulence genes of *Helicobacter pylori* by random insertion mutagenesis," *Infection and Immunity*, vol. 67, no. 5, pp. 2433–2440, 1999.

[9] G. Bode, F. Mauch, and P. Malfertheiner, "The coccoid forms of *Helicobacter pylori*. Criteria for their viability," *Epidemiology and Infection*, vol. 111, no. 3, pp. 483–490, 1993.

[10] L. Cellini, N. Allocati, E. Di Campli, and B. Dainelli, "*Helicobacter pylori*: a fickle germ," *Microbiology and Immunology*, vol. 38, no. 1, pp. 25–30, 1994.

[11] H. Mizoguchi, T. Fujioka, and M. Nasu, "Evidence for viability of coccoid forms of *Helicobacter pylori*," *Journal of Gastroenterology*, vol. 34, supplement 11, pp. 32–36, 1999.

[12] D. J. Reynolds and C. W. Penn, "Characteristics of *Helicobacter pylori* growth in a defined medium and determination of its amino acid requirements," *Microbiology*, vol. 140, no. 10, pp. 2649–2656, 1994.

[13] M. Shahamat, U. Mai, C. Paszko-Kolva, M. Kessel, and R. R. Colwell, "Use of autoradiography to assess viability of *Helicobacter pylori* in water," *Applied and Environmental Microbiology*, vol. 59, no. 4, pp. 1231–1235, 1993.

[14] R. M. Stark, G. J. Gerwig, R. S. Pitman et al., "Biofilm formation by *Helicobacter pylori*," *Letters in Applied Microbiology*, vol. 28, no. 2, pp. 121–126, 1999.

[15] S. P. Cole, J. Harwood, R. Lee, R. She, and D. G. Guiney, "Characterization of monospecies biofilm formation by *Helicobacter pylori*," *Journal of Bacteriology*, vol. 186, no. 10, pp. 3124–3132, 2004.

[16] L. Cellini, R. Grande, E. di Campli et al., "Characterization of an *Helicobacter pylori* environmental strain," *Journal of Applied Microbiology*, vol. 105, no. 3, pp. 761–769, 2008.

[17] M. A. Carron, V. R. Tran, C. Sugawa, and J. M. Coticchia, "Identification of *Helicobacter pylori* biofilms in human gastric

mucosa," *Journal of Gastrointestinal Surgery*, vol. 10, no. 5, pp. 712–717, 2006.

[18] J. M. Coticchia, C. Sugawa, V. R. Tran, J. Gurrola, E. Kowalski, and M. A. Carron, "Presence and density of *Helicobacter pylori* biofilms in human gastric mucosa in patients with peptic ulcer disease," *Journal of Gastrointestinal Surgery*, vol. 10, no. 6, pp. 883–889, 2006.

[19] L. Cellini, R. Grande, E. D. Campli et al., "Dynamic colonization of *Helicobacter pylori* in human gastric mucosa," *Scandinavian Journal of Gastroenterology*, vol. 43, no. 2, pp. 178–185, 2008.

[20] A. García, M. J. Salas-Jara, C. Herrera, and C. González, "Biofilm and *Helicobacter pylori*: from environment to human host," *World Journal of Gastroenterology*, vol. 20, no. 19, pp. 5632–5638, 2014.

[21] M. R. Parsek and P. K. Singh, "Bacterial biofilms: an emerging link to disease pathogenesis," *Annual Review of Microbiology*, vol. 57, pp. 677–701, 2003.

[22] C. J. Whittaker, C. M. Klier, and P. E. Kolenbrander, "Mechanisms of adhesion by oral bacteria," *Annual Review of Microbiology*, vol. 50, pp. 513–552, 1996.

[23] C. B. Whitchurch, T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick, "Extracellular DNA required for bacterial biofilm formation," *Science*, vol. 295, no. 5559, p. 1487, 2002.

[24] J. W. Costerton, P. S. Stewart, and E. P. Greenberg, "Bacterial biofilms: a common cause of persistent infections," *Science*, vol. 284, no. 5418, pp. 1318–1322, 1999.

[25] G. O'Toole, H. B. Kaplan, and R. Kolter, "Biofilm formation as microbial development," *Annual Review of Microbiology*, vol. 54, pp. 49–79, 2000.

[26] T.-F. C. Mah and G. A. O'Toole, "Mechanisms of biofilm resistance to antimicrobial agents," *Trends in Microbiology*, vol. 9, no. 1, pp. 34–39, 2001.

[27] D. G. Davies, A. M. Chakrabarty, and G. G. Geesey, "Exopolysaccharide production in biofilms: Substratum activation of alginate gene expression by *Pseudomonas aeruginosa*," *Applied and Environmental Microbiology*, vol. 59, no. 4, pp. 1181–1186, 1993.

[28] V. Sperandio, A. G. Torres, B. Jarvis, J. P. Nataro, and J. B. Kaper, "Bacteria-host communication: the language of hormones," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 15, pp. 8951–8956, 2003.

[29] W. C. Fuqua, S. C. Winans, and E. P. Greenberg, "Quorum sensing in bacteria: the LuxR-LuxI family of cell density—responsive transcriptional regulators," *Journal of Bacteriology*, vol. 176, no. 2, pp. 269–275, 1994.

[30] C. Fuqua, M. R. Parsek, and E. P. Greenberg, "Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing," *Annual Review of Genetics*, vol. 35, pp. 439–468, 2001.

[31] M. G. Surette and B. L. Bassler, "Regulation of autoinducer production in *Salmonella typhimurium*," *Molecular Microbiology*, vol. 31, no. 2, pp. 585–595, 1999.

[32] X. Chen, S. Schauder, N. Potier et al., "Structural identification of a bacterial quorum-sensing signal containing boron," *Nature*, vol. 415, no. 6871, pp. 545–549, 2002.

[33] H. Yonezawa, T. Osaki, S. Kurata, C. Zaman, T. Hanawa, and S. Kamiya, "Assessment of *in vitro* biofilm formation by *Helicobacter pylori*," *Journal of Gastroenterology and Hepatology*, vol. 25, supplement 1, pp. S90–S94, 2010.

[34] H. Yonezawa, T. Osaki, S. Kurata et al., "Outer membrane vesicles of *Helicobacter pylori* TK1402 are involved in biofilm formation," *BMC Microbiology*, vol. 9, article 197, 2009.

[35] E.-Y. Lee, D.-S. Choi, K.-P. Kim, and Y. S. Gho, "Proteomics in Gram-negative bacterial outer membrane vesicles," *Mass Spectrometry Reviews*, vol. 27, no. 6, pp. 535–555, 2008.

[36] T. J. Beveridge, "Structures of gram-negative cell walls and their derived membrane vesicles," *Journal of Bacteriology*, vol. 181, no. 16, pp. 4725–4733, 1999.

[37] S. R. Schooling and T. J. Beveridge, "Membrane vesicles: an overlooked component of the matrices of biofilms," *Journal of Bacteriology*, vol. 188, no. 16, pp. 5945–5957, 2006.

[38] Y. Tashiro, H. Uchiyama, and N. Nomura, "Multifunctional membrane vesicles in *Pseudomonas aeruginosa*," *Environmental Microbiology*, vol. 14, no. 6, pp. 1349–1362, 2012.

[39] S. Inagaki, S. Onishi, H. K. Kuramitsu, and A. Sharma, "*Porphyromonas gingivalis* vesicles enhance attachment, and the leucine-rich repeat BspA protein is required for invasion of epithelial cells by *Tannerella forsythia*," *Infection and Immunity*, vol. 74, no. 9, pp. 5023–5028, 2006.

[40] M. Yamaguchi, K. Sato, H. Yukitake, Y. Noiri, S. Ebisu, and K. Nakayama, "A *Porphyromonas gingivalis* mutant defective in a putative glycosyltransferase exhibits defective biosynthesis of the polysaccharide portions of lipopolysaccharide, decreased gingipain activities, strong autoaggregation, and increased biofilm formation," *Infection and Immunity*, vol. 78, no. 9, pp. 3801–3812, 2010.

[41] R. Fiocca, V. Necchi, P. Sommi et al., "Release of *Helicobacter pylori* vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium," *The Journal of Pathology*, vol. 188, no. 2, pp. 220–226, 1999.

[42] J. I. Keenan, R. A. Allardyce, and P. F. Bagshaw, "Dual silver staining to characterise *Helicobacter* spp. outer membrane components," *Journal of Immunological Methods*, vol. 209, no. 1, pp. 17–24, 1997.

[43] A. Olofsson, A. Vallström, K. Petzold et al., "Biochemical and functional characterization of *Helicobacter pylori* vesicles," *Molecular Microbiology*, vol. 77, no. 6, pp. 1539–1555, 2010.

[44] H. Yonezawa, T. Osaki, T. Woo et al., "Analysis of outer membrane vesicle protein involved in biofilm formation of *Helicobacter pylori*," *Anaerobe*, vol. 17, no. 6, pp. 388–390, 2011.

[45] R. Grande, M. di Giulio, L. J. Bessa et al., "Extracellular DNA in *Helicobacter pylori* biofilm: a backstairs rumour," *Journal of Applied Microbiology*, vol. 110, no. 2, pp. 490–498, 2011.

[46] F.-L. Yang, A. M. Hassanbhai, H.-Y. Chen et al., "Proteomannans in Biofilm of *Helicobacter pylori* ATCC 43504," *Helicobacter*, vol. 16, no. 2, pp. 89–98, 2011.

[47] R. Grande, E. Di Campli, S. Di Bartolomeo et al., "*Helicobacter pylori* biofilm: a protective environment for bacterial recombination," *Journal of Applied Microbiology*, vol. 113, no. 3, pp. 669–676, 2012.

[48] M. H. Forsyth and T. L. Cover, "Intercellular communication in *Helicobacter pylori*: *luxS* is essential for the production of an extracellular signaling molecule," *Infection and Immunity*, vol. 68, no. 6, pp. 3193–3199, 2000.

[49] E. A. Joyce, B. L. Bassler, and A. Wright, "Evidence for a signaling system in *Helicobacter pylori*: detection of a *luxS*-encoded autoinducer," *Journal of Bacteriology*, vol. 182, no. 13, pp. 3638–3643, 2000.

[50] W. K. Lee, K. Ogura, J. T. Loh, T. L. Cover, and D. E. Berg, "Quantitative effect of *luxS* gene inactivation on the fitness of *Helicobacter pylori*," *Applied and Environmental Microbiology*, vol. 72, no. 10, pp. 6615–6622, 2006.

[51] B. A. Rader, C. Wreden, K. G. Hicks, E. G. Sweeney, K. M. Ottemann, and K. Guillemin, "*Helicobacter pylori* perceives the quorum-sensing molecule AI-2 as a chemorepellent via the chemoreceptor TlpB," *Microbiology*, vol. 157, no. 9, pp. 2445–2455, 2011.

[52] F. Shen, L. Hobley, N. Doherty et al., "In *Helicobacter pylori* autoinducer-2, but not LuxS/MccAB catalysed reverse transsulphuration, regulates motility through modulation of flagellar gene transcription," *BMC Microbiology*, vol. 10, article 210, 2010.

[53] T. Osaki, T. Hanawa, T. Manzoku et al., "Mutation of *luxS* affects motility and infectivity of *Helicobacter pylori* in gastric mucosa of a Mongolian gerbil model," *Journal of Medical Microbiology*, vol. 55, no. 11, pp. 1477–1485, 2006.

[54] N. C. Doherty, F. Shen, N. M. Halliday et al., "In *Helicobacter pylori*, LuxS is a key enzyme in cysteine provision through a reverse transsulfuration pathway," *Journal of Bacteriology*, vol. 192, no. 5, pp. 1184–1192, 2010.

[55] J. E. Thomas, G. R. Gibson, M. K. Darboe, A. Dale, and L. T. Weaver, "Isolation of *Helicobacter pylori* from human faeces," *The Lancet*, vol. 340, no. 8829, pp. 1194–1195, 1992.

[56] I. M. Madinier, T. M. Fosse, and R. A. Monteil, "Oral Carriage of *Helicobacter pylori*: a review," *Journal of Periodontology*, vol. 68, no. 1, pp. 2–6, 1997.

[57] J. Parsonnet, H. Shmuely, and T. Haggerty, "Fecal and oral shedding of *Helicobacter pylori* from healthy infected adults," *The Journal of the American Medical Association*, vol. 282, no. 23, pp. 2240–2245, 1999.

[58] T. Osaki, M. Okuda, J. Ueda et al., "Multilocus sequence typing of DNA from faecal specimens for the analysis of intrafamilial transmission of *Helicobacter pylori*," *Journal of Medical Microbiology*, vol. 62, no. 5, pp. 761–765, 2013.

[59] Y. Akcan, S. Ersan, M. Alper, Z. Bjcjk, and N. Aytug, "The transmission of *Helicobacter pylori* via exposure to common sources outweighs the person-to-person contact among spouses in developing countries," *The American Journal of Gastroenterology*, vol. 95, no. 1, pp. 317–319, 2000.

[60] P. D. Klein, D. Y. Graham, A. Gaillour et al., "Water source as risk factor for *Helicobacter pylori* infection in Peruvian children," *The Lancet*, vol. 337, no. 8756, pp. 1503–1506, 1991.

[61] C. L. Watson, R. J. Owen, B. Said et al., "Detection of *Helicobacter pylori* by PCR but not culture in water and biofilm samples from drinking water distribution systems in England," *Journal of Applied Microbiology*, vol. 97, no. 4, pp. 690–698, 2004.

[62] J. P. Hegarty, M. T. Dowd, and K. H. Baker, "Occurrence of *Helicobacter pylori* in surface water in the United States," *Journal of Applied Microbiology*, vol. 87, no. 5, pp. 697–701, 1999.

[63] T. Horiuchi, T. Ohkusa, M. Watanabe, D. Kobayashi, H. Miwa, and Y. Eishi, "*Helicobacter pylori* DNA in drinking water in Japan," *Microbiology and Immunology*, vol. 45, no. 7, pp. 515–519, 2001.

[64] Y. Imanishi, T. Ogata, A. Matsuzuka et al., "Possibility for the presence of *Helicobacter pylori* in drinking well water," *Kansenshogaku Zasshi*, vol. 77, no. 1, pp. 18–23, 2003.

[65] Y. Lu, T. E. Redlinger, R. Avitia, A. Galindo, and K. Goodman, "Isolation and genotyping of *Helicobacter pylori* from untreated municipal wastewater," *Applied and Environmental Microbiology*, vol. 68, no. 3, pp. 1436–1439, 2002.

[66] Y. Moreno, S. Botella, J. L. Alonso, M. A. Ferrús, M. Hernández, and J. Hernández, "Specific detection of *Arcobacter* and *Campylobacter* strains in water and sewage by PCR and fluorescent in situ hybridization," *Applied and Environmental Microbiology*, vol. 69, no. 2, pp. 1181–1186, 2003.

[67] U. Szewzyk, R. Szewzyk, W. Manz, and K. H. Schleifer, "Microbiogical safety of drinking water," *Annual Review of Microbiology*, vol. 54, pp. 81–127, 2000.

[68] N. F. Azevedo, N. Guimarães, C. Figueiredo, C. W. Keevil, and M. J. Vieira, "A new model for the transmission of *Helicobacter pylori*: role of environmental reservoirs as gene pools to increase strain diversity," *Critical Reviews in Microbiology*, vol. 33, no. 3, pp. 157–169, 2007.

[69] M. S. Gião, N. F. Azevedo, S. A. Wilks, M. J. Vieira, and C. W. Keevil, "Persistence of *Helicobacter pylori* in heterotrophic drinking-water biofilms," *Applied and Environmental Microbiology*, vol. 74, no. 19, pp. 5898–5904, 2008.

[70] S. L. Percival and L. Suleman, "Biofilms and *Helicobacter pylori*: dissemination and persistence within the environment and host," *World Journal of Gastrointestinal Pathophysiology*, vol. 15, pp. 122–132, 2014.

[71] G. Cammarota, G. Branca, F. Ardito et al., "Biofilm demolition and antibiotic treatment to eradicate resistant *Helicobacter pylori*: a clinical trial," *Clinical Gastroenterology and Hepatology*, vol. 8, no. 9, pp. 817.e3–820.e3, 2010.

[72] M. Asaka, M. Kato, S.-I. Takahashi et al., "Guidelines for the management of *Helicobacter pylori* infection in Japan: 2009 revised edition," *Helicobacter*, vol. 15, no. 1, pp. 1–20, 2010.

[73] B. J. Egan, L. Marzio, H. O'Connor, and C. O'Morain, "Treatment of *Helicobacter pylori* infection," *Helicobacter*, vol. 13, supplement 1, pp. 35–40, 2008.

[74] P. Malfertheiner, F. Megraud, C. A. O'Morain et al., "Management of *Helicobacter pylori* infection—the Maastricht IV/Florence consensus report," *Gut*, vol. 61, no. 5, pp. 646–664, 2012.

[75] D. Y. Graham, W. A. de Boer, and G. N. J. Tytgat, "Choosing the best anti-*Helicobacter pylori* therapy: effect of antimicrobial resistance," *The American Journal of Gastroenterology*, vol. 91, no. 6, pp. 1072–1076, 1996.

[76] R. J. Adamek, S. Suerbaum, B. Pfaffenbach, and W. Opferkuch, "Primary and acquired *Helicobacter priori* resistance to clarithromycin, metronidazole, and amoxicillin—influence on treatment outcome," *American Journal of Gastroenterology*, vol. 93, no. 3, pp. 386–389, 1998.

[77] F. Mégraud and H. P. Doermann, "Clinical relevance of resistant strains of *Helicobacter pylori*: a review of current data," *Gut*, vol. 43, supplement 1, pp. S61–S65, 1998.

[78] N. Horiki, F. Omata, M. Uemura et al., "Annual change of primary resistance to clarithromycin among *Helicobacter pylori* isolates from 1996 through 2008 in Japan," *Helicobacter*, vol. 14, no. 5, pp. 86–90, 2009.

[79] J. Versalovic, M. S. Osato, K. Spakovsky et al., "Point mutations in the 23S rRNA gene of *Helicobacter pylori* associated with different levels of clarithromycin resistance," *Journal of Antimicrobial Chemotherapy*, vol. 40, no. 2, pp. 283–286, 1997.

[80] B. L. T. Prosser, D. Taylor, B. A. Dix, and R. Cleeland, "Method of evaluating effects of antibiotics on bacterial biofilm," *Antimicrobial Agents and Chemotherapy*, vol. 31, no. 10, pp. 1502–1506, 1987.

[81] J. C. Nickel, I. Ruseska, J. B. Wright, and J. W. Costerton, "Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material," *Antimicrobial Agents and Chemotherapy*, vol. 27, no. 4, pp. 619–624, 1985.

[82] A. G. Gristina, C. D. Hobgood, L. X. Webb, and Q. N. Myrvik, "Adhesive colonization of biomaterials and antibiotic resistance," *Biomaterials*, vol. 8, no. 6, pp. 423–426, 1987.

[83] R. C. Evans and C. J. Holmes, "Effect of vancomycin hydrochloride on *Staphylococcus epidermidis* biofilm associated with silicone elastomer," *Antimicrobial Agents and Chemotherapy*, vol. 31, no. 6, pp. 889–894, 1987.

[84] J. W. Costerton, Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott, "Microbial biofilms," *Annual Review of Microbiology*, vol. 49, pp. 711–745, 1995.

[85] J. L. Adams and R. J. C. McLean, "Impact of *rpoS* deletion on *Escherichia coli* biofilms," *Applied and Environmental Microbiology*, vol. 65, no. 9, pp. 4285–4287, 1999.

[86] J. N. Anderl, M. J. Franklin, and P. S. Stewart, "Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin," *Antimicrobial Agents and Chemotherapy*, vol. 44, no. 7, pp. 1818–1824, 2000.

[87] M. Desai, T. Bühler, P. H. Weller, and M. R. W. Brown, "Increasing resistance of planktonic and biofilm cultures of *Burkholderia cepacia* to ciprofloxacin and ceftazidime during exponential growth," *Journal of Antimicrobial Chemotherapy*, vol. 42, no. 2, pp. 153–160, 1998.

[88] W. M. Dunne Jr., E. O. Mason Jr., and S. L. Kaplan, "Diffusion of rifampin and vancomycin through a *Staphylococcus epidermidis* biofilm," *Antimicrobial Agents and Chemotherapy*, vol. 37, no. 12, pp. 2522–2526, 1993.

[89] H. Yonezawa, T. Osaki, T. Hanawa, S. Kurata, K. Ochiai, and S. Kamiya, "Impact of *Helicobacter pylori* biofilm formation on clarithromycin susceptibility and generation of resistance mutations," *PLoS ONE*, vol. 8, no. 9, Article ID e73301, 2013.

[90] M. Nakamura, R. C. Spiller, D. A. Barrett et al., "Gastric juice, gastric tissue and blood antibiotic concentrations following omeprazole, amoxicillin and clarithromycin triple therapy," *Helicobacter*, vol. 8, no. 4, pp. 294–299, 2003.

[91] U. Römling and C. Balsalobre, "Biofilm infections, their resilience to therapy and innovative treatment strategies," *Journal of Internal Medicine*, vol. 272, no. 6, pp. 541–561, 2012.

[92] R. M. Donlan and J. W. Costerton, "Biofilms: survival mechanisms of clinically relevant microorganisms," *Clinical Microbiology Reviews*, vol. 15, no. 2, pp. 167–193, 2002.

[93] G. Cammarota, M. Sanguinetti, A. Gallo, and B. Posteraro, "Review article: biofilm formation by *Helicobacter pylori* as a target for eradication of resistant infection," *Alimentary Pharmacology and Therapeutics*, vol. 36, no. 3, pp. 222–230, 2012.

[94] M. F. Parry and H. C. Neu, "Effect of N-acetylcysteine on antibiotic activity and bacterial growth in vitro," *Journal of Clinical Microbiology*, vol. 5, no. 1, pp. 58–61, 1977.

[95] A. Marchese, M. Bozzolasco, L. Gualco, E. A. Debbia, G. C. Schito, and A. M. Schito, "Effect of fosfomycin alone and in combination with N-acetylcysteine on *E. coli* biofilms," *International Journal of Antimicrobial Agents*, vol. 22, no. 2, pp. S95–S100, 2003.

[96] C. Pérez-Giraldo, A. Rodriguez-Benito, F. J. Morán, C. Hurtado, M. T. Blanco, and A. C. Gómez-Garcí, "Influence of N-acetylcysteine on the formation of biofilm by *Staphylococcus epidermidis*," *Journal of Antimicrobial Chemotherapy*, vol. 39, no. 5, pp. 643–646, 1997.

[97] L. Q. Schwandt, R. van Weissenbruch, I. Stokroos, H. C. van der Mei, H. J. Busscher, and F. W. J. Albers, "Prevention of biofilm formation by dairy products and N-acetylcysteine on voice prostheses in an artificial throat," *Acta Oto-Laryngologica*, vol. 124, no. 6, pp. 726–731, 2004.

[98] A.-C. Olofsson, M. Hermansson, and H. Elwing, "*N*-acetyl-L-cysteine affects growth, extracellular polysaccharide production, and bacterial biofilm formation on solid surfaces," *Applied and Environmental Microbiology*, vol. 69, no. 8, pp. 4814–4822, 2003.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 304768, 2 pages
http://dx.doi.org/10.1155/2015/304768

Editorial
Helicobacter pylori and Pathogenesis

Akio Tomoda,[1] Shigeru Kamiya,[2] and Hidekazu Suzuki[3]

[1]*Tokyo Medical University, Tokyo 160-8402, Japan*
[2]*Kyorin University School of Medicine, Mitaka 181-8611, Japan*
[3]*Keio University School of Medicine, Tokyo 160-8582, Japan*

Correspondence should be addressed to Akio Tomoda; tomoda@tokyo-med.ac.jp

Received 9 March 2015; Accepted 9 March 2015

By calling for manuscripts for this special issue, many manuscripts were submitted to the editorial office. After careful reviewing by expert referees, highly qualified papers concerning the topics were accepted as review and research articles for publication in the journal. From the accepted articles, some interesting ones are introduced as follows.

S. K. Pachathundikandi et al. reviewed an interplay of *H. pylori* with toll-like receptors (TLRs). TRL2 is able to recognize various different pathogen associated molecular patterns (PAMPs) including lipoproteins, lipoteichoic acid, and peptidoglycan. *H. pylori* activated NF-κB primarily through TLR2 and induced chemokine expression. Lipopolysaccharide (LPS) of *H. pylori* was identified as the ligand for TLR4, and *H. pylori* induced the secretion of IL-12 and IL-10 in mouse macrophages through TLR4/MyD88. It was also shown that *H. pylori* LPS can promote proliferation and progression of gastric cancer cells via a TLR4-dependent pathway. Flagellin from *H. pylori* is the ligand for TLR5, and the involvement of TLR5 in the recognition and further inflammatory processes is important for establishing a persistent infection of *H. pylori* at the mucosal surface. A chimeric flagellin composed of terminal regions from *Escherichia coli* and the middle region from *H. pylori* was reported to activate TLR5, suggesting that the chimeric flagellin might be a vaccine candidate with significant protection against *H. pylori* infection. Correlation between TLR8/9 sensing nucleic acids and *H. pylori* infection is also discussed in the review article.

T. Nishizawa and H. Suzuki reviewed recent findings on gastric carcinogenesis and underlying molecular mechanisms. Reactive oxygen species (ROS) induced by *H. pylori* can bind with nucleic acids, turning them into mutated forms that play a role in multistep carcinogenesis. Correlation of CD44 variant, cell-surface marker of cancer stem-like cells with ROS defense system was reported. The important roles of CagA and activation-induced cytidine deaminase (AID) in carcinogenesis are also reviewed. *H. pylori* infection up- or downregulates expression of microRNAs that is linked to gastric tumorigenesis. Activation of epidermal growth factor receptor (EGFR) and erythroblastic leukemia-associated viral oncogene B (ErbB2) induced by *H. pylori* infection results in survival of gastric epithelial cells with DNA damage. In addition, recent advances in molecular targeting therapies by anti-EGRF are introduced.

H. Tsugawa et al. identified novel FecA1-binding compounds *in silico* and examined the effect of NDGA (nordihydroguaiaretic acid) that is one of the above compounds, on SodB activity, metronidazole (Mtz) susceptibility, and H_2O_2 sensitivity of *H. pylori*. NDGA reduced SodB activity and increased both H_2O_2 sensitivity and Mtz susceptibility. These results suggest that NDGA might be effective for the development of a novel eradication therapy.

Y. Shan et al. reported that outer membrane protein 18 (Hp1125) of *H. pylori* is involved in persistent colonization by evading interferon- (IFN-) gamma signaling. It was shown that IFN-gamma induced higher expression of *H. pylori* Omp18 and reduced the expression of CagA and NapA. By mouse infection model, isogenic omp18 mutant strain showed defective colonization and increased inflammatory changes in gastric mucosa. It was also shown that the isogenic mutant strain induced more production of cytokine, chemokine, and NO, indicating that Omp18 is involved in bacterial survival against oxidative stress and phagocytosis by

macrophages. Comment on this paper was sent from A. T. B. Abadi and E. Ierardi. They hypothesize that more factors except Omp18 are contributing to long term infection of *H. pylori* in gastric mucosa as the connection of a unique factor to the drive of the final pattern of this phenomenon could be too speculative.

O. Feliciano et al. reported the prevalence of *vacA*, *cagA*, and *iceA* genotypes of *H. pylori* strains isolated from Cuban patients with upper gastrointestinal diseases. It was shown that the *vacA* s1 allele, *cagA* gene, and *iceA2* allele were the most prevalent (72.0%, 56.0%, and 57.3%, resp.). Significant statistical association was observed between *iceA2* allele and patients with nonpeptic ulcer dyspepsia as well as virulence genotypes (*s1*, *s1m2*) and patients over 40 years old. Although the total number ($n = 75$) of the isolates was not enough to conclude clearly, it was indicated that a high prevalence of main virulence factors was detected in Cuban isolates similar to that observed in other Western populations.

Since the discovery of *H. pylori* in 1983 (first isolation in 1982), many research studies were performed to clarify the mechanisms by which this microorganism causes not only gastroduodenal diseases including gastric cancer but also extragastric diseases such as idiopathic thrombocytopenic purpura and iron-deficiency anemia. However, the details on the correlation between *H. pylori* infection and gastric/extragastric pathogenesis in human remain to be determined. The review and research articles published in this special issue may give us a hint to resolve the above question, but further studies on pathogenesis of *H. pylori* infection need to continue to be done.

Akio Tomoda
Shigeru Kamiya
Hidekazu Suzuki

Journal of Medical Microbiology (2015), 64, 67–73 DOI 10.1099/jmm.0.080507-0

Analysis of intra-familial transmission of *Helicobacter pylori* in Japanese families

Takako Osaki,[1] Mutsuko Konno,[2] Hideo Yonezawa,[1] Fuhito Hojo,[1] Cynthia Zaman,[1] Michiko Takahashi,[2] Shinichi Fujiwara[2] and Shigeru Kamiya[1]

Correspondence
Takako Osaki
osaki@ks.kyorin-u.ac.jp

[1]Department of Infectious Diseases, Kyorin University School of Medicine, Mitaka, Tokyo, Japan
[2]Department of Pediatrics, Sapporo Kosei General Hospital, Sapporo, Japan

Intra-familial infection is considered to be one of the main routes of transmission for *Helicobacter pylori* in Japan. We assessed the genomic profiles of *H. pylori* isolates from family members by multi-locus sequence typing (MLST) and identified the original strain infecting the index child. A total of 19 isolates from five families were analysed by MLST using seven housekeeping genes and by random amplification of polymorphic DNA (RAPD)-PCR. Phylogenetic analysis was performed using nucleotide sequences of the seven loci. Two or more different types of *H. pylori* strains were indicated in three (K-1, K-2 and K-5) out of five families. Independent genotypes of *H. pylori* strains were detected from all members of the other two families suggesting that these strains (K26-28 and K29-33) may be dominant. Mother-to-child transmission of *H. pylori* was demonstrated in four out of five families, whilst transmission from father-to-child and sibling-to-sibling were demonstrated in two families and one family, respectively.

Received 4 July 2014
Accepted 17 October 2014

INTRODUCTION

Helicobacter pylori is a curved Gram-negative bacterium that has been implicated in chronic gastritis, peptic ulcers, gastric adenocarcinoma and mucosal-associated lymphoid tissue lymphoma (Ernst & Gold, 2000; Marshall *et al.*, 1985; Uemura *et al.*, 2001). It was first discovered in the stomachs of gastritis patients by Marshall & Warren (1984). The infection prevalence of *H. pylori* has decreased in the industrialized world (Genta, 2002; Blaser & Atherton, 2004). Improved hygiene, housing conditions and the elimination of *H. pylori* from the population have resulted in a lower prevalence in children (Kosunen *et al.*, 1997; Rehnberg-Laiho *et al.*, 2001; Roosendaal *et al.*, 1997). It is considered that *H. pylori* will have been transmitted to an individual before the age of 5 years in many countries including developed (Weyermann *et al.*, 2009) and developing countries with high infection prevalence (Fiedorek *et al.*, 1991). However, in industrialized countries, the prevalence of *H. pylori* infection is low early in childhood and slowly rises with increasing age (Kuipers *et al.*, 1993). New infections are thought to occur as a consequence of direct human-to-human transmission by the oral–oral or faecal–oral route or both (Allaker *et al.*, 2002; Ferguson *et al.*, 1999; Leung *et al.*, 1999; Parsonnet *et al.*, 1999). In a recent study in Dutch children, relatively high *H. pylori* colonization rates in children of non-Dutch ethnicity who were born and raised in a western city were demonstrated. However, decreased colonization rates were also found in all ethnic groups in the study, implying the importance of environmental factors in *H. pylori* transmission in modern cities (den Hollander *et al.*, 2014).

In 1993, it was estimated that approximately 0.4 % of the 60 million Japanese who were infected with *H. pylori* have been diagnosed with gastric cancer (Asaka *et al.*, 1993). It has been reported that *H. pylori* infection rates gradually increased with age (Asaka *et al.*, 1992). Although this increase with age was similarly reported in more recent work (Asaka, 2002; Shiota *et al.*, 2013), the total prevalence of *H. pylori* infection is decreasing continuously in Japan (Shiota *et al.*, 2013). It can be implied that the detection rate of the pathogen originating from the environment has recently declined due to improving water provision and sewer services, and therefore one of the main transmission routes for this pathogen is unlikely to be a major factor in Japan. Several epidemiological studies reported that *H. pylori*-infected family member(s) are the risk factor for paediatric infection with *H. pylori* (Goodman & Correa, 2000; Vincent *et al.*, 1994). *H. pylori*-infected parents, particularly mothers, are suspected as the infectious sources. Although it was proven that other family members could transmit *H. pylori* infection, more precise analysis is needed to clarify the origin of the pathogen in each case. Multi-locus sequence typing (MLST) analysis of *H. pylori*

Abbreviations: MLST, multi-locus sequence typing; RAPD, random amplification of polymorphic DNA; SNP, single nucleotide polymorphism.

Two supplementary figures and two supplementary tables are available with the online Supplementary Material.

by comparing seven housekeeping genes (*atpA*, *efp*, *trpC*, *ppa*, *mutY*, *yphC* and *ureI*) has been reported (Falush *et al.*, 2003; Kennemann *et al.*, 2011; Yamaoka, 2009), and we have used this method while investigating intra-familial transmission of *H. pylori* using faecal samples (Osaki *et al.*, 2013). In this study, to further investigate intra-familial transmission of *H. pylori*, the strains isolated from several family members including index children were cultured and analysed by MLST.

METHODS

Study design and definitions. This study was undertaken with approval from the ethics committees of Kyorin University, Tokyo and Sapporo Kosei General Hospital. Children attending clinic with gastric disorders and/or iron deficiency anaemia during the period April 2011 to December 2012 at Sapporo Kosei General Hospital were recruited for the study. Gastric biopsy specimens or gastric juice samples from all participants were obtained before eradication of *H. pylori* infection. Family members recruited to participate in this study were then tested for status of *H. pylori* infection by the presence of *H. pylori* IgG in their serum (HM-CAP; Enteric Products) or presence of antigen in their stool (Premier Platinum HpSA; Meridian Diagnostics) using commercially prepared kits and according to manufacturers' instructions. Gastric biopsy specimens were then collected only from *H. pylori*-positive family members.

Isolation of *H. pylori* from gastric specimens. The biopsies or gastric juice samples from patients or their family members were inoculated onto *H. pylori* selective agar media (Nissui Pharmaceutical) and cultured for 5 days at 37 °C under microaerobic conditions (AnaeroPack; Mitsubishi Gas Chemical Company) as described previously (Konno *et al.*, 2005; Osaki *et al.*, 2008). Single colonies were picked from the plates and sub-cultured on *Brucella* medium (Becton, Dickinson and Company) supplemented with 1.5 % (w/v) agar and 7 % horse serum (BHS medium). The isolates were identified by colony morphological analysis and urease-positive characterization. Each isolate was suspended in brain heart infusion broth (Nissui Pharmaceuticals) and stocked at −80 °C until use.

DNA extraction from *H. pylori* isolates. *H. pylori* strains were inoculated onto BHS medium and cultured for 48 h at 37 °C under microaerobic conditions. The DNA was extracted using the Wizard Genomic DNA purification kit (Promega) according to manufacturer's instructions.

MLST analysis. The extracted DNA (10 ng) was used as template for the amplification of seven housekeeping genes (*atpA*, *efp*, *trpC*, *ppa*, *mutY*, *yphC* and *ureI*) using targeted primer pairs (Table S1; available in the online Supplementary Material). PCR amplicons were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions for sequence studies.

The purified PCR amplicons were sequenced in a Bio-Rad DNA Engine Dyad PTC-220 Peltier Thermal Cycler using ABI BigDye Terminator v3.1 Cycle Sequencing kits with AmpliTaq DNA polymerase (FS enzyme, Applied Biosystems), according to the manufacturer's instructions. Single-pass sequencing was performed on each template using a second primer (forward or reverse; Table S1). The fluorescently labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (ABI). The DNA sequence of each gene locus was registered on the MLST website (http://pubmlst.org/helicobacter/) (Jolley & Maiden, 2010). The allele number corresponds to the exact matching sequence for each gene to the strain listed in the database. In the case that the sequence had one or more base differences a new allele number(s) was listed on the MLST website.

Phylogenetic analysis. Phylogenetic analysis was carried out to compare nucleotide arrangement. The gene sequences for the seven loci were combined into one linear arrangement. The sequences were aligned and the maximum-likelihood tree was obtained by using MEGA5.1 (Arizona State University software) (Tamura *et al.*, 2011). The evolutionary history was inferred using the unweighted pair group method with arithmetic mean (UPGMA) method. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. As a control strain, the genome sequence of Japanese *H. pylori* strain F32 was obtained from Pubmed (http://www.ncbi.nlm.nih.gov/genome) and used for comparison.

Random amplification of polymorphic DNA (RAPD)-PCR. RAPD-PCR was carried out as described previously (Akopyanz *et al.*, 1992; Konno *et al.*, 2005). The extracted genomic DNA of *H. pylori* isolates was assessed by RAPD-PCR using the D1254 primer (Akopyanz *et al.*, 1992).

RESULTS

The five index children aged 6–10 years, their siblings aged 5–17 years, and their parents aged 28–51 years participated in this study (Fig. 1, Table S2). A total of five families were recruited for the detection of intra-familial transmission

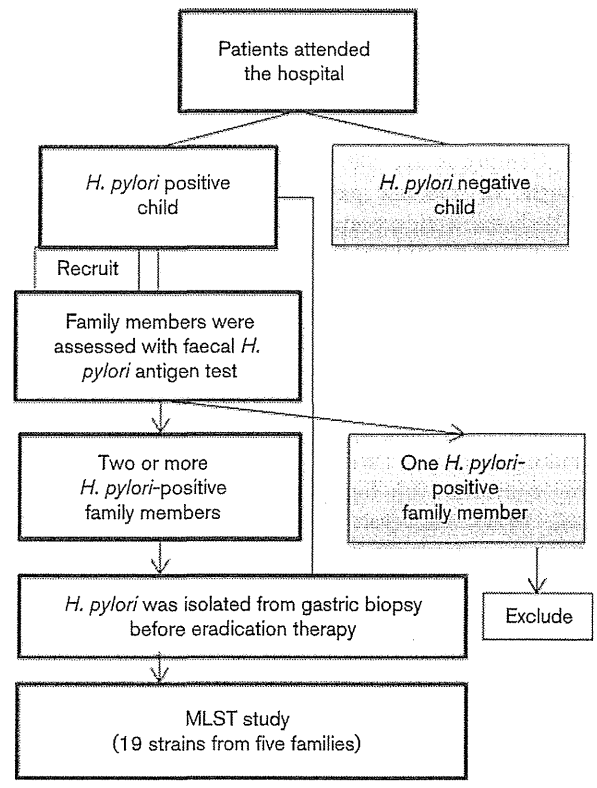

Fig. 1. Flow chart of this study.

and for determination of the family member harbouring the original strain.

In family K-1, three *H. pylori* isolates from the father (K16), mother (K17) and index child (K15) were compared. The alleles of seven loci (*atpA*, *efp*, *mutY*, *ppa*, *trpC* and *ureI*) in the isolates matched between K15 and K17 (Table 1). In family K-2, the alleles in the isolate (K37) from the index child were exactly the same as those from the mother (K35) and the sibling (K36), but not the same as those of the father (K34) (Table 1). The phylogenetic analysis of both families is shown in Fig. 2. There is large sequence diversity between the father's *H. pylori* strain and the index child's strain or mother's strain in both families. The genotype of *H. pylori* (K36) isolated from the sibling in family K-2 also matched that of the isolates from the mother and the index child, but not the isolate from the father (Fig. 2b). We could not find any nucleotide arrangement differences between K15 and K17 for family K-1 and between K37 and K36 for family K-2. These results indicate that these infections in families K-1 and K-2 may have occurred relatively recently.

The MLST results from families K-3 and K-4 are shown in Table 1. The alleles of all seven loci in *H. pylori* isolates matched almost those of all family members including the father and the sibling in each family. In addition, in the phylogenetic analysis the nucleotide arrangement was markedly similar, implying that only one original strain colonized all members in each family (Fig. 3). However, since the mother's isolates (K27 and K30) were located upstream from the other two isolates on the phylogeny tree (K26 and K28 in K-3, K29 and K32 in K-4, except K33), this suggested that the mother was infected with *H. pylori* first and then other family members were infected with the mother's *H. pylori* strain. The K33 (index child) strain was located at the same position as the mother's isolate (K30) on the phylogenetic tree, suggesting that the infection between the mother and the index child may have occurred relatively recently. The order of intra-familial infections between parents and child (sibling) cannot be determined.

For family K-5, the same sequence type strains were detected by MLST in all three children, but different types were detected in their mother and father (Table 1, Fig. 4). In total, three different types of *H. pylori* strains were detected in family K-5. This implied that the *H. pylori* strain was therefore transmitted between the index child (K24) and the two siblings (K23 and K25) and that the strains isolated from either mother (K22) or father (K21) were not the source of intra-familial transmission. The phylogenetic study supports this implication, as the genotype of the index child's isolate (K24) was divided from the nodes of its father's isolate and mother's isolate (K21 and K22) (Fig. 4). Sibling-to-sibling transmission(s) of *H. pylori* was clearly illustrated in family K-5.

Table 1. MLST analysis of *H. pylori* strains isolated from family members

Family	Member	Strain	Allele type number							
			atpA	*efp*	*mutY*	*ppa*	*trpC*	*ureI*	*yphC*	ST
K-1	Father	K16	2305	2129	2342	2153	2418	2390	2307	2747
	Mother	K17	2309	2170	2347	1125	2419	2391	2345	2748
	Index child	K15	2309	2170	2347	1125	2419	2391	2345	2748
K-2	Father	K34	2313	2199	2359	942	458	1968	2401	2749
	Mother	K35	2324	2202	2360	2235	2433	2406	2404	2750
	Sibling	K36	2324	2202	2360	2235	2433	2406	2404	2750
	Index child	K37	2324	2202	2360	2235	2433	2406	2404	2750
K-3	Father	K26	1760	2185	2354	936*	457	2393*	457	2753
	Mother	K27	1760	2185	2354	2232*	457	2393*	457	2754
	Index child	K28	1760	2185	2354	2232*	457	2400*	457	2755
K-4	Father	K29	2319	2186†	2355†	945	954	36	957	2756
	Mother	K30	2319	2191†	2355†	945	954	36	957	2757
	Sibling	K32	2319	2191	2358†	945	954	36	957	2758
	Index child	K33	2319	2191	2355†	945	954	36	957	2757
K-5	Father	K21	2313	2182	459	2155	2420	1968	2393	2760
	Mother	K22	2315	2184	2351	445	2426	1968	2394	2761
	Sibling no. 1	K23	951	909	950	2230	2427	2392	2400	2762
	Index child	K24	951	909	950	2230	2427	2392	2400	2762
	Sibling no. 2	K25	951	909	950	2230	2427	2392	2400	2762

ST, Sequencing type.

Allele numbers over 2000 were given for this study from the MLST website (http://pubmlst.org/helicobacter/)

*One SNP difference between *ppa*936 and *ppa* 2232, *ureI*2393 and *ureI*2400 in family K-3.

†One SNP difference between *efp*2186 and *efp*2191, *mutY*2355 and *mutY* 2358 in family K-4.

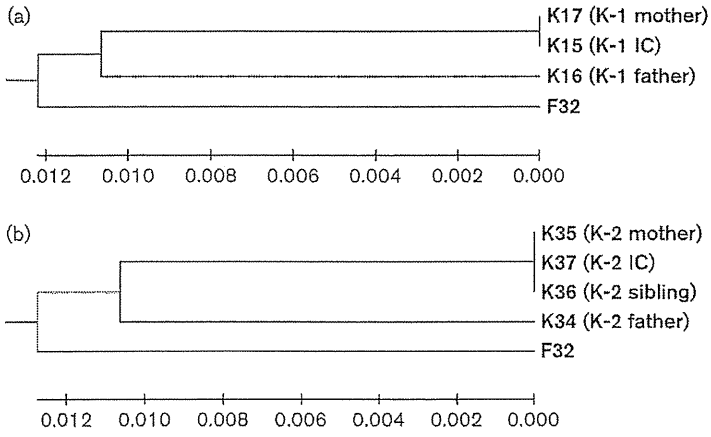

Fig. 2. Phylogenetic tree showing the correlation of isolates from family K-1 (a) and family K-2 (b) based on sequence arrangements of seven loci. Paternal isolates are K17 and K34 displayed as K-1 father and K-2 father, respectively. Maternal isolates are K17 and K35 displayed as K-1 mother and K-2 mother, respectively. The K-2 sibling isolate is K37 displayed as K-2 sibling. The index child (IC) isolates are K15 and K37 displayed as K-1 IC and K-2 IC. The genome sequence of Japanese *H. pylori* strain F32 was used for the comparison as a control and displayed as F32. The scale bar indicates distance between the strains tested (substitutions/site).

The MLST results were confirmed by RAPD fingerprinting (Fig. S1). There was no discrepancy between the results from MLST and RAPD-PCR.

DISCUSSION

MLST is a standard method of molecular typing for pathogenic and non-pathogenic bacteria. We have conducted this study using MLST to clarify the mode of intrafamilial transmission among several *H. pylori*-positive family members with an index child.

In this study, four cases (families K-1, K-2, K-3 and K-4) of mother-to-child transmission were demonstrated in five families. The alleles of all loci in *H. pylori* isolates matched almost those of all family members including the father and the sibling in family K-3 and K-4, therefore the possibility of paternal or sibling infection cannot be excluded in two of the cases. There have been several studies concerning the intra-familial transmission of *H. pylori* evaluated by molecular analysis (Georgopoulos *et al.*, 1996; Han *et al.*, 2000; Kivi *et al.*, 2003; Konno *et al.*, 2005, 2008; Nahar *et al.*, 2009; Nwokolo *et al.*, 1992). The major causative mode of infection was suggested to be mother-to-child transmission in these studies and our previous study (Osaki *et al.*, 2013). Father-to-child transmission was not detected clearly, as the father and the mother had similar genotypes of *H. pylori* in families K-3 and K-4. It has been previously reported that maternal, paternal and sibling infection are all strongly and significantly related to infection of the child with *H. pylori* in bivariate analyses (Weyermann *et al.*, 2009). Phylogenetic analysis revealed *H. pylori* isolated from the father of the K-3 (K26) and K-4 (K29) family were located upstream from the each node compared to *H. pylori* strains from other family members (family K-3, K27 and K28; family K-4, K30 and K33), implying that the father was infected with the original *H. pylori* strain.

In two families (K-3 and K-4), the original *H. pylori* strain was not only related to infection in the children but also inter-spousally. A previous study reported this type of transmission previously by using *H. pylori* 16S rRNA ribotyping, showing that eight of 18 couples were colonized with a single *H. pylori* strain (Georgopoulos *et al.*, 1996). An additional study reported six cases of inter-spousal infection using RAPD-fingerprinting analysis (Konno *et al.*, 2008). It is difficult to clarify the time when inter-spousal infection occurred, but it is unlikely that the couples were

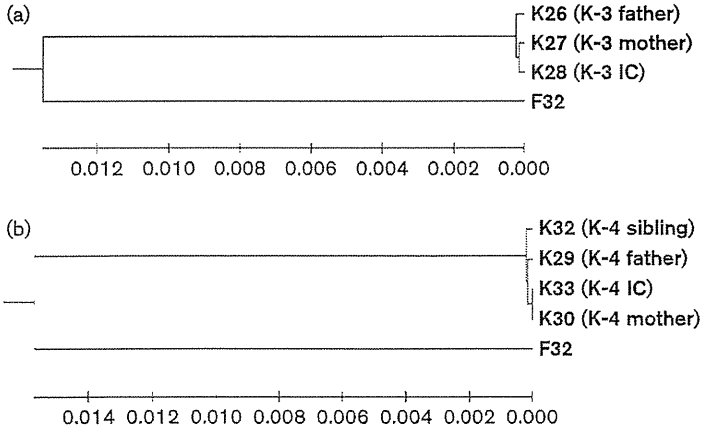

Fig. 3. Phylogenetic trees showing the correlation of the isolates from family K-3 (a) and family K-4 (b) based on sequence arrangements of seven loci. Paternal isolates are K26 and K29, displayed as K-3 father and K-4 father. Maternal isolates are K27 and K30 displayed as K-3 mother and K-4 mother. The K-4 sibling isolate is K32 displayed as K-4 sibling. The index child isolates (IC) are K28 and K33 displayed as K-3 IC and K-4 IC. The genome sequence of Japanese *H. pylori* strain F32 was used for the comparison as a control and displayed as F32. The scale bar indicates distance between the strains tested (substitutions/site).

single *H. pylori* clone (Toita *et al.*, 2013). As we used single colony isolates of *H. pylori* from each family member and MLST analysis was performed for this isolate, mixed infection of *H. pylori* could not be detected. Two different strains of *H. pylori* were isolated from the same individuals at different sampling times, but all isolates derived from a single individual showed the same MLST in this study (data not shown). The dominant population of *H. pylori* may be determined by microbiota environmental factors. In our previous study, the composition of gastric indigenous microbiota in Mongolian gerbils may be disturbed by long-term infection with *H. pylori*, and that these changes may in fact inhibit *H. pylori* infection (Osaki, *et al.*, 2012). Further study is necessary to clarify the mechanism of intra-familial infection.

In conclusion, person-to-person transmission between family members was detected frequently in this study, with mother-to-child, parent-to-child, intra-spousal and sibling(s)-to-sibling transmissions being demonstrated. This may be the predominant mode of *H. pylori* transmission in Japan.

ACKNOWLEDGEMENTS

This study was undertaken with approval from the ethics committees of Kyorin University, Tokyo (No. 537) and Sapporo Kosei General Hospital (H24-104). We thank the members of the Sapporo Kosei General Hospital and clinical microbiology laboratory for their excellent technical support for the collection of isolates. We thank also Dr Yoshikazu Furuta and Professor Ichizo Kobayashi who belong to Department of Medical Genome Sciences, Graduate School of Frontier Sciences and Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan for their support for the sequencing analysis. This project was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science in Japan (grant nos. 20590455 and 21590492).The authors report no conflicts of interest.

REFERENCES

Akopyanz, N., Bukanov, N. O., Westblom, T. U., Kresovich, S. & Berg, D. E. (1992). DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res* **20**, 5137–5142.

Allaker, R. P., Young, K. A., Hardie, J. M., Domizio, P. & Meadows, N. J. (2002). Prevalence of *Helicobacter pylori* at oral and gastrointestinal sites in children: evidence for possible oral-to-oral transmission. *J Med Microbiol* **51**, 312–317.

Asaka, M. (2002). *Helicobacter pylori* infection and gastric cancer. *Intern Med* **41**, 1–6.

Asaka, M., Kimura, T., Kudo, M., Takeda, H., Mitani, S., Miyazaki, T., Miki, K. & Graham, D. Y. (1992). Relationship of *Helicobacter pylori* to serum pepsinogens in an asymptomatic Japanese population. *Gastroenterology* **102**, 760–766.

Asaka, M., Kato, M., Kudo, M., Meguro, T., Kimura, T., Miyazaki, T. & Inoue, K. (1993). The role of *Helicobacter pylori* in peptic ulcer disease. *Gastroenterol Jpn* **28** (*Suppl. 5*), 163–167.

Blaser, M. J. & Atherton, J. C. (2004). *Helicobacter pylori* persistence: biology and disease. *J Clin Invest* **113**, 321–333.

den Hollander, W. J., Holster, I. L., van Gilst, B., van Vuuren, A. J., Jaddoe, V. W., Hofman, A., Perez-Perez, G. I., Kuipers, E. J., Moll, H. A. & Blaser, M. J. (2014). Intergenerational reduction in *Helicobacter pylori* prevalence is similar between different ethnic groups living in a Western city. *Gut* doi: 10.1136/gutjnl-2014-307689 [Epub ahead of print].

Ernst, P. B. & Gold, B. D. (2000). The disease spectrum of *Helicobacter pylori*: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. *Annu Rev Microbiol* **54**, 615–640.

Falush, D., Wirth, T., Linz, B., Pritchard, J. K., Stephens, M., Kidd, M., Blaser, M. J., Graham, D. Y., Vacher, S. & other authors (2003). Traces of human migrations in *Helicobacter pylori* populations. *Science* **299**, 1582–1585.

Ferguson, D. A., Jr, Jiang, C., Chi, D. S., Laffan, J. J., Li, C. & Thomas, E. (1999). Evaluation of two string tests for obtaining gastric juice for culture, nested-PCR detection, and combined single- and double-stranded conformational polymorphism discrimination of *Helicobacter pylori*. *Dig Dis Sci* **44**, 2056–2062.

Fialho, A. M., Braga, A. B., Braga Neto, M. B., Carneiro, J. G., Rocha, A. M., Rodrigues, M. N., Queiroz, D. M. & Braga, L. L. (2010). Younger siblings play a major role in *Helicobacter pylori* transmission among children from a low-income community in the Northeast of Brazil. *Helicobacter* **15**, 491–496.

Fiedorek, S. C., Malaty, H. M., Evans, D. L., Pumphrey, C. L., Casteel, H. B., Evans, D. J., Jr & Graham, D. Y. (1991). Factors influencing the epidemiology of *Helicobacter pylori* infection in children. *Pediatrics* **88**, 578–582.

Fujimura, S., Kato, S. & Watanabe, A. (2008). Water source as a *Helicobacter pylori* transmission route: a 3-year follow-up study of Japanese children living in a unique district. *J Med Microbiol* **57**, 909–910.

Garg, P. K., Perry, S., Sanchez, L. & Parsonnet, J. (2006). Concordance of *Helicobacter pylori* infection among children in extended-family homes. *Epidemiol Infect* **134**, 450–459.

Genta, R. M. (2002). Review article: after gastritis–an imaginary journey into a *Helicobacter*-free world. *Aliment Pharmacol Ther* **16** (Suppl. 4), 89–94.

Georgopoulos, S. D., Mentis, A. F., Spiliadis, C. A., Tzouvelekis, L. S., Tzelepi, E., Moshopoulos, A. & Skandalis, N. (1996). *Helicobacter pylori* infection in spouses of patients with duodenal ulcers and comparison of ribosomal RNA gene patterns. *Gut* **39**, 634–638.

Goh, K. L., Chan, W. K., Shiota, S. & Yamaoka, Y. (2011). Epidemiology of *Helicobacter pylori* infection and public health implications. *Helicobacter* **16** (Suppl. 1), 1–9.

Goodman, K. J. & Correa, P. (2000). Transmission of *Helicobacter pylori* among siblings. *Lancet* **355**, 358–362.

Han, S. R., Zschausch, H. C., Meyer, H. G., Schneider, T., Loos, M., Bhakdi, S. & Maeurer, M. J. (2000). *Helicobacter pylori*: clonal population structure and restricted transmission within families revealed by molecular typing. *J Clin Microbiol* **38**, 3646–3651.

Hirschl, A. M., Richter, M., Makristathis, A., Prückl, P. M., Willinger, B., Schütze, K. & Rotter, M. L. (1994). Single and multiple strain colonization in patients with *Helicobacter pylori*-associated gastritis: detection by macrorestriction DNA analysis. *J Infect Dis* **170**, 473–475.

Kennemann, L., Didelot, X., Aebischer, T., Kuhn, S., Drescher, B., Droege, M., Reinhardt, R., Correa, P., Meyer, T. F. & other authors (2011). *Helicobacter pylori* genome evolution during human infection. *Proc Natl Acad Sci U S A* **108**, 5033–5038.

Jolley, K. A. & Maiden, M. C. (2010). BIGSad: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* **11**, 595.

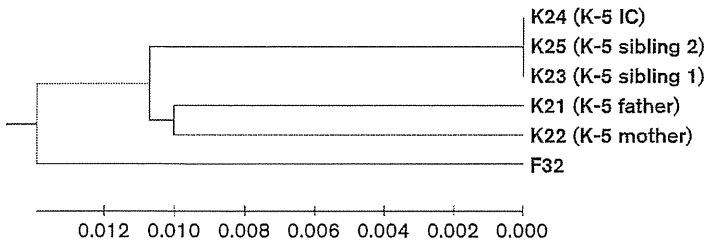

Fig. 4. Phylogenetic tree showing the correlation of isolates from family K-5 based on sequence arrangements of seven loci. Paternal isolate is K21 displayed as K-5 father. Maternal isolate is K22 displayed as K-5 mother. The K-5 sibling isolates are K23 and K25 displayed as K5 sibling 1 and K5 sibling 2. The index child isolate is K24 displayed as K-5 IC. The genome sequence of Japanese *H. pylori* strain F32 was used for the comparison as a control and displayed as F32. The scale bar indicates distance between the strains tested (substitutions/site).

independently infected with the same *H. pylori* strain. It is well known that recombination occurs frequently in the *H. pylori* genome (Suerbaum *et al.*, 1998). If the couple were infected with same *H. pylori* strain before marriage, their *H. pylori* strains would not keep the same genotype and therefore it is reasonable to assume the transmission of *H. pylori* between parents occurred recently.

Sibling-to-sibling(s) transmission of *H. pylori* was found in family K-5, but parent-to-child transmission was not. In this case, it is suggested that the isolates from the children originated from outside the family. There are several hypotheses as to the sources, including a study in which *H. pylori* DNA was detected in drinking water (Fujimura *et al.*, 2008). In this family, the origin of infection and the reason for the lack of parent-to-child transmission were not clear.

In Japan, oral transmission through saliva and gastric juice is thought to be the main cause of *H. pylori* infection. Japanese families have customarily fed their children pre-chewed food. It has been reported that use of soothers or bottle teats is closely related with *H. pylori* transmission in Canadian children (Sinha *et al.*, 2004). In contrast, it was reported that feeding infants food first chewed by a parent did not affect *H. pylori* status (Kurosawa *et al.*, 2000). On the other hand, children vomit more frequently and *H. pylori* strains from a young child with vomiting can be transmitted to other family members. Siblings may therefore play an important role in *H. pylori* transmission among children (Fialho *et al.*, 2010). Faecal–oral transmission is another route of infection related to sanitary conditions; water supply and sewage are considered important factors for *H. pylori* infection (Goh *et al.*, 2011). After the Second World War, sanitary conditions improved in Japan and the prevalence of *H. pylori* infection decreased (Shiota *et al.*, 2010).

In this study, sibling-to-sibling transmission of *H. pylori* without maternal or paternal infection was detected. The type of transmission of *H. pylori* has been reported in several articles (Garg *et al.*, 2006; Miehlke *et al.*, 1999). However, mother-to-child transmission was also found in these cases, and it was therefore difficult to clarify the transmission route of *H. pylori* in these families. Households with many children have been shown to be one of the risk factors for *H. pylori* infection (Fiedorek *et al.*, 1991). In Japan, the average number of children per household is 1.70 in 2010, and the number of children in family K-5 was higher than the average. Although *H. pylori* infection from the elder-to-younger sibling was most likely, the origin of the strain isolated from the index child was unclear.

Several SNPs (single nucleotide polymorphisms) were found by comparing all MLST gene sequences. Deletion and transformation of other sequences was not detected. It was shown that the SNPs were derived from the original strain during a long infection period after transmission of *H. pylori* (Raymond *et al.*, 2004). According to Graphical review of Japanese households-from comprehensive survey of living conditions, 2010, edt. Japanese Ministry of Health, Labour and Welfare (http://www.mhlw.go.jp/tokei/list/dl/20-21-01.pdf).

It is well known that clinical strains of *H. pylori* have numeric diversity. Although the genotype of intra-familial transmitted strains were closely related each other, it was shown that K16 (K-1 family), K34 (K-2 family), K21 and K22 (K-5 family) had different allele types in the seven loci tested, compared to the isolates from other family members. These results showed that similar molecular type strains were detected in the cases of intra-familial transmission than in cases with other infection routes. In the phylogenetic study, we found similar strains were present in each family (Figs 2, 3, 4 and S2). These results support the notion that the same original strain was transmitted to family members.

Another possibility is that family members were infected with two or more strains at the same time. There have been several reports of multiple infections (Hirschl *et al.*, 1994; Fiedorek *et al.*, 1991; Miehlke *et al.*, 1999; Raymond *et al.*, 2004) with different types of *H. pylori* strains in a single individual. On study reported the examination of clonal diversity by RAPD fingerprinting method (Toita *et al.*, 2013); the isolates obtained from several patients at 5 to 9 year intervals showed identical or very similar RAPD patterns. It was concluded that each Japanese individual of an urban population is predominantly infected with a

Kivi, M., Tindberg, Y., Sörberg, M., Casswall, T. H., Befrits, R., Hellström, P. M., Bengtsson, C., Engstrand, L. & Granström, M. (2003). Concordance of *Helicobacter pylori* strains within families. *J Clin Microbiol* **41**, 5604–5608.

Konno, M., Fujii, N., Yokota, S., Sato, K., Takahashi, M., Sato, K., Mino, E. & Sugiyama, T. (2005). Five-year follow-up study of mother-to-child transmission of *Helicobacter pylori* infection detected by a random amplified polymorphic DNA fingerprinting method. *J Clin Microbiol* **43**, 2246–2250.

Konno, M., Yokota, S., Suga, T., Takahashi, M., Sato, K. & Fujii, N. (2008). Predominance of mother-to-child transmission of *Helicobacter pylori* infection detected by random amplified polymorphic DNA fingerprinting analysis in Japanese families. *Pediatr Infect Dis J* **27**, 999–1003.

Kosunen, T. U., Aromaa, A., Knekt, P., Salomaa, A., Rautelin, H., Lohi, P. & Heinonen, O. P. (1997). *Helicobacter* antibodies in 1973 and 1994 in the adult population of Vammala, Finland. *Epidemiol Infect* **119**, 29–34.

Kuipers, E. J., Peña, A. S., Pels, N. F. M., Kurz-Pohlmann, E., Meuwissen, S. G. M., van Kamp, G., Uyterlinde, A. M., Pals, G. & (1993). Seroconversion for *Helicobacter pylori*. *Lancet* **342**, 328–331.

Kurosawa, M., Kikuchi, S., Inaba, Y., Ishibashi, T. & Kobayashi, F. (2000). *Helicobacter pylori* infection among Japanese children. *J Gastroenterol Hepatol* **15**, 1382–1385.

Leung, W. K., Siu, K. L., Kwok, C. K., Chan, S. Y., Sung, R. & Sung, J. J. (1999). Isolation of *Helicobacter pylori* from vomitus in children and its implication in gastro-oral transmission. *Am J Gastroenterol* **94**, 2881–2884.

Marshall, B. J. & Warren, J. R. (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **323**, 1311–1315.

Marshall, B. J., Armstrong, J. A., McGechie, D. B. & Glancy, R. J. (1985). Attempt to fulfil Koch's postulates for pyloric *Campylobacter*. *Med J Aust* **142**, 436–439.

Miehlke, S., Genta, R. M., Graham, D. Y. & Go, M. F. (1999). Molecular relationships of *Helicobacter pylori* strains in a family with gastroduodenal disease. *Am J Gastroenterol* **94**, 364–368.

Nahar, S., Kibria, K. M., Hossain, M. E., Sultana, J., Sarker, S. A., Engstrand, L., Bardhan, P. K., Rahman, M. & Endtz, H. P. (2009). Evidence of intra-familial transmission of *Helicobacter pylori* by PCR-based RAPD fingerprinting in Bangladesh. *Eur J Clin Microbiol Infect Dis* **28**, 767–773.

Nwokolo, C. U., Bickley, J., Attard, A. R., Owen, R. J., Costas, M. & Fraser, I. A. (1992). Evidence of clonal variants of *Helicobacter pylori* in three generations of a duodenal ulcer disease family. *Gut* **33**, 1323–1327.

Osaki, T., Mabe, K., Hanawa, T. & Kamiya, S. (2008). Urease-positive bacteria in the stomach induce a false-positive reaction in a urea breath test for diagnosis of *Helicobacter pylori* infection. *J Med Microbiol* **57**, 814–819.

Osaki, T., Matsuki, T., Asahara, T., Zaman, C., Hanawa, T., Yonezawa, H., Kurata, S., Woo, T. D., Nomoto, K. & Kamiya, S. (2012). Comparative analysis of gastric bacterial microbiota in Mongolian gerbils after long-term infection with *Helicobacter pylori*. *Microb Pathog* **53**, 12–18.

Osaki, T., Okuda, M., Ueda, J., Konno, M., Yonezawa, H., Hojo, F., Yagyu, K., Lin, Y., Fukuda, Y. & other authors (2013). Multilocus sequence typing of DNA from faecal specimens for the analysis of intra-familial transmission of *Helicobacter pylori*. *J Med Microbiol* **62**, 761–765.

Parsonnet, J., Shmuely, H. & Haggerty, T. (1999). Fecal and oral shedding of *Helicobacter pylori* from healthy infected adults. *JAMA* **282**, 2240–2245.

Raymond, J., Thiberg, J. M., Chevalier, C., Kalach, N., Bergeret, M., Labigne, A. & Dauga, C. (2004). Genetic and transmission analysis of *Helicobacter pylori* strains within a family. *Emerg Infect Dis* **10**, 1816–1821.

Rehnberg-Laiho, L., Rautelin, H., Koskela, P., Sarna, S., Pukkala, E., Aromaa, A., Knekt, P. & Kosunen, T. U. (2001). Decreasing prevalence of *helicobacter* antibodies in Finland, with reference to the decreasing incidence of gastric cancer. *Epidemiol Infect* **126**, 37–42.

Roosendaal, R., Kuipers, E. J., Buitenwerf, J., van Uffelen, C., Meuwissen, S. G., van Kamp, G. J. & Vandenbroucke-Grauls, C. M. (1997). *Helicobacter pylori* and the birth cohort effect: evidence of a continuous decrease of infection rates in childhood. *Am J Gastroenterol* **92**, 1480–1482.

Shiota, S., Matsunari, O., Watada, M. & Yamaoka, Y. (2010). Serum *Helicobacter pylori* CagA antibody as a biomarker for gastric cancer in east-Asian countries. *Future Microbiol* **5**, 1885–1893.

Shiota, S., Murakawi, K., Suzuki, R., Fujioka, T. & Yamaoka, Y. (2013). *Helicobacter pylori* infection in Japan. *Expert Rev Gastroenterol Hepatol* **7**, 35–40.

Sinha, S. K., Martin, B., Gold, B. D., Song, Q., Sargent, M. & Bernstein, C. N. (2004). The incidence of *Helicobacter pylori* acquisition in children of a Canadian First Nations community and the potential for parent-to-child transmission. *Helicobacter* **9**, 59–68.

Suerbaum, S., Smith, J. M., Bapumia, K., Morelli, G., Smith, N. H., Kunstmann, E., Dyrek, I. & Achtman, M. (1998). Free recombination within *Helicobacter pylori*. *Proc Natl Acad Sci U S A* **95**, 12619–12624.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Toita, N., Yokota, S., Fujii, N. & Konno, M. (2013). Clonality analysis of *Helicobacter pylori* in patients isolated from several biopsy specimens and gastric juice in a Japanese urban population by random amplified polymorphic DNA fingerprinting. *Gastroenterol Res Pract* **2013**, 721306.

Uemura, N., Okamoto, S., Yamamoto, S., Matsumura, N., Yamaguchi, S., Yamakido, M., Taniyama, K., Sasaki, N. & Schlemper, R. J. (2001). *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med* **345**, 784–789.

Vincent, P., Gottrand, F., Pernes, P., Husson, M. O., Lecomte-Houcke, M., Turck, D. & Leclerc, H. (1994). High prevalence of *Helicobacter pylori* infection in cohabiting children. Epidemiology of a cluster, with special emphasis on molecular typing. *Gut* **35**, 313–316.

Weyermann, M., Rothenbacher, D. & Brenner, H. (2009). Acquisition of *Helicobacter pylori* infection in early childhood: independent contributions of infected mothers, fathers, and siblings. *Am J Gastroenterol* **104**, 182–189.

Yamaoka, Y. (2009). *Helicobacter pylori* typing as a tool for tracking human migration. *Clin Microbiol Infect* **15**, 829–834.

Original Article

Rebamipide protects small intestinal mucosal injuries caused by indomethacin by modulating intestinal microbiota and the gene expression in intestinal mucosa in a rat model

Satoshi Kurata,[1,†] Takako Nakashima,[2,†] Takako Osaki,[1] Naoya Uematsu,[2] Masafumi Shibamori,[2] Kazushi Sakurai[2] and Shigeru Kamiya[1,*]

[1]Department of Infectious Diseases, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan
[2]Third Institute of New Drug Discovery, Otsuka Pharmaceutical Co., Ltd., 463-10, Kagasuno, Kawauchi-cho, Tokushima 771-0192, Japan

(Received 15 May, 2014; Accepted 5 June, 2014; Published online 4 October, 2014)

The effect of rebamipide, a mucosal protective drug, on small intestinal mucosal injury caused by indomethacin was examined using a rat model. Indomethacin administration (10 mg/kg, p.o.) induced intestinal mucosal injury was accompanied by an increase in the numbers of intestinal bacteria particularly *Enterobacteriaceae* in the jejunum and ileum. Rebamipide (30 and 100 mg/kg, p.o., given 5 times) was shown to inhibit the indomethacin-induced small intestinal mucosal injury and decreased the number of *Enterococcaceae* and *Enterobacteriaceae* in the jejunal mucosa to normal levels. It was also shown that the detection rate of segmented filamentous bacteria was increased by rebamipide. PCR array analysis of genes related to inflammation, oxidative stress and wound healing showed that indomethacin induced upregulation and downregulation of 14 and 3 genes, respectively in the rat jejunal mucosa by more than 5-fold compared to that of normal rats. Rebamipide suppressed the upregulated gene expression of TNFα and Duox2 in a dose-dependent manner. In conclusion, our study confirmed that disturbance of intestinal microbiota plays a crucial role in indomethacin-induced small intestinal mucosal injury, and suggests that rebamipide could be used as prophylaxis against non-steroidal anti-inflammatory drugs -induced gastrointestinal mucosal injury, by modulating microbiota and suppressing mucosal inflammation in the small intestine.

Key Words: **non-steroidal anti-inflammatory drugs, small intestine, oxidative stress, rebamipide, intestinal flora**

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in clinical medicine, and their use is reported to be associated with a broad spectrum of adverse reactions in the liver, kidney, skin and gastrointestinal tract.[1] NSAIDs have toxic effects in the small and large intestine as well as stomach.[2] The NSAID loxoprofen sodium was reported to induce erosions and ulcers along the mesenteric margin of the distal jejunum,[3] and it was also demonstrated that the number of both aerobic and anaerobic bacteria markedly increased at the sites of the lesions. Other similar studies show that NSAID induced small intestinal mucosal injury is closely associated with dysbiosis of the small intestinal microbiota.[4,5]

Use of acid-suppressing drugs of the proton pump inhibitors (PPI) and histamine 2-receptor antagonist (H2) families has been associated with a 1.5- to 2-fold increased risk of community-acquired pneumonia.[6,7] According to a recent study,[8] 12% (71 out of 608 cases) of current PPI/H2 users had recurrent pneumonia, compared with 8% of nonusers, indicating that acid-suppressing drug use substantially increased the likelihood of recurrent pneumonia in high-risk elderly patients.

Rebamipide 2-{4-cholorobenzoylamino-3-[2(1*H*)-quinolinon-4-yl]} propionic acid, CAS 11911-87-6, is a gastric mucosal stabilizer and gastric mucosal prostaglandin inducer, and is used for treatment of gastric ulcers and gastritis.[9] It has been reported that rebamipide has various biological effects including increasing the production of mucus and prostaglandins, scavenging hydroxyl radicals, inhibition of neutrophil activation and suppression of gastric mucosal inflammation.[10–12] In addition to these effects, rebamipide has been reported to prevent NSAID-induced peptic ulcer in patients on long-term NSAID therapy.[13,14]

Wallace *et al.*[15] reported that PPIs (omeprazole and lansoprazole) significantly exacerbated naproxen- and celecoxib-induced intestinal ulceration and bleeding in rats. It was also indicated that omeprazole treatment resulted in significant increase in the number of aerobic bacteria and significant decrease in the number of Actinobacteria including *Bifidobacterium* spp. Colonization of germ-free mic with jejunal bacteria from PPI-treated rats increased the severity of NSAID-induced intestinal injury.

In the present study, the effects of rebamipide on small intestinal microbiota including aerobic and anaerobic bacteria were examined by both culture and real-time PCR techniques. In addition, the effect of rebamipide on the expression of genes in the jejunal mucosa after indomethacin administration was analyzed by quantitative real-time RT (reverse-transcription)-PCR assay.

Methods

Ethics statement. This study was carried out in accordance with Guidelines for Animal Care and Use in Otsuka Pharmaceutical Co., Ltd. And this study was approved by the Institutional Animal Care and Use Committee of Kyorin University School of Medicine (Approval No. 107-1, 107-2).

Animals. Specific-pathogen-free male Wistar/ST strain rats (6-weeks old) were purchased from Japan SLC Co. (Hamamatsu, Japan), and used after one week habituation. The animals were given CRF-1 (Oriental Yeast Co., Ltd., Osaka, Japan) and tap water *ad libitum*.

*To whom correspondence should be addressed.
E-mail: skamiya@ks.kyorin-u.ac.jp
†Equally contributing authors.

doi: 10.3164/jcbn.14-67

Indomethacin-induced small intestinal injury. To induce small intestinal injury, indomethacin 10 mg/kg suspended in 0.5% carboxylmethylcellulose sodium salt (CMC) was orally administered in a volume of 4 ml/kg to non-fasted animals that were sacrificed 24 h later. Under the isoflurane gas anesthesia, 1 ml of blood was obtained from the abdominal vein and hematologic analysis was performed using an automated hematology analyzer (Sysmex XT-2000iV, Sysmex Co., Kobe, Japan). The animals were killed by cutting the abdominal aorta. The small intestine was removed and cut opened along the anti-mesenteric border, and gently rinsed with saline to remove fecal contents. The small intestine was divided into 4 segments named proximal, mid and distal jejunum and ileum. Image were captured by a digital camera for macroscopic evaluation of the mucosal lesion area. This was calculated using computer-assisted image analyzer software (WinROOF ver. 5, Mitani Corporation, Fukui, Japan) and summed per small intestine. The examiner was unaware of animal treatment.

Experimental groups. The rats were allocated to five experimental groups using stratified random sampling on the basis of weight on the experiment day. Rebamipide (30 and 100 mg/kg, Otsuka Pharmaceutical Co., Ltd.) was orally administered five times, 24 h, 16 h and 1 h prior to, and 3 h and 7 h after indomethacin treatment. Ampicillin (800 mg/kg, Sigma-Aldrich Co., St. Louis, MO) was orally administered twice, 24 h and 1 h before indomethacin treatment. Multiple oral administrations of rebamipide were given to ensure adequate intestinal mucosal distribution, due to the relatively short half-life of the drug.[9] In the control group, 0.5% CMC was orally administered according to the same dosing regimen as the rebamipide groups. Untreated animals were also prepared as normal group. Each group consisted of 10 animals except the normal group, which contained 6 animals.

Examination of microbiota in the small intestinal mucosa. Immediately after capturing the macroscopic image, the intestinal mucosa was obtained by scraping each specimen with a glass slide and weighed. A small amount of the mucosa was frozen in liquid nitrogen and stored at −80°C for mRNA extraction.

The mucosal samples were added in a 9 fold volume of anaerobic dilution buffer and homogenized by a homogenizer. The diluted mucosal samples were divided into several tubes and stored at −80°C until use for culture and extraction of DNA.

DNA extraction from mucosal samples. The total DNA of the microbiota contained in the mucosa of each rat was extracted from diluted mucosal samples using the QIAamp DNA Stool kit (QIAGEN Valencia, CA). Two-hundred µl of each sample was added to 1.4 ml of buffer ASL (QIAGEN). Consequent steps were performed according to manufacture documentation. Finally, a total of 100 µl of DNA solution was obtained.

Analysis of viable bacterial flora in small intestine. Small intestinal mucosal samples from three jejunal and ileal sites were homogenized and 10-fold diluted in anaerobic diluent (KH_2PO_4 4.5 g, Na_2HPO_4, 6.0 g, L-cysteine hydrochloride 0.5 g, Tween 80 0.5 g and agar 0.5 g in 1 L of distilled water),[14] and stored at −80°C for later analysis.

The total viable aerobic and facultative anaerobic bacterial counts were determined by culture method on MacConkey agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and Brain Heart Infusion (BHI) agar (Difco, Detroit, MI) containing 5% horse blood as described previously.[16] The viable obligate anaerobe counts were cultured on Gifu Anaerobic Medium (GAM) agar (Nissui Pharmaceutical Co., Ltd.).

DNA extraction from standard bacterial strains. Standard strains of *Lactobacillus casei* (*L. casei*) ss. casei JCM 1134^T, *Enterococcus faecalis* (*E. faecalis*) ATCC 19433^T and *Escherishia coli* (*E. coli*) ATCC 25922 were used in this study. These microorganisms were obtained from the Japan Collection of Microorganisms, RIKEN BioResource Center (Saitama, Japan) (JCM/ATCC). *E. faecalis* ATCC 19433^T and *E. coli* were inoculated into Brain Heart Infusion broth (Difco) and cultured under aerobic conditions at 37°C. *L. casei* ss. *casei* JCM 1134^T was cultured anaerobically in GAM broth supplemented with 1% glucose at 37°C for 18 to 48 h. The bacterial samples were diluted and plated onto BHI or GAM agar. The plates were subsequently incubated at 37°C for 3 to 5 days in an incubator (SANYO Electric Co., Ltd., Tokyo, Japan) or anaerobic chamber (Hirasawa Works Inc., Tokyo, Japan), and cultural counts (in CFU) were determined in triplicate. Extraction of the standard DNA from the cultures (1 ml, $<5 \times 10^8$ CFU) of these bacteria was performed by using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). After centrifugation (21,000 × g for 2 min) of the cultures and removing the supernatant, each pellet was resuspended in 480 µl of 50 mmol/L EDTA. Then 120 µl of lysozyme (400 µg/ml for Gram negative bacteria or 1 mg/ml for Gram positive bacteria) was added to the suspension followed by incubation at 37°C for 60 min. According to the manufacturer's instruction, purified DNA was finally eluted by 100 µl of nuclease-free water. Segmented filamentous bacteria (SFB) was not culturable under *in vitro* condition. The SFB DNA (3 µg/ml, 8.4×10^4 bacterial number/ml) for this experiment was kindly provided by Dr. Koji Sawada, Yakult Central Institute.

Quantitative (qt) real-time PCR. qt-PCR amplification and detection was performed with an ABI PRISM 7500 sequence detection system (Applied Biosystems (ABI), Foster City, CA) as previously reported.[17] Each reaction mixture (10 µl) was prepared with 5 µl of SYBR Green I Mater mix (Takara Bio. Inc., Otsu, Japan), 1 µl of each of the specific primers (0.05 µmol/L) and 1 µl of 1× or 10× diluted template DNA. PCR was performed by one cycle of denaturing at 94°C for 5 min and annealing at 60, 58, 55 or 50°C for 20 s (Supplemental Table 1*)[18–21] and extension of the DNA chain at 72°C for 35 s, followed by final denaturing at 94°C for 15 s. The fluorescent products were detected at the last step of each cycle in the reactions.

According to the standard curves made by amplifying serial dilutions of a known quantity of amplicon, bacterial numbers in gastric samples were calculated based on the PCR kinetics of target genes in the sample.

In parallel, different amounts of chromosomal DNA of standard strain were used for PCR with each primer set. DNA extracted from *L. casei* ss. *casei* JCM 1134^T, *E. coli* ATCC25922, *E. faecalis* ATCC 19433^T and SFB were used as real-time PCR standards.

The standard ABI PRISM 7500 quantification software (Applied Biosystems) was used for data analysis. After amplification to distinguish the target product from non-specific annealing, the melting curve analysis was used for confirming the specificity of the PCR product. The melting curves were made by heating at a range of 60 to 95°C at a slow rate of 0.2°C/s, with continuous fluorescence collection. To confirm the specificity of PCR, the melting temperature (Tm) value of standard strains was determined, and the Tm obtained with standard Tm ± 1°C was considered to be identical to the target product.

As the genus *Lactobacillus* exhibited a wider range of PCR products between species, Tm ± 2°C was used for the above determination.

PCR array for the analysis of intestinal mucosal gene expression. Small intestinal mucosa from mid jejunum immersed in RNAlater (Ambion Inc., Austin, TX) was dissolved in Nucleic Acid Purification Lysis Solution (Applied Biosystems). Total RNA was purified by using the ABI PRISM 6100 Nucleic Acid PrepStation System (Applied Biosystems) according to the manufacturer's instructions. The RT2 Profiler PCR Array System was used to quantify mRNA expression with common rat cytokines and chemokines-, oxidative stress-, and wound healing-related genes (84 genes of each, Qiagen). The same quantity of RNA from each sample (10 samples per group except for 6 samples from the normal group) was mixed to make a pooled

*See online. https://www.jstage.jst.go.jp/article/jcbn/56/1/56_14-67/_article/supplement

RNA up to a total of 100 ng, which was reverse-transcribed with the RT2 First Strand Kit (Qiagen). cDNA was mixed with the RT2 SYBR Green/ROX qPCR Master Mix (Qiagen) and amplified on an ABI 7500 Fast 96-well real-time PCR machine (Applied Biosystems) according to the manufacturer's protocol. Results were analyzed with the RT2 Profiler Array Data Analysis ver. 3.5 at the manufacturer's website (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

RT-PCR for the analysis of intestinal mucosal gene expression. Small intestinal mucosa immersed in RNAlater (Ambion) was dissolved in Nucleic Acid Purification Lysis Solution (Applied Biosystems). Total RNA was purified using the ABI PRISM 6100 Nucleic Acid PrepStation System (Applied Biosystems) according to manufacturer's instructions. cDNA was synthesized with MultiScribe Reverse Transcriptase (Applied Biosystems) and real-time PCR was performed using the Applied Biosystems 7500 Fast real-time PCR System. The reaction mixture was prepared according to the manufacturer's protocol using TaqMan® Gene Expression Primer & Probe (Applied Biosystems). The thermal cycling conditions were 95°C for 20 s, followed by 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. The expression levels of mRNA for TNFα, NADP oxidase type 2 (Nox2), dual oxidase 1 (Duox1) and dual oxidase 2 (Duox2) were standardized to β-actin mRNA and the relative expression of each gene was quantified by the comparative Ct (ΔΔCt) method as ratios to the mean value for normal intestinal tissue.

Statistical analysis. The data were expressed as the mean ± SEM for all rats in each group. Statistical analysis was performed on the raw data using the two-tailed *t* test for the comparison between two groups. One-way analysis of variance followed by two-tailed Dunnett multiple comparison test was performed between control and rebamipide-treated groups. Differences with *p* values less than 0.05 were considered significant. The Student's *t* test for unpaired values was used to compare the differences between the three groups.

Results

Effects of rebamipide and ampicillin on small intestinal mucosal injury after indomethacin administration. Macroscopically, multiple mucosal injuries characterized by segmental round ulcerations extending along the mesenteric border of the jejunum (Fig. 1A, arrows) and ileum were observed 24 h after administration of indomethacin 10 mg/kg p.o. Intestinal lesion area in the control group (0.5% CMC, p.o.) was 175.6 ± 19.2 mm^2 (Fig. 1B). Rebamipide 30 and 100 mg/kg, p.o. significantly reduced the intestinal lesion area in a dose-dependent manner, by 41% ($p<0.01$) and 51% ($p<0.01$), respectively. Ampicillin 800 mg/kg, p.o. also significantly reduced the lesion area by 56% ($p<0.01$).

The most severe lesions were observed in the mid jejunum. (63.8 ± 19.2 mm^2) followed by distal jejunum (55.8 ± 10.1 mm^2), proximal jejunum (33.9 ± 20.1 mm^2) and ileum (22.1 ± 5.1 mm^2) (Fig. 2A–D). Rebamipide dose-dependently reduced the lesion at the sites of proximal, mid and distal jejunum, and the reduction rates at 100 mg/kg were 54% (not significant), 79% ($p<0.01$) and 45% ($p<0.05$), respectively. Ampicillin also significantly inhibited mucosal injury in the mid-jejunum (64%, $p<0.05$), distal jejunum (44%, $p<0.05$) and ileum (76%, $p<0.01$).

Suppression of indomethacin-induced small intestinal bleeding by rebamipide and ampicillin administration. Indomethacin-induced small intestinal injury is often accompanied by mucosal bleeding. In this study, severity of small intestinal bleeding was diagnosed by a blood test. As shown in Table 1, red blood cell number was significantly reduced by 27% ($p<0.01$), accompanied by significant reductions in hemoglobin level (27% reduction, $p<0.01$) and hematocrit ratio (22% reduction, $p<0.01$) 24 h after indomethacin administration. Anemia was inhibited by rebamipide 30 and 100 mg/kg in a dose-dependent manner, and was completely abolished by ampicillin 800 mg/kg. The indomethacin treatment also significantly reduced the platelet number by 35% ($p<0.01$) and the white blood cell number by 6% ($p<0.01$). Rebamipide 100 mg/kg and ampicillin recovered the platelet and the white blood cell numbers.

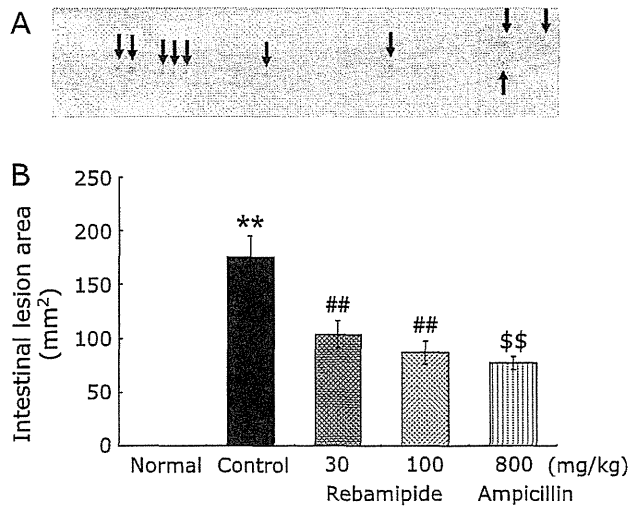

Fig. 1. Effect of rebamipide and ampicillin on indomethacin-induced small intestinal mucosal injury in rats. (A) Macroscopic appearance of the mucosal injuries (arrows) in the mid-jejunum 24 h after indomethacin 10 mg/kg p.o. (control, 0.5% CMC). (B) Rebamipide was orally administered five times, 24, 16 and 1 h prior to, and 3 and 7 h after indomethacin administration. Ampicillin was orally administered twice, 24 and 1 h prior to indomethacin administration. $^{**}p<0.01$ Normal vs Control by *t* test (two-tailed), $^{\#\#}p<0.01$ Rebamipide vs control by Dunnett test (two-tailed), $^{\$\$}p<0.01$ ampicillin vs control by *t* test (two-tailed).

Evaluation of total bacterial number by culture. The total viable number of aerobes, facultative anaerobes and obligate anaerobes were increased in the jejunum of control rats by oral administration of indomethacin (Supplemental Fig. 1 and 2*). Furthermore, the number of *Enterobacteriaceae* was markedly increased by 1,000–10,000 times in the jejunum of indomethacin-treated control rats compared with normal rats untreated with indomethacin (Supplemental Fig. 3*).

The numbers of total aerobes and facultative anaerobes in proximal and central jejunum were decreased by treatment with rebamipide 100 mg/kg (Supplemental Fig. 1a and b*), and the number of total obligate anaerobes was decreased in mid jejunum by treatment with rebamipide 100 mg/kg (Supplemental Fig. 2b*). However, the numbers of aerobes, facultative anaerobes and obligate anaerobes were not decreased by treatment with rebamipide in the distal jejunum and ileum (Supplemental Fig. 1c, 1d, 2c and 2d). The number of *Enterobacteriaceae* was significantly decreased by treatment with rebamipide 100 mg/kg in all 4 small intestinal sites (Supplemental Fig. 3*). In particular, the number of *Enterobacteriaceae* was markedly reduced by treatment with rebamipide 100 mg/kg in the proximal jejunum (Supplemental Fig. 3a*).

Ampicillin treatment extensively decreased the number of total aerobes, facultative anaerobes and obligate anaerobes compared with normal rats (Supplemental Figs. 1 and 2*). It was also shown that the number of *Enterobacteriaceae* in jejunum and ileum of the ampicillin-treated rats was significantly lower than that in control rats (Supplemental Fig. 3*).

*See online. https://www.jstage.jst.go.jp/article/jcbn/56/1/56_14-67/_article/supplement

doi: 10.3164/jcbn.14-67

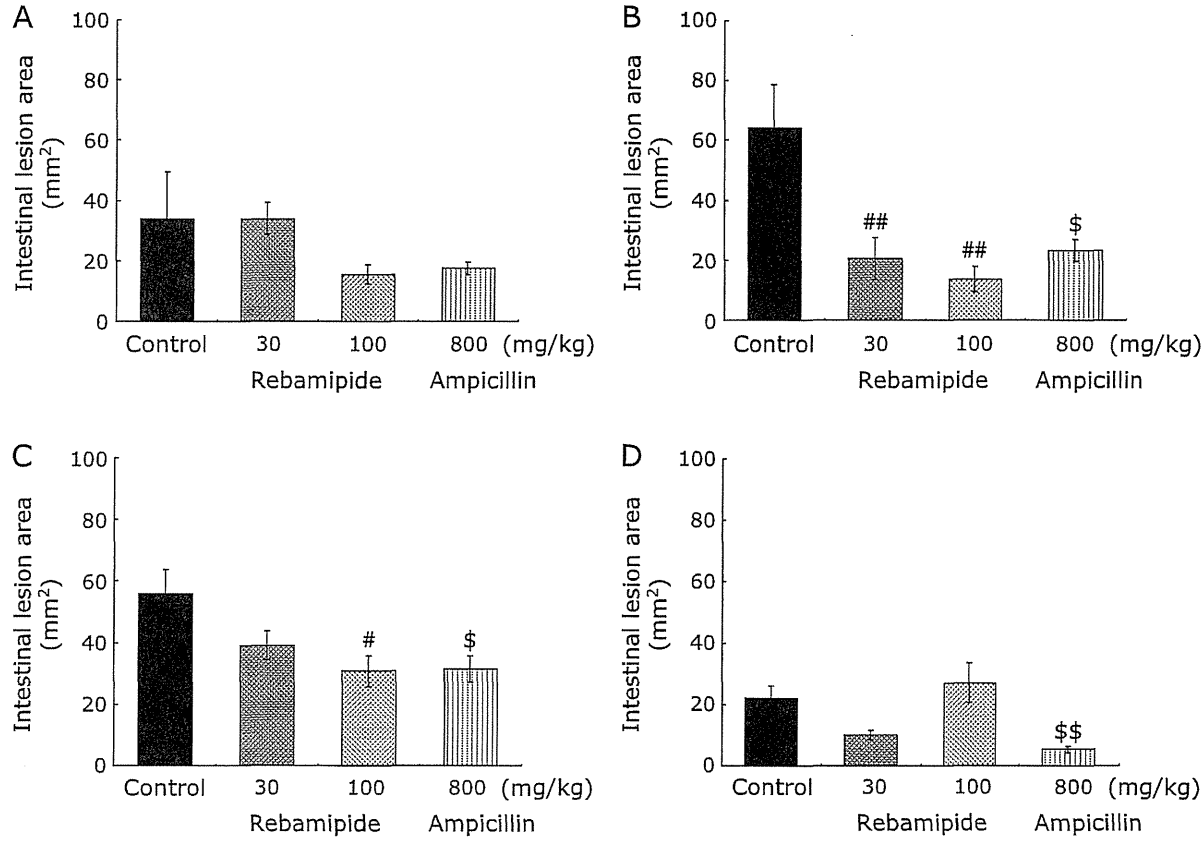

Fig. 2. Site-dependent-small intestinal mucosal injury caused by indomethacin administration and inhibition by rebamipide and ampicillin. (A) Proximal jejunum, (B) mid-jejunum, (C) distal jejunum, (D) ileum. $^{\#}p<0.05$, $^{\#\#}p<0.01$ rebamipide vs control by Dunnett test (two-tailed), $^{\$}p<0.05$, $^{\$\$}p<0.01$ ampicillin vs control by *t* test (two-tailed).

Table 1. Effect of rebamipide and ampicillin on various blood cell numbers 24 h after oral administration of indomethacin in the rat

Group	*n*	RBC (10^7/ml)	Hemoglobin (g/dl)	Hematocrit (%)	Platelet (10^7/ml)	WBC (10^5/ml)
Normal	6	735.5 ± 17.1	14.2 ± 0.3	44.3 ± 0.8	87.0 ± 5.7	101.2 ± 7.6
Control	10	539.3 ± 32.7**	10.4 ± 0.6**	34.5 ± 1.9**	56.3 ± 2.2**	95.0 ± 5.9**
Rebamipide 30 mg/kg	10	614.6 ± 33.6$^{\#}$	11.9 ± 0.6$^{\#}$	38.6 ± 1.9	63.5 ± 3.8	96.7 ± 9.4
Rebamipide 100 mg/kg	10	694.9 ± 17.2$^{\#\#}$	13.4 ± 0.3$^{\#\#}$	42.9 ± 0.9$^{\#\#}$	89.0 ± 5.5$^{\#\#}$	113.1 ± 9.9
Ampicillin 800 mg/kg	10	742.4 ± 28.3$^{\$\$}$	14.4 ± 0.5$^{\$\$}$	44.5 ± 1.6$^{\$\$}$	83.6 ± 9.2$^{\$\$}$	107.7 ± 9.6

The blood was collected at 24 h after the indomethacin 10 mg/kg p.o. administration. Rebamipide at the doses of 30 and 100 mg/kg were orally administered 5 times during the experiment at 24, 16 and 1 h before, and 3 and 7 h after treatment of indomethacin. Ampicillin with 800 mg/kg was orally administered 24 and 1 h prior to the indomethacin administration. The data are expressed as the mean ± SEM from 10 rats except the Normal group as 6 rats. **$p<0.01$ Normal vs Control (0.5% CMC-treated). $^{\#}p<0.05$, $^{\#\#}p<0.01$ Control vs Rebamipide groups. $^{\$\$}p<0.01$ Control vs Ampicillin. RBC, red blood cell; WBC, white blood cell.

Analysis of microbiota in mucosa of the small intestine by real-time PCR. For identification of microbiota, 10 bacterial strains cultured on MacConkey, BHI and GAM agar by aerobic or anaerobic culture were identified by 16S rRNA sequencing in normal rats. Eight strains of *Lactobacillus* sp., one strain of *Enterococcus* sp. (*E. faecalis*) and one strain of *Enterobacteriaceae* (*E. coli*) were detected (Supplemental Table 2*). We analyzed each member quantitatively by real-time PCR using 16S or 23S specific RNA gene targeted primers for the detection of *Lactobacillus* sp., *Enterococcus* sp., and *Enterobacteriaceae*. For the detection of non-culturable bacteria, quantitative real-time PCR using SFB (segmented filamentous bacteria) specific primers was performed.

The average numbers of jejunal and ileal bacteria of each rat group analyzed by real-time PCR are shown in Table 2. In normal rats, *Enterobacteriaceae* was dominant and *Lactobacillus* spp. was subdominant in proximal and mid-jejunum. The higher numbers ($10^{7.73-7.87}$/g mucosa) of *Lactobacillus* spp. were detected in distal jejunum and ileum of normal rats, and *Enterobacteriaceae* and SFB were subdominant in this area. In control rats treated with indomethacin, the numbers of *Enterococcaceae* and *Enterobacteriaceae* were significantly higher in mid-jejunum compared to normal rats. SFB were not detected in the mid-jejunum of any control rats, and the number of SFB in the distal jejunum was

*See online. https://www.jstage.jst.go.jp/article/jcbn/56/1/56_14-67/_article/supplement