TABLE 1 Participant characteristics and predictive accuracy of treatment outcome

Parameter[a]	No. (%) with infection: Eradicated (n = 61)	Not eradicated (n = 12)	OR (95% CI)[b]
Demographic information			
Age (yr [mean ± SD])	51.4 ± 13.4	51.2 ± 10.8	1.00 (0.95–1.05)
Gender			
Men	31 (50.8)	4 (33.3)	0.48 (0.13–1.78)
Women	30 (49.2)	8 (66.7)	
Smokers	17 (27.9)	4 (33.3)	1.65 (0.48–5.74)
Alcohol drinkers	23 (37.7)	6 (50.0)	1.29 (0.34–4.87)
BMI (kg/m^2 [mean ± SD])	22.1 ± 2.9	21.0 ± 2.4	0.85 (0.66–1.10)
H. pylori status			
MIC of STFX (μg/ml [mean ± SD]	0.09 ± 0.13	0.17 ± 0.14	40.55 (0.76–2172.43)
Presence of mutation in *gyrA*	32 (52.5)	11 (91.7)	**9.97 (1.21–82.05)**
Presence of *gyrA* mutation at:			
D91	19 (31.1)	3 (25.0)	0.74 (0.18–3.03)
N87	13 (21.3)	8 (66.7)	**7.39 (1.92–28.42)**
Specific change at D91 or N87:			
D91N	6 (9.8)	0 (0)	
D91G	6 (9.8)	0 (9.8)	
D91Y	7 (11.5)	3 (25.0)	2.57 (0.56–11.82)
N87T	0 (0)	1 (8.3)	
N87K	12 (19.7)	5 (41.7)	2.92 (0.79–10.81)
N87I	1 (1.6)	2 (16.7)	12.00 (0.99–145.03)
CLR resistance (MIC, ≥1 μg/ml)	54 (88.5)	11 (91.7)	1.01 (0.98–1.03)
MNZ resistance (MIC, ≥8 μg/ml)	48 (78.7)	9 (75.0)	1.00 (0.97–1.03)

[a] BMI, body mass index; STFX, sitafloxacin; CLR, clarithromycin; MNZ, metronidazole.

[b] Boldface values indicate significant association with the treatment outcome, analyzed using a univariable logistic regression model.

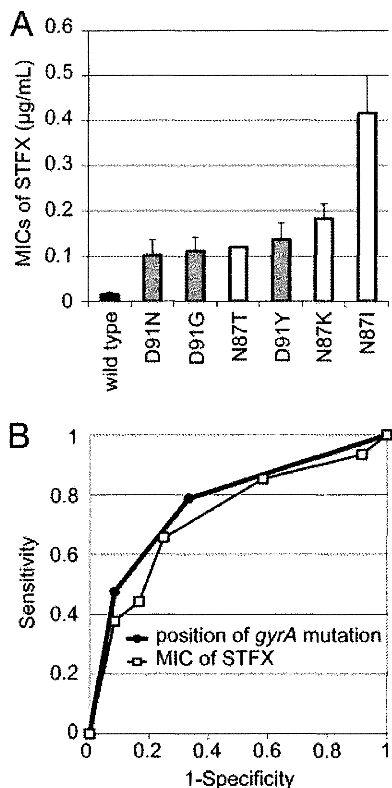

FIG 1 (A) The average MICs of sitafloxacin were higher in *H. pylori* strains with a *gyrA* mutation at N87 than in those with a *gyrA* mutation at D91. (B) Receiver-operating characteristic (ROC) curves show that the position of the *gyrA* mutation is a better marker than MIC levels for predicting outcomes of sitafloxacin-based treatment.

tions and MICs of STFX demonstrated that the diagnostic accuracy of the position of *gyrA* mutations (area under the curve [AUC], = 0.773 ± 0.070) for predicting eradication success is higher than that of MICs of STFX (AUC = 0.725 ± 0.076) (Fig. 1B). When the cutoff value for the MICs of STFX was defined as more than 0.12 μg/ml, an odds ratio (OR) of 5.7 (95% confidence interval [CI], 1.4 to 23.4), a positive predictive value (PPV) of 93.0%, a negative predictive value (NPV) of 30.0%, and an accuracy of 67.1% were yielded for predicting eradication success. On the other hand, the presence of N87 mutations achieved an OR of 7.4 (95% CI, 1.9 to 28.4), a PPV of 92.3%, an NPV of 38.1%, and an accuracy of 76.7%. These results show that prediction of treatment outcomes was better using the positions of *gyrA* mutations than using the MICs of STFX.

According to a systematic review and meta-analysis, the mean eradication rate with 7-day levofloxacin-based rescue therapies was 73% (1). The present study showed that the STFX-based therapy is a marked improvement on quinolone-based rescue therapy, especially for *H. pylori* strains with a *gyrA* mutation. We also discovered that the position of the *gyrA* mutation affects the outcome of the STFX-based therapy. The MICs of the other quinolones, such as levofloxacin and moxifloxacin, have also been demonstrated to be different between N87 and D91 mutations (2, 4). This suggests that detection of N87 mutations will be useful for predicting eradication failure in broad-spectrum quinolone-based therapies and not just in STFX-based therapy. Since stool specimens can be used noninvasively to obtain *H. pylori* DNA, the position of the *gyrA* mutation can be checked more easily and rapidly than drug susceptibility testing in clinical practice. In conclusion, the STFX-based rescue therapy was highly effective even in patients infected with *gyrA* mutation-positive *H. pylori* and is a promising

candidate for third-line therapy. Furthermore, the *gyrA* mutation at N87 is a better marker than MIC levels for predicting outcomes of quinolone-based treatment. To confirm the efficacy of the STFX-based rescue therapy, randomized controlled trials are warranted.

ACKNOWLEDGMENTS

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EDUCATION AND IMAGING

Gastrointestinal: Capsule endoscopy assists in the complete deworming of parasites

A 67-year-old man presented with the complaint of ribbon-like substances in the feces. In the beginning of August 2010, he was referred to our hospital for treatment of parasitic worm infection. The patient did not have symptoms such as diarrhea, anemia, or weight loss; the results of physical and hematological examinations and of thoracoabdominal radiography were normal. Although he did not have a history of parasitic worm infection and had never traveled abroad, he had eaten raw salmon 1 month ago. On the first day of the hospital visit, the patient was diagnosed with diphyllobothriasis after examination of the helminth eggs found in the feces. After diatrizoic acid swallow on the second day, the presence of the worm body was confirmed by colonoscopy, and the worm body was extirpated from the anus by using grasping forceps. Although approximately 1 m of the worm body together with most of the neck portion was successfully extirpated, removal of the scolex was not confirmed. On the fourth day, after obtaining the patient's consent, we performed capsule endoscopy because of possible incomplete deworming. Capsule endoscopy confirmed parasitization by a cestode and detected the scolex attached to the jejunal mucosa (Figure 1). Parasite-specific drugs, i.e., praziquantel and magnesium citrate, were administered, and complete deworming was subsequently confirmed by microscopic identification of the scolex (Figure 2). The worm body was pathologically confirmed as *Diphyllobothrium nihonkaiense* by performing trichrome and carmine staining (Figure 3).

For the successful treatment of diphyllobothriasis, it is essential to remove the scolex of the parasite. The use of capsule endoscopy now allows for (1) easy capture of images of parasites, such as that of the scolex of *Diphyllobothrium nihonkaiense*, in the small intestine, which was previously considered difficult; and (2) provides information critical for therapeutic decision making before administering anthelmintics.

Contributed by
Shinsuke Kobayashi*, Yukishige Okamura*, Hidekazu Suzuki†, Nobuhito Taniki*, Jun Uehara*, Keisuke Ojiro*, Ken Teramoto*, Masashi Kirinoki‡, Yuichi Chigusa‡, Kuninori Shiwaku§, Toshifumi Hibi†
**Department of Gastroenterology, Sano Kosei General Hospital, Sano, Tochigi, †Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, ‡Laboratory of Tropical Medicine and Parasitology, Dokkyo Medical University, Tochigi and §Department of Environmental Medicine, Shimane University Faculty of Medicine, Izumo, Japan*

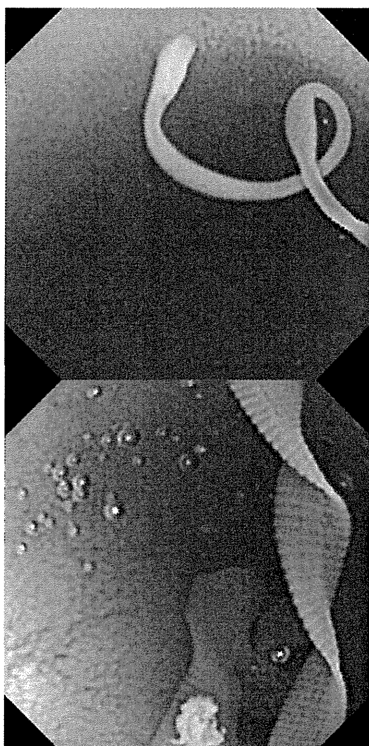

Figure 1 The worm's scolex was clearly detected and found to be attached to the upper small intestinal mucosa, by using capsule endoscopy.

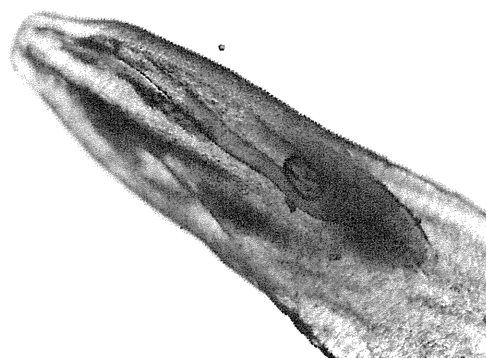

Figure 2 The presence of the discharged scolex was confirmed by using a microscope (magnification, 400×), but the scolex was not stained.

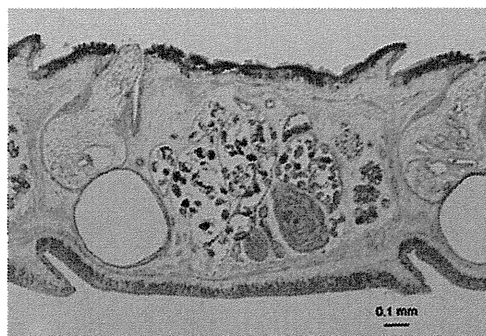

Figure 3 The worm body was stained with trichrome stain and was morphologically confirmed as *Diphyllobothrium nihonkaiense*.

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Classification of functional dyspepsia based on concomitant bowel symptoms

J. MATSUZAKI,* H. SUZUKI,* K. ASAKURA,† Y. FUKUSHIMA,‡ J. M. INADOMI,§ T. TAKEBAYASHI† & T. HIBI*

*Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan
†Department of Preventive Medicine and Public Health, Keio University School of Medicine, Tokyo, Japan
‡Department of Internal Medicine, Tokyo-Eki Center-Building Clinic, Tokyo, Japan
§Division of Gastroenterology, Department of Medicine, University of Washington, Seattle, WA, USA

Abstract

Background *Functional dyspepsia (FD) is a heterogeneous disease, and categorized into postprandial distress syndrome (PDS) and epigastric pain syndrome (EPS). However, many FD patients have overlap of both PDS and EPS. The present study was designed to examine whether FD could be categorized based on the presence of concomitant gastrointestinal symptoms.* ***Methods*** *A web survey comprised of the Gastrointestinal Symptom Rating Scale (GSRS), Rome III criteria of FD, and demographic information was sent to public participants who have no history of severe illness. Factor and cluster analyses were conducted to identify sub-categories of FD based on GSRS.* ***Key Results*** *A total of 8038 participants completed the survey. A total of 563 participants met the criteria for FD, whereas 6635 participants did not have dyspepsia symptoms. The remainder had either organic disease (377) or uninvestigated dyspepsia (463). The cluster analysis categorized participants as constipation predominant (cluster C), diarrhea predominant (cluster D), or having neither diarrhea nor constipation (cluster nCnD). Cluster C and D were significantly associated with the presence of FD [odds ratio (OR) 2.57, 95% confidence interval (CI) 2.06–3.21; OR 2.80; 95% CI 2.27–3.45, respectively]. In FD, especially in PDS cases, the scores of upper gastrointestinal symptoms were higher in cluster C or D than in cluster nCnD.* ***Conclusions & Inferences*** *The severity of dyspepsia symptoms is associated with the presence of bowel symptoms especially in PDS. This novel categorization of FD based on concomitant constipation or diarrhea may improve classification of patients.*

Keywords *cluster analysis, constipation, diarrhea, dyspepsia, factor analysis.*

Address for Correspondence
Hidekazu Suzuki, MD, PhD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.
Tel: 81 3 5363 3914; fax: 81 3 5363 3967;
e-mail: hsuzuki@a6.keio.jp
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INTRODUCTION

Functional dyspepsia (FD) is a common clinical syndrome characterized by chronic and recurrent gastroduodenal symptoms in the absence of any organic or metabolic disease that is likely to explain the symptoms.[1,2] FD is a heterogeneous condition consisting of different subgroups. According to Rome III criteria of FD, FD is divided into two subgroups: postprandial distress syndrome (PDS) and epigastric pain syndrome (EPS), to distinguish between meal-induced symptoms and meal-unrelated symptoms believed to be pathophysiologically and clinically relevant.[1,3] Although it has been postulated that symptom subgroups could be used to identify more homogenous subgroups that would respond to targeted medical therapy, up to half of FD patients have overlap of both PDS and EPS.[4] In addition, to the best of our knowledge, there is no evidence that PDS or EPS should be treated differently.

Overlap among functional gastrointestinal disorders (FGIDs) is extremely common. Specifically, dyspepsia and bowel symptoms, such as diarrhea and constipation, often coexist.[5] A recent meta-analysis reported that the prevalence of irritable bowel syndrome (IBS) among participants of dyspepsia was 37% compared with 7% in those without dyspepsia.[6] In addition, the prevalence of esophageal symptoms, such as heartburn, is also high in FD patients, although esophageal reflux

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symptoms may co-exist, but are not consider typical FD symptoms in the Rome III criteria.[1] Savarino *et al.*[7] showed that patients with functional heartburn had more frequent postprandial fullness, bloating, early satiety, and nausea than patients with non-erosive reflux disease (NERD). These recent studies reinforce the concept that FGIDs extend beyond the boundaries suggested by the anatomical location of symptoms.

Although subclasses of FD based on symptom clusters have been proposed,[1,8] subclustering based on bowel symptoms or esophageal symptoms has not been investigated. The aim of this study was to use factor and cluster analyses to determine whether FD could be characterized based on the presence of concomitant other gastrointestinal (GI) symptoms, including bowel symptoms and esophageal symptoms.

MATERIALS AND METHODS

Study participants

The protocol for this study was approved by the ethics committee of Tokyo Ekimae Building Clinic (TEC-0801, September 24, 2008). We conducted a web-based cross-sectional study. Participants were solicited from a list of public participants who are invited previously to participate in the clinical studies conducted by the Tokyo Ekimae Building Clinic with informed consent. No participants in the list have a severe chronic or life-threating illness such as malignancy or systemic autoimmune diseases, and a serious mental illness, such as major depression or schizophrenia. Participants who used prescribed medicines or over-the-counter (OTC) drugs were not excluded from the present study. The questionnaires were comprised of items including the Japanese version of the Gastrointestinal Symptom Rating Scale (GSRS)[9] and the Rome III criteria of FD;[1] in addition, prior receipt of upper GI screening examination was elicited. If either of the latter two were identified, the presence/absence of structural disease was also abstracted. The Japanese version of GSRS questionnaire is a validated, self-administered questionnaire that includes 15 questions, which assess severity of GI symptoms, including esophageal reflux symptoms, dyspepsia symptoms, and bowel symptoms, using a 7-point Likert scale.[10] Demographic information, such as age, gender, smoking habit, alcohol habit, height, and weight, were also obtained, and body mass index (BMI) (weight height^{-2}) was calculated. Smoking was categorized into 'none', 'light' (1–15 cigarettes day^{-1}), and 'heavy' (>16 cigarettes day^{-1}) according to a number of cigarettes consumed per day. Alcohol intake was also categorized into 'none', 'light' (1–3 days week^{-1}), and 'heavy' (4–7 days week^{-1}) according to a number of days of alcohol consumption per week.

Definition of FD cases and non-dyspepsia controls

Based on Rome III criteria, participants were defined as having dyspepsia when they have one or more of symptoms, such as postprandial fullness, early satiation, or epigastric pain or burning for at least 6 months prior to the survey. Participants without dyspepsia symptoms were defined as a 'non-dyspepsia' control group. Participants with dyspepsia who had undergone the upper GI examination and had no evidence of structural disease in the stomach and duodenum were defined as 'FD' cases. Participants with dyspepsia who had not undergone upper GI examination were classified as 'uninvestigated dyspepsia'. Participants with dyspepsia who had undergone upper GI examination and had structural disease were classified as 'organic disease' patients.

The FD subjects with postprandial fullness or early satiation were defined as those with PDS, whereas FD subjects with epigastric pain or burning were defined as those with epigastric pain syndrome (EPS). Using these definitions, FD subjects were subcategorized into three groups as follows: subjects with PDS alone, subjects with EPS alone, and subjects with both PDS and EPS.

Statistical analysis

Exploratory factor analysis was conducted in all web responders to identify the latent pathologic conditions, named 'symptom factors', and to reduce the dimensionality of subsequent analyses. Principal factor method with Varimax rotation was used. Subsequently, a non-hierarchical *k*-means cluster analysis for a three-cluster solution was performed using the symptom factor scores derived from the preceding factor analysis. Three 'symptom clusters' were extracted. The differences in the prevalence of FD between three symptom clusters were evaluated using univariable and multivariable logistic regression. In the multivariable model, age, gender, smoking, alcohol use, and BMI were included. The differences of the symptom factor scores between three symptom clusters in FD cases were examined using one-way ANOVA and Tukey's *post hoc* analysis. The differences of life-style characteristics between three symptom clusters were examined for each gender separately, as participant characteristics were significantly different between men and women. The differences between the three symptom clusters in age and BMI were examined using one-way ANOVA and Tukey's *post hoc* analysis. The differences between the three symptom clusters in smoking and alcohol habits were examined using Fisher's exact test.

All statistical analyses were conducted using the SPSS Statistics version 18.0 for Windows software (SPSS Japan, Tokyo, Japan; SPSS Inc., Chicago, IL, USA). The data in the tables were expressed as mean ± standard deviation. Two-sided *P*-values were considered as statistically significant at a level of 0.05.

RESULTS

Participant characteristics

A total of 8038 participants (3462 men and 4576 women; mean age 40.8 ± 9.7 years) completed the questionnaire. A total of 563 participants were defined as FD cases, whereas 6635 participants without dyspepsia symptoms were identified as non-dyspepsia controls. A total of 463 participants were classified as uninvestigated dyspepsia, and 377 had organic disease (Fig. 1). Participant characteristics are shown in Table 1. Mean age was higher in FD cases than in non-dyspepsia controls. There was greater proportion of women among FD cases than among non-dyspepsia controls. Alcohol consumption and smoking was greater in FD than in non-dyspepsia. BMI was lower in FD than in non-dyspepsia. BMI was especially lower

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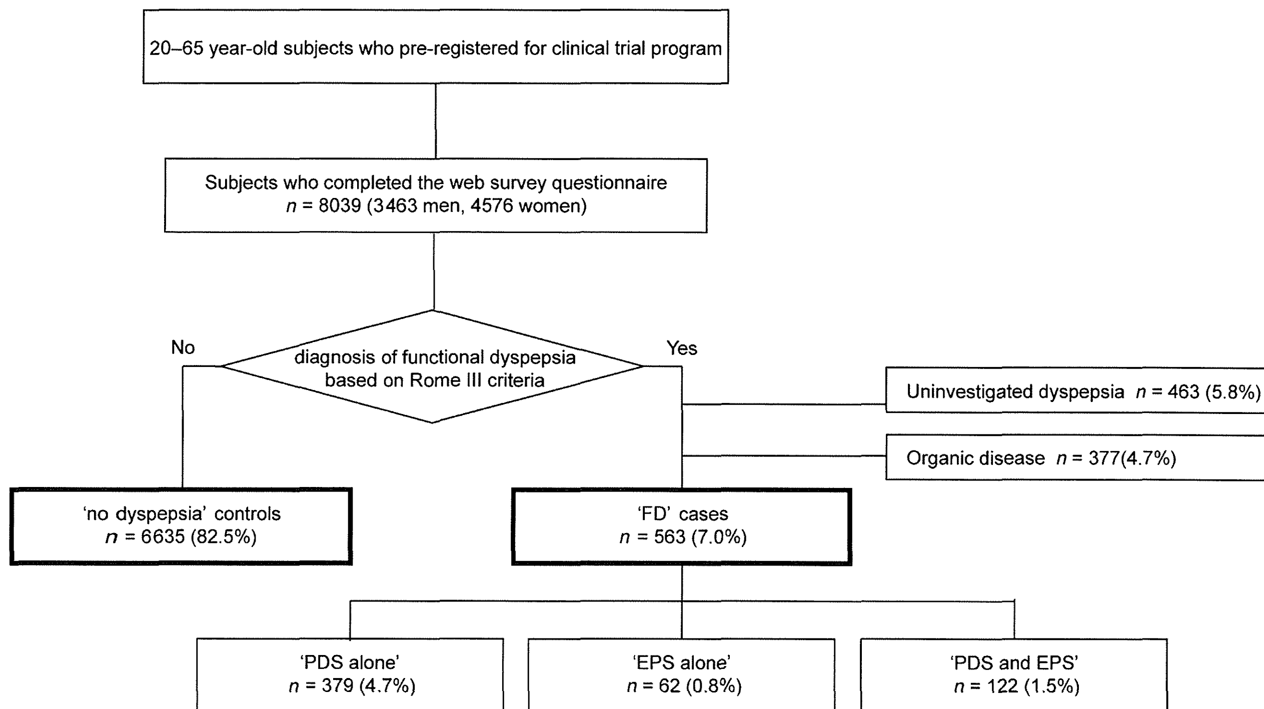

Figure 1 The study population.

Table 1 Participant characteristics

	All (n = 8038)	Non-dyspepsia (n = 6635)	FD (n = 563)	Uninvestigated dyspepsia (n = 463)	Organic disease (n = 377)	P value (non-dyspepsia vs FD)
Age						
Mean ± SD (years) (Mean ± SD)	40.8 ± 9.7	40.9 ± 9.8	42.8 ± 7.8	34.4 ± 9.0	44.0 ± 8.7	<0.001*
20–29 [No. (%)]	1028	842 (12.7)	21 (3.7)	148 (32.0)	17 (4.5)	
30–39 [No. (%)]	2639	2183 (32.9)	157 (27.9)	199 (43.0)	100 (26.5)	
40–49 [No. (%)]	2894	2372 (35.7)	282 (50.1)	80 (17.3)	160 (42.4)	
50–59 [No. (%)]	1201	999 (15.1)	90 (16.0)	33 (7.1)	79 (21.0)	
60–65 [No. (%)]	276	239 (3.6)	13 (2.3)	3 (0.6)	21 (5.6)	
Gender [No. (%)]						
Men	3462	2920 (44.0)	228 (40.5)	142 (30.7)	172 (45.6)	0.111†
Women	4576	3715 (56.0)	335 (59.5)	321 (69.3)	205 (54.4)	
Smoking habit [No. (%)] (number of consumptions per day)						
None (0)	5924	4947 (74.6)	392 (69.6)	332 (71.7)	253 (67.1)	0.037†
Light (1–15)	1062	848 (12.8)	85 (15.1)	74 (16.0)	55 (14.6)	
Heavy (>16)	1052	840 (12.7)	86 (15.3)	57 (12.3)	69 (18.3)	
Alcohol habit [No. (%)] (number of days of consumption per week)						
None (0)	2743	2281 (34.4)	181 (32.1)	159 (34.3)	122 (32.4)	<0.001
Light (1–3)	2956	2482 (37.4)	178 (31.6)	186 (40.2)	110 (29.2)	
Heavy (4–7)	2339	1872 (28.2)	204 (36.2)	118 (25.5)	145 (38.5)	
BMI (kg m^{-2}) (Mean ± SD)	22.6 ± 3.9	22.7 ± 3.9	22.2 ± 3.8	21.7 ± 3.9	22.6 ± 4.5	0.008*

BMI, body mass index; FD, functional dyspepsia.
*Analyzed by unpaired Student's *t*-test.
†Analyzed by Pearson's Chi-squared test.

in 'PDS alone' group (22.1 ± 3.7 kg m^{-2}) than in non-dyspepsia (22.7 ± 3.9 kg m^{-2}, $P = 0.003$). This suggests that participants with PDS alone may avoid food because it precipitates their symptoms.

The differences between FD cases and non-dyspepsia controls in the average scores of the 15 GI symptom assessed by GSRS were compared using unpaired Student's *t*-test. All of the 15 GI symptoms were

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significantly more severe in FD cases than in non-dyspepsia controls (See Table S1 online). Scores in participants with uninvestigated dyspepsia or organic disease were also higher than in non-dyspepsia. These results showed that not only upper GI symptoms, but also bowel symptoms and esophageal symptoms were more severe in participants with dyspepsia.

Factor analysis

Factor analysis revealed that the 15 items could be reduced to three GI symptom factors, namely factor EGD (esophagogastroduodenal symptoms), factor C (constipation), and factor D (diarrhea) (Table 2). Factor EGD mainly reflects the severity of upper GI symptoms, such as heartburn, abdominal pains, and abdominal distension. Factor C reflects constipation-related symptoms. Factor D reflects diarrhea-related symptoms.

To examine potential associations between demographic factors (exposure variables) and the three symptom factors (outcome variables), linear regression analyses were performed (See Table S2 online). Younger age was associated with increased scores of all three symptom factors. Factor EGD and factor C scores were greater in women, whereas factor D scores were greater in men. Smoking was associated with factor EGD score in a dose-dependent manner. Heavy smoking was also associated with factor D score. Heavy alcohol consumption positively associated with factor EGD and factor D scores; conversely it was inversely correlated with factor C score. BMI was inversely associated with factor C score.

Table 2 Factor loading of the severity of 15 gastrointestinal symptoms (n = 8038)

	Factor EGD	Factor C	Factor D
Heartburn	**0.718**	0.139	0.132
Acid regurgitation	**0.701**	0.092	0.165
Abdominal pains	**0.681**	0.157	0.150
Sucking sensations in the epigastrium	**0.651**	0.200	0.166
Nausea and vomiting	**0.591**	0.169	0.228
Abdominal distension	**0.555**	0.335	0.181
Eructation	**0.498**	0.233	0.218
Borborygmus	0.396	0.314	0.276
Increased flatus	0.306	0.399	0.280
Feeling of incomplete evacuation	0.251	**0.624**	0.307
Urgent need for defecation	0.237	0.192	**0.668**
Increased passage of stools	0.227	0.064	**0.835**
Loose stools	0.209	0.088	**0.818**
Hard stools	0.188	**0.772**	0.047
Decreased passage of stools	0.161	**0.820**	0.010

Bold values indicate the loading values of higher than 0.5 for each symptom factor.
Factor EGD: the severity of upper gastrointestinal symptoms.
Factor C: the severity of constipation-related symptoms.
Factor D: the severity of diarrhea-related symptoms.

Cluster analysis

Cluster analysis based on the three symptom factor scores showed that FGIDs could be categorized into three clusters, namely cluster nCnD (non-constipation and non-diarrhea), cluster C (constipation), and cluster D (diarrhea). Cluster C was characterized by high scores of factor C (factor EGD 0.31; factor C 1.31; factor D −0.38). Cluster D was characterized by high scores of factor D (factor EGD 0.28; factor C −0.08; factor D 1.34). Cluster nCnD was not associated with any of the three symptom factors (factor EGD −0.22; factor C −0.43; factor D −0.39). The scores of the three symptom factors are plotted on the 3D coordinate systems to illustrate the distribution of three clusters in Fig. 2.

Based on the result of cluster analysis, FD cases and non-dyspepsia controls could be categorized into three clusters. Among 6635 non-dyspepsia controls, 4101 (61.8%) were categorized to cluster nCnD, 1218 (18.4%) were to cluster C, and 1316 (19.8%) were to cluster D. On the other hand, among 563 FD cases, 217 (38.5%) were categorized to cluster nCnD, 160 (28.4%) were to cluster C, and 186 (33.0%) were to cluster D. Multivariable logistic regression analysis revealed that both cluster C and D were significantly associated with the presence of FD (Table 3). Association between cluster C and FD were almost same level as association between cluster D and FD, suggesting that constipation and diarrhea were equally contributed to the onset of FD.

The prevalence of PDS or EPS was similar among the three symptom clusters: 217 FD participants in cluster nCnD were 146 (67.3%) with PDS alone, 26 (12.0%) with EPS alone, and 45 (20.7%) with both PDS and EPS; 160 in cluster C were 113 (70.6%) with PDS alone, 16 (10.0%) with EPS alone, and 31 (19.3%) with both PDS and EPS; 186 in cluster D were 120 (64.5%) with PDS alone, 20 (10.8%) with EPS alone, and 46 (24.7%) with both PDS and EPS. This illustrates that overlap of constipation or diarrhea was not associated with the presence/absence of PDS or EPS. In 'PDS alone' and 'PDS and EPS' groups, factor EGD score was higher in cluster C or D than in cluster nCnD. These results showed that upper GI symptoms, such as reflux or dyspepsia, were more severe in participants with bowel symptoms than without bowel symptoms especially in participants with PDS. On the other hand, in 'EPS alone' group, factor EGD score was not significantly different among the three symptom clusters (Fig. 3).

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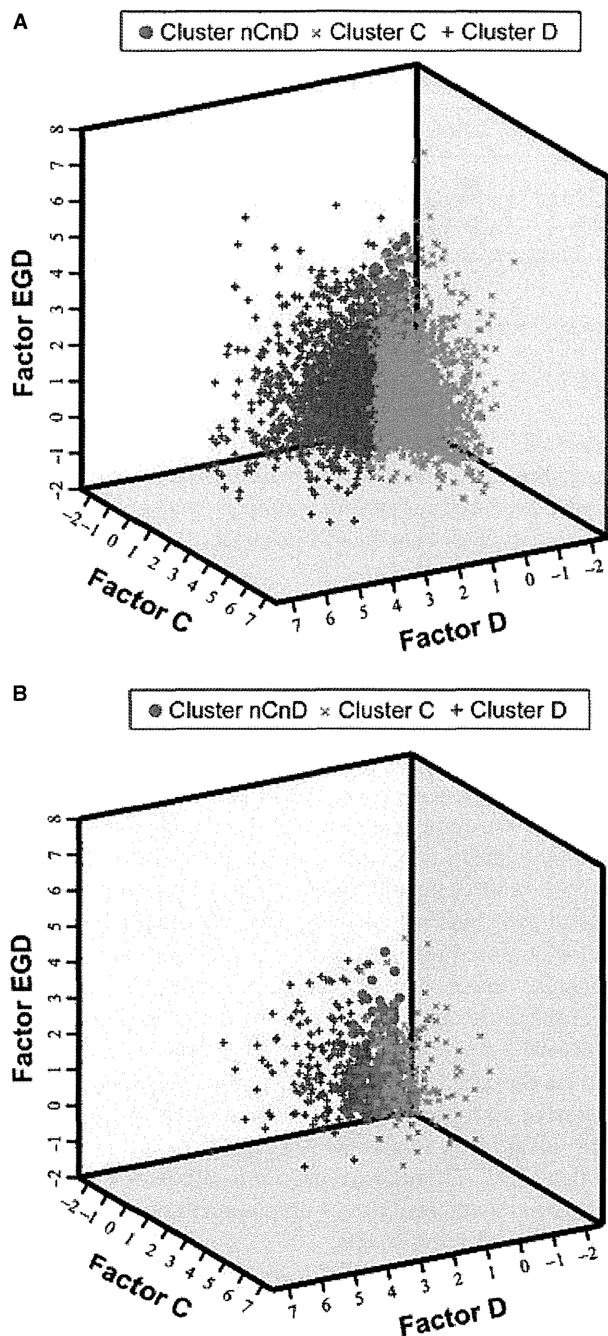

Figure 2 Distribution of cluster nCnD, cluster C, and cluster D. The 3D spatial distribution of overall 8038 participants (A) and 563 functional dyspepsia participants (B) with three symptom factor scores derived from factor analysis showed that the three symptom clusters were well separated.

Demographic factors in FD cases were significantly different between the three clusters (See Table S3 online). As there was a greater proportion of women in cluster C, subsequent analyses were examined for each gender separately. In both genders, alcohol consumption was associated with cluster nCnD and cluster D, but not with cluster C. In women, lower BMI was associated with cluster C.

DISCUSSION

This population based, large-scale cross-sectional study was conducted to identify GI symptom clusters in FGIDs. Cluster analysis in the present study revealed that all FGIDs, including FD, could be subcategorized based on concomitant bowel symptoms. As IBS is classified as constipation predominant IBS (IBS-C), diarrhea predominant IBS (IBS-D), and mixed IBS (IBS-M) in Rome III criteria,[11] FD could be categorized into three clusters: absence of bowel symptoms (cluster nCnD), constipation predominant (cluster C), and diarrhea predominant (cluster D). Esophageal reflux symptoms, postprandial distress, and epigastric pain symptoms could not be separated using factor analysis, suggesting that overlaps between functional esophageal disorders, PDS, and EPS occur frequently. Classification of FD based on concomitant lower GI symptoms is a novel concept and may improve our ability to discriminate between subgroups of FD. Recent study showed that psychosocial factors, such as anxiety, depression, and somatization are also important variables for subgrouping FD.[12] Classification of FD based on a combination of bowel symptoms and psychosocial factors would be a promising alternative for gastroduodenal symptom-based classification as proposed by the Rome III criteria.

In the present study, FD was more prevalent in participants with bowel symptoms (cluster C or cluster D) than those without bowel symptoms (cluster nCnD). This result is consistent with the observed high frequency of overlap between FD and IBS. Moreover, concomitant bowel symptoms were associated with demographic factors, such as gender, alcohol consumption, and BMI, among FD participants. These results suggest that the etiology of dyspepsia symptoms may differ among participants classified as cluster nCnD, cluster C, and cluster D. Corsetti *et al.*[13] showed that FD-IBS overlap is more prevalent among women and is associated with a greater weight loss, overall symptom severity, and with hypersensitivity to distention than FD alone. The present study confirmed that FD with constipation is more prevalent among women, and is associated with lower BMI among women. On the other hand, these associations were not observed in FD with diarrhea (See Table S3 online).

When FD subjects were subcategorized into 'PDS alone', 'EPS alone', and 'PDS and EPS' groups, a significant association between these three groups

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Table 3 Relationship between the three symptom clusters and diagnosis of FD

	Non-dyspepsia (n = 6635)	FD (n = 563)	Univariable analysis*	Multivariable analysis†
	No. (%)	No. (%)	Odds ratio (95% CI)	Odds ratio (95% CI)
Cluster nCnD (n = 4318)	4101 (95.0)	217 (5.0)	Ref.	Ref.
Cluster C (n = 1378)	1218 (88.4)	160 (11.6)	2.48 (2.00–3.08)	2.57 (2.06–3.21)
Cluster D (n = 1502)	1316 (87.6)	186 (12.4)	2.67 (2.18–3.28)	2.80 (2.27–3.45)

CI, confidence interval; FD, functional dyspepsia.
*Analyzed by univariable logistic regression model.
†Analyzed by multivariable logistic regression model with adjustment for cluster C, cluster D, age, gender, smoking habit, alcohol habit, and body mass index.

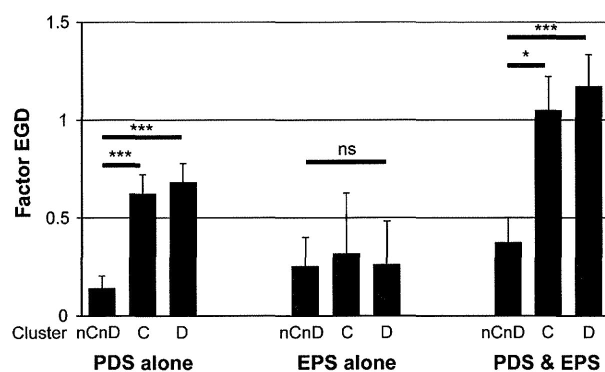

Figure 3 Associations between upper gastrointestinal symptoms and the three symptom clusters in each subgroup of functional dyspepsia. ***P < 0.001, *P < 0.05 significant difference using one-way ANOVA and Tukey's *post hoc* analysis. EPS, epigastric pain syndrome; ns, not significant; PDS, postprandial distress syndrome.

and the three symptom clusters was not observed. However, the association between the severity of upper GI symptoms (factor EGD score) and concomitant bowel symptoms among PDS participants differed from the association among participants with EPS alone. Some previous studies also demonstrated that FD-IBS overlap patients have worse quality of life than FD-alone and IBS-alone patients.[14,15] Results of the present study revealed that FD participants with bowel symptoms have greater symptoms severity than those without bowel symptoms especially in PDS, but not in EPS alone. This suggests that while PDS might be associated with the bowel symptoms, EPS without PDS might be independent of the presence/absence of bowel symptoms. Patients with constipation or diarrhea tend to have a general motor disturbance throughout the GI tract, including abnormal colonic transit and delayed gastric emptying, especially in patients with concomitant FD and IBS.[16–18] GI motility disorders are likely to induce symptoms of PDS rather than those of EPS.[19] The other study showed that patients with both FD and IBS are associated with hypersensitivity to distention of the stomach using gastric barostat.[13] Gastric hypersensitivity was more prevalent when patients suffered from both EPS and PDS.[20] These previous reports also support that concomitant constipation or diarrhea is associated with PDS, but not EPS alone.

Criticisms of the present study include possible differences between web-survey responder population and general population (generalizability). Web-based assessment may select participants from comparatively young and socially advantaged groups characterized by high literacy, and high internet access.[21] In the present study, mean age in FD cases were older than that in non-dyspepsia controls. This might be because our population contains a higher proportion of young people (<40 years old) than general population. This participant bias might affect the prevalence of FD, as FD was more prevalent in those with lower household income, lower educational levels, larger household membership, and those who were unemployed.[22–25] However, a previous study showed that participation bias is thought to have little effect on associations with putative risk factors.[21] In addition, web-based survey has advantages related to the speed and cost of data collection.[21] Therefore, it would be a powerful tool for studying characteristics of diseases and overlaps of the other disorders in FGIDs.

The disadvantage of the k-means cluster analysis is that the number of clusters must be supplied as a parameter. In the present study, we selected a three-cluster solution, as the results in three-cluster solution were the most understandable not only for gastroenterologists but also general practitioners. This categorization of FD can be determined just by the presence/absence of constipation or diarrhea which can be obtained from medical history taking. Whether treatments for bowel symptoms would improve dyspepsia symptoms in FD patients with constipation or diarrhea has not been examined[26], warranting future research.

In conclusion, GI symptoms, including FD, can be categorized into three clusters based on the presence

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and type of bowel symptoms, suggesting differences in etiology between FD patients with constipation, with diarrhea, or neither. Constipation and diarrhea contribute almost equally to the presence of FD. PDS patients with bowel symptoms have greater symptoms severity than those without bowel symptoms. This categorization of FD is easy to use for general practice, and may improve classification of patients and identify subgroups that have differing pathophysiology or who may respond differently to treatment.

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AUTHOR CONTRIBUTIONS

HS & YF designed the research study. HS & YF conducted the web survey and collected the data. JM, HS & KA analyzed and interpreted the data. JM & HS drafted the article. KA & JMI revised the manuscript. TT & TH supervised and approved to be published.

CONFLICT OF INTEREST

The authors have no competing interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Average scores of 15 gastrointestinal symptoms.

Table S2. Associations between demographic factors and symptom factor scores (n = 8038).

Table S3. Difference of life-style characteristics between three symptom clusters in FD cases.

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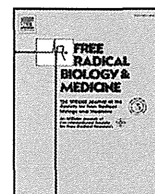

Original Contribution

FecA1, a bacterial iron transporter, determines the survival of *Helicobacter pylori* in the stomach

Hitoshi Tsugawa, Hidekazu Suzuki *, Juntaro Matsuzaki, Kenro Hirata, Toshifumi Hibi

Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan

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ABSTRACT

Helicobacter pylori encodes a single iron-cofactored superoxide dismutase (SodB), which is regulated by the ferric uptake regulator (Fur). Ferrous ion (Fe^{2+}) is necessary for the activation of SodB. The activity of SodB is an important determinant of the capability of *H. pylori* for long-term colonization of the stomach and of the development of metronidazole (Mtz) resistance of the bacterium. This study is conducted to characterize the Fe^{2+}-supply mechanisms for the activation of SodB in *H. pylori*, which, as mentioned above, is associated with the host-colonization ability and Mtz resistance of *H. pylori*. In this study, we demonstrate that *fecA1*, a Fe^{3+}-dicitrate transporter homolog, is an essential gene for SodB activation, but not for the biogenic activity of *H. pylori*. *H. pylori* with SodB inactivation by *fecA1* deletion showed reduced resistance to H_2O_2, reduced gastric mucosal-colonization ability in Mongolian gerbils, and also reduced resistance to Mtz. Our experiment demonstrated that FecA1 is an important determinant of the host-colonization ability and Mtz resistance of *H. pylori* through Fe^{2+} supply to SodB, suggesting that FecA1 may be a possible target for the development of a novel bactericidal drug.

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Helicobacter pylori is a spiral-shaped, flagellated, microaerophilic gram-negative bacterium that colonizes the gastric epithelium of humans and is a major cause of peptic ulcers and also a key risk factor for gastric cancer and gastric MALT lymphoma [1]. *H. pylori* encodes only one single iron-cofactored superoxide dismutase (Fe-SOD; SodB) (HP0389), which catalyzes the conversion of superoxide anions to hydrogen peroxide, with the latter degraded into oxygen and water by catalase [2-4]. The *sodB* mRNA expression in *H. pylori* is directly regulated by the ferric uptake regulator (Fur) protein [5]. Recently, we reported that clinically isolated metronidazole (Mtz)-resistant strains (KS0048 and KS0145) showed derepression of *sodB* mRNA expression by amino acid mutations of Fur (C78Y and P114S; mutant Fur), which led to the development of Mtz resistance [6]. In addition, it has been reported that *sodB* deletion in *H. pylori* causes the bacterium to lose its ability to colonize the gastric mucosa in mice [7]. These results demonstrate that SodB is an important determinant of Mtz resistance and of the host-colonization ability of *H. pylori*.

Ferrous ion (Fe^{2+}) is necessary for the basal functioning of all cells, as a cofactor for enzymes and metalloproteins, and is also required for SodB activation [3,8]. On the other hand, iron (Fe^{2+} and Fe^{3+}) overload produces toxic oxygen radicals in the presence of oxygen [9]. Therefore, there exists an ingenious regulatory system for intracellular iron (Fe^{2+} and Fe^{3+}) uptake. *H. pylori* has three *fecA*-like genes (HP0686, *fecA1;* HP0807, *fecA2;* and HP1400, *fecA3*), each encoding a high-affinity transporter of Fe^{3+}-dicitrate. The expression of *fecA1* and *fecA2* is regulated by Fur, while the transcription of *fecA3* is regulated by nickel-responsive regulator (NikR) [10-12]. Additionally, Danielli et al. [13] reported that the expression patterns of *fecA1* and *fecA2* throughout the period of growth were different: whereas *fecA1* showed sustained expression over time, *fecA2* expression was derepressed only in the late phase of growth. This report suggested that the role of FecA1 was different from that of FecA2, although they were both regulated by Fur [13].

Fur dimers, as global transcriptional regulators, are formed by binding of Fur to Fe^{2+} (iron-bound Fur), and these dimers bind to the Fur-binding consensus sequences (Fur-box) of the target genes [14,15]. The expression of the *fecA1* and *fecA2* genes is repressed by binding of iron-bound Fur to each Fur-box under iron-replete conditions (normal cultivation conditions; +Fe) (Fig. 1). On the other hand, under iron-restricted conditions (−Fe), iron-bound Fur is absent; thus, the expression is derepressed by a decrease in the binding of iron-free Fur (apo-Fur) to each Fur-box (Fig. 1) [10,15].

The mechanisms of detoxification of ROS by antioxidant enzymes are of particular interest in understanding the development of Mtz resistance and also the capability of *H. pylori* for long-term gastric mucosal colonization [2,6,16,17]. Therefore, examination of the SodB activation process is required for understanding the mechanisms of detoxification of ROS in *H. pylori*. This study was designed to examine

Abbreviations used: FecA1, ferric citrate transporter homolog protein; Fur, ferric uptake regulator; Mtz, metronidazole; ROS, reactive oxygen species; SodB, iron-cofactored superoxide dismutase.

* Corresponding author. Fax: +81 3 5363 3967.
E-mail address: hsuzuki@a6.keio.jp (H. Suzuki).

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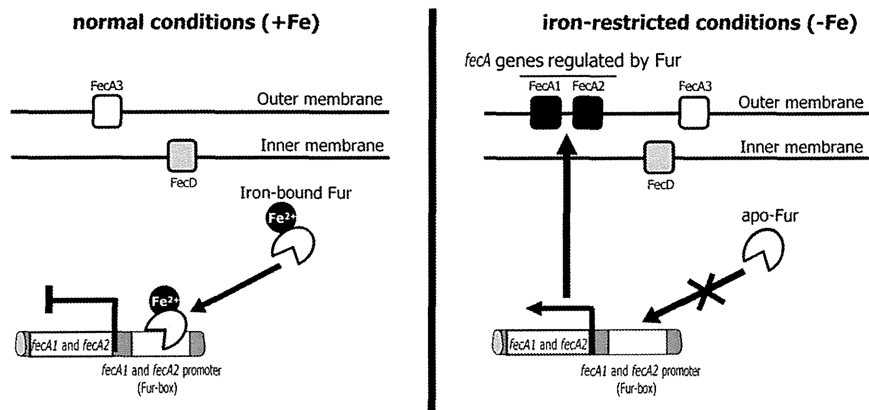

Fig. 1. Regulation of the *fecA* genes for the Fe^{3+}-dicitrate transporter by Fur in *H. pylori*. The transcription of *fecA3* is not regulated by Fur. Iron-bound Fur binds to the promoter of *fecA1* and *fecA2*, leading to transcriptional repression under normal cultivation conditions (iron-replete conditions). Under iron-restricted conditions, iron-bound Fur is absent; thus the *fecA1* and *fecA2* genes are derepressed by iron-free Fur (apo-Fur).

the Fe^{2+} supply system associated with SodB activation, by analyzing the *H. pylori* strains KS0048 and KS0145.

Materials and methods

Bacterial strains and culture conditions

H. pylori strains ATCC700392 and KS0189 were used as the Mtz-susceptible strains with wild-type Fur; ATCC430504 was used as the Mtz-resistant strain with deletion of the oxygen-insensitive NADPH nitroreductase (RdxA) [18]; the KS0048 and KS0145 strains were used as the Mtz-resistant strains with mutant Fur. KS0048 showed an amino acid mutation of Fur (Pro 114 replaced by Ser and Asn 118 replaced by His; mutant Fur); KS0145 also showed mutant Fur (Cys 78 replaced by Tyr and Asn 118 replaced by His) [6]. The KS strains were clinically isolated strains and were kept at −80 °C in *Brucella* broth (Becton–Dickinson, Franklin Lakes, NJ, USA) containing 25% (vol/vol) glycerol. The bacteria were cultured on *Brucella* agar containing 7% sheep blood and 7% fetal bovine serum (FBS) for 2 days at 37 °C under microaerobic conditions maintained with AnaeroPack MicroAero (Mitsubishigas, Tokyo, Japan).

Construction of SodB-overexpressing and fecA1-deletion mutants

The construction of a SodB-overexpressing strain of *H. pylori* using the shuttle vector pHel3 [19] has been described previously [6]. Briefly, the pHel3::sodB construct was electroporated into *H. pylori*, which was grown on 30 μg/ml kanamycin to obtain a SodB-overexpressing strain (ATCC700392 pHel3::sodB). Only the pHel3 vector was electroporated into *H. pylori*, which was grown on 30 μg/ml kanamycin to obtain a control strain (ATCC700392 pHel3 control).

The target-region gene cassette (5′*fecA1*-chloramphenicol acetyltransferase (*cat*)-3′*fecA1*) for construction of a *fecA1*-deletion mutant was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA), and the sequences were determined (target vector). The target-region gene cassette was constructed for insertion into the open reading frame of FecA1. The construction of the target-region gene cassette was carried out using the PCR-based overlap extension method [20]. The primer sequences used for construction of the target-region gene cassette were as follows: 5′*fecA1* region, forward 5′-ATGAAAAGAATTTTAGTCTCTTTGGCTG and reverse 5′-GGTGGTATATCCAGTGATTTTTTTCTCCATATTCATCATGCCCCCTGTTCTTAAG; *cat* region, forward 5′-ACTTAAGAACAGGGGGCATGATGAATATGGAGAAAAAAATCACTGGATATACCACC and reverse 5′-CCATACCGCTGTATAGTTGTTAAACAGTTACGCCCCGCCCTGCCACTCATCGC; 3′*fecA1* region, forward 5′-GCGATGAGTGGCAGGGCGGGGCGTAACTGTTTAACAACTATACAGCGGTATGG and reverse 5′-AATACCAAAAGTCGTGTGCTTGTAAG. For the overlap extension, the forward primer was the 5′*fecA1* region forward primer and the reverse primer was the 3′*fecA1* region reverse primer. The target vector was electroporated into *H. pylori* ATCC700392, ATCC43504, KS0048, and KS0145, respectively, which were grown on 20 μg/ml chloramphenicol to obtain the *fecA1*-deletion mutants (ATCC700392 *fecA1*-deletion mutant, ATCC43504 *fecA1*-deletion mutant, KS0048 *fecA1*-deletion mutant, and KS0145 *fecA1*-deletion mutant).

Expression and purification of Fur

The expression and purification methods for recombinant Fur protein from *Escherichia coli* BL21(DE3) using a pET-30b(+) vector (Novagen, Madison, WI, USA) (*E. coli* pET::Fur) have been described previously [6]. Briefly, the expression of Fur protein was induced by 0.5 mM IPTG for 6–8 h at 30 °C, and then the Fur protein expressed in the *E. coli* pET::Fur was purified using the MagneHis protein purification system (Promega, Madison, WI, USA).

RNA isolation and quantitative reverse transcription–polymerase chain reaction (RT-PCR)

The bacteria, normalized to an OD_{600} of 1.0, were incubated under normal cultivation conditions (*Brucella* broth containing 7% FBS) and iron-restricted conditions (normal cultivation conditions with 20 μM deferoxamine mesylate, which is a ferric-iron chelator) for 5 h. Because *fecA* genes (*fecA1* and *fecA2*) were derepressed by apo-Fur under iron-restricted conditions, the bacteria were incubated under normal cultivation conditions. The total RNA of the bacteria was isolated using the SV total RNA isolation system (Promega). The reverse transcription was performed using the PrimeScript RT reagent kit (TaKaRa, Ohtsu, Japan). The quantitative RT-PCR was performed using the SYBR Premix Ex Taq Perfect Real-Time Kit (TaKaRa) in a Dice thermal cycler real-time system (TaKaRa). The primer sequences used were as follows: *fecA1* mRNA, forward 5′-GGTGGAAGCTTCAGGGGTG and reverse 5′-GCTTCTTCAATGCTCTGATTGG; *fecA2* mRNA, forward 5′-AGCTCTCGCACGGTGATTTCCAAC and reverse 5′-CTATGCCCGTTACCGCCCC; *pfr* mRNA, forward 5′-TTGATCATGCGGCTGAAGAATACG and reverse 5′-TGATGTGTTGCTCATGTTCATAGGC. The 16S rRNA gene mRNA primers used as the internal control for the quantitative RT-PCR have been described in detail previously [21].

Binding assays by surface plasmon resonance assay (BIAcore2000)

A BIAcore2000 instrument (Biacore AB, Uppsala, Sweden) was used to perform the surface plasmon resonance assay in accordance with the manufacturer's guidelines. First of all, each promoter region

of *fecA1* and *fecA2* was PCR-amplified with specific biotinylated primers (*fecA1* promoter, forward 5′-Bio-GAAGCTTCCACCCTTTC-CAAATTATG and reverse 5′-CTTGATAGCTTTTTATGCGACTCAAATT; *fecA2* promoter, forward 5′-Bio-CATTCATTGTGATAACCTTTCTC and reverse 5′-AATAAATAACGCATTCTAAAACTAACAT). Biotinylated PCR products of the *fecA1* or *fecA2* promoter were immobilized onto Sensor Chip SA (GE Healthcare, Piscataway, NJ, USA). At least five concentrations of each purified Fur protein were applied to the *fecA1* or *fecA2* promoter-immobilized Sensor Chip SA in HBS-EP running buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid, 0.005% surfactant P20) at a flow rate of 10 μl/min. The response value of the reference cell (flow cell 3, blank) was subtracted from the response values for each flow cell 4 (*fecA1* promoter-immobilized or *fecA2* promoter-immobilized) to correct for nonspecific binding. The measured values were expressed in resonance units proportional to the concentration of each Fur protein. The data were analyzed and the dissociation constant (K_d) values were calculated using BIAevaluation software (Biacore).

Measurement of SOD activity

The bacteria normalized to an OD_{600} of 1.0 were incubated under normal cultivation conditions or iron-restricted conditions (normal cultivation conditions with 20 μM deferoxamine mesylate) for 5 h. After sonication (1.5 min at 25% power) of the bacteria, the bacterial lysates were centrifuged, and then the SOD activity was measured using a SOD assay kit (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's guidelines.

DNA sequencing of H. pylori sodB

The complete *sodB* gene was PCR-amplified with specific primers (forward 5′-ATTAACTTTTAAAAAATTTAAAAAGAATTTG and reverse 5′-TTAAGCTTTTTTATGCACC) using Ex Taq DNA polymerase (TaKaRa). The specific PCR products were direct-sequenced using the BigDye terminator version 1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the deduced amino acid sequences were aligned using GENETYX version 5.1.

Disk assays for H_2O_2 susceptibility

The bacteria, normalized to an OD_{600} of 0.1, were plated for confluent growth on *Brucella* agar with or without 20 μM deferoxamine mesylate. Sterile 5-mm disks saturated with 10 μl of 5 M H_2O_2 were placed onto the plates. After 3 days, the zone of inhibition around the disks was measured.

Measurement of the MICs of Mtz

The bacteria, normalized to an OD_{600} of 0.1, were inoculated onto an agar plate containing Mtz in serial twofold dilutions (0.5–128 μg/ml) with or without 20 μM deferoxamine mesylate. After 3 days, the minimum inhibitory concentration (MIC) values were determined [22].

Measurement of the intracellular iron (Fe^{2+} and Fe^{3+}) concentration

The bacteria, normalized to an OD_{600} of 1.0, were incubated under normal cultivation conditions and iron-restricted conditions (normal cultivation conditions with 20 μM deferoxamine mesylate) for 5 h. After sonication (1.5 min at 25% power) of the bacteria, the bacterial lysates were centrifuged, and then the intracellular iron (Fe^{2+} and Fe^{3+}) concentration was measured using a Metalloassay Kit Fe (AKJ Global Technology Co., Chiba, Japan) in accordance with the manufacturer's guidelines. The data for the intracellular iron (Fe^{2+} and Fe^{3+}) concentration were corrected for total cellular protein.

Mongolian gerbil colonization studies

All experiments and procedures were carried out by the Keio University Animal Research Committee (08080-10). Six-week-old male specific-pathogen-free Mongolian gerbils (MON/Jms/Gbs Slc) ($n=42$) were purchased from Japan SLC. Seven-week-old animals were inoculated with one of the *fecA1* mutant *H. pylori* strains (ATCC700392, ATCC700392 *fecA1*-deletion mutant, KS0048, KS0048 *fecA1*-deletion mutant, KS0145, and KS0145 *fecA1*-deletion mutant); 0.6 ml of each bacterial suspension at a concentration of 10^9CFU/ml was administered using an orogastric catheter. Twelve weeks after the inoculation, the animals were sacrificed after 12 h of food deprivation and their stomachs were excised. One half of the tissues were weighed and homogenized in sterile saline, and the number of viable colony-forming units was determined by plating portions on Nissui *Helicobacter* agar (Nissui, Tokyo, Japan).

Statistical analysis

All values were expressed as means ± SD. The statistical significance of differences between the two groups was evaluated using the Student *t* test. The analysis was performed using the JSTAT statistical software (Version 8.2). Statistical significance was accepted at $P<0.05$, unless otherwise indicated.

Results

Enhancement of SodB activity and derepression of fecA1 mRNA expression in Mtz-resistant strains carrying mutant Fur

The SodB activity was significantly higher in the KS0048 and KS0145 strains compared with that in the ATCC700392 and KS0189 strains (Mtz-susceptible strains with wild-type Fur) under normal cultivation conditions (Fig. 2). Subsequently, in order to assess if amino acid mutation of SodB would contribute in the enhancement of its enzymatic activity, we aligned the predicted amino acid sequences of SodB for ATCC700392, KS0189, KS0048, and KS0145. No distinct amino acid mutation of the SodB protein was observed in the KS0048 and KS0145 strains (Supplementary Fig. 1). Based on these results, it is conceivable that KS0048 and KS0145 have an altered Fe^{2+} supply system for the SodB protein that enhances its enzymatic activity. Therefore, we next examined the iron (Fe^{2+} and Fe^{3+})-transport mechanisms of KS0048 and KS0145 in relation to the SodB activity. Initially, the mRNA expression of the *fecA* genes (*fecA1* and *fecA2*)

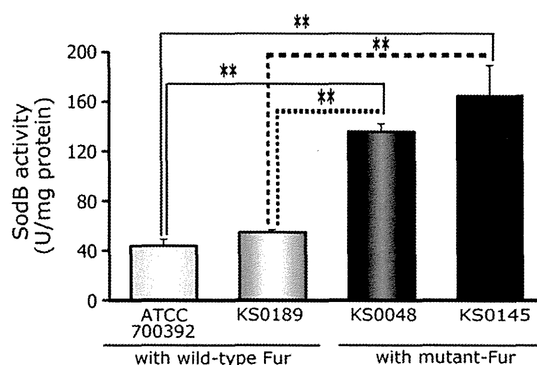

Fig. 2. Enhancement of SodB activity in the Mtz-resistant strains with mutant Fur under normal cultivation conditions. Under normal cultivation conditions, the SodB activity in ATCC700392 and KS0189 (Mtz-susceptible strains with wild-type Fur) and KS0048 and KS0145 (Mtz-resistant strains with mutant Fur) was measured by the method described under Materials and methods. Results are means ± SD of three independent assays. **$P<0.01$, statistically significant difference compared with the SodB activity in ATCC700392 and KS0189.

regulated by Fur was evaluated to assess whether expression of the Fe^{3+}-dicitrate transporter contributed to the increase in the SodB activity. The *fecA1* mRNA expression in KS0048 and KS0145 was significantly derepressed compared with that in ATCC700392 and KS0189 under normal cultivation conditions (Fig. 3A). Interestingly, the *fecA2* mRNA expression was not derepressed under normal cultivation conditions (Fig. 3B). These results suggest that the enhanced SodB activities of KS0048 and KS0145 were related to the increase in the Fe^{3+}-dicitrate uptake mediated by the *fecA1* gene. Next, to assess the mechanisms of *fecA1* mRNA derepression in KS0048 and KS0145, we aligned the nucleic acid sequences of the Fur-binding consensus sequence (Fur-box: AACTAATAATGGTTATT) of the *fecA1* promoter [15] and then examined the binding affinity of the iron-bound wild-type Fur and iron-bound mutant Fur to the promoters of *fecA1* and *fecA2* by surface plasmon resonance assay (BIAcore2000). No distinct mutation in the *fecA1* promoter was observed in KS0048 and KS0145 (data not shown). The K_d value of the binding of iron-bound mutant Fur to the *fecA1* and *fecA2* promoters as control was measured in comparison with that of iron-bound wild-type Fur. The results of the BIAcore assay revealed a significant increase in the K_d value for binding of iron-bound mutant Fur to the *fecA1* promoter compared with that of iron-bound wild-type Fur to the *fecA1* promoter (Fig. 3C), indicating a significantly reduced affinity of iron-bound mutant Fur for the *fecA1* promoter; therefore, *fecA1* expression was derepressed to a greater extent in KS0048 and KS0145 than in ATCC700392 and KS0189. On the other hand, the K_d value of iron-bound mutant Fur binding to the *fecA2* promoter did not increase (Fig. 3D), indicating that the amino acid mutations in Fur did not influence binding affinity to the *fecA2* promoter.

H_2O_2 sensitivity and Mtz resistance in the Mtz-resistant strains with mutant Fur under iron-restricted conditions

Next, we expected that the enhanced SodB activity in KS0048 and KS0145 might be repressed by iron-restricted conditions, to increase the H_2O_2 sensitivity and decrease Mtz resistance. First of all, to characterize the H_2O_2 sensitivity under iron-restricted conditions, we used an inhibition zone assay to comparatively examine the sensitivity of the ATCC700392, KS0189, KS0048, KS0145, and SodB-overexpressing mutants (ATCC700392 pHel3::sodB). The H_2O_2 sensitivity of KS0048, KS0145, and ATCC700392 pHel3::sodB was significantly decreased compared with that of ATCC700392 under normal cultivation conditions (Table 1). Under iron-restricted conditions, on the other hand, whereas the H_2O_2 sensitivity of ATCC700392 pHel3::sodB increased to the same level as that of ATCC700392, that of KS0048 and KS0145 was significantly lower compared with that of ATCC700392 (Table 1). Similarly, although the Mtz resistance of ATCC700392 pHel3::sodB (MIC = 32 μg/ml) [6] decreased to the level of Mtz sensitivity (MIC < 8 μg/ml) under iron-restricted conditions (MIC = 4 μg/ml), no decrease in the Mtz resistance of KS0048 and KS0145 was observed (Table 1). A possible reason for this finding is that the SodB activity was significantly higher in KS0048 and KS0145 compared with that in ATCC700392 and KS0189 under iron-

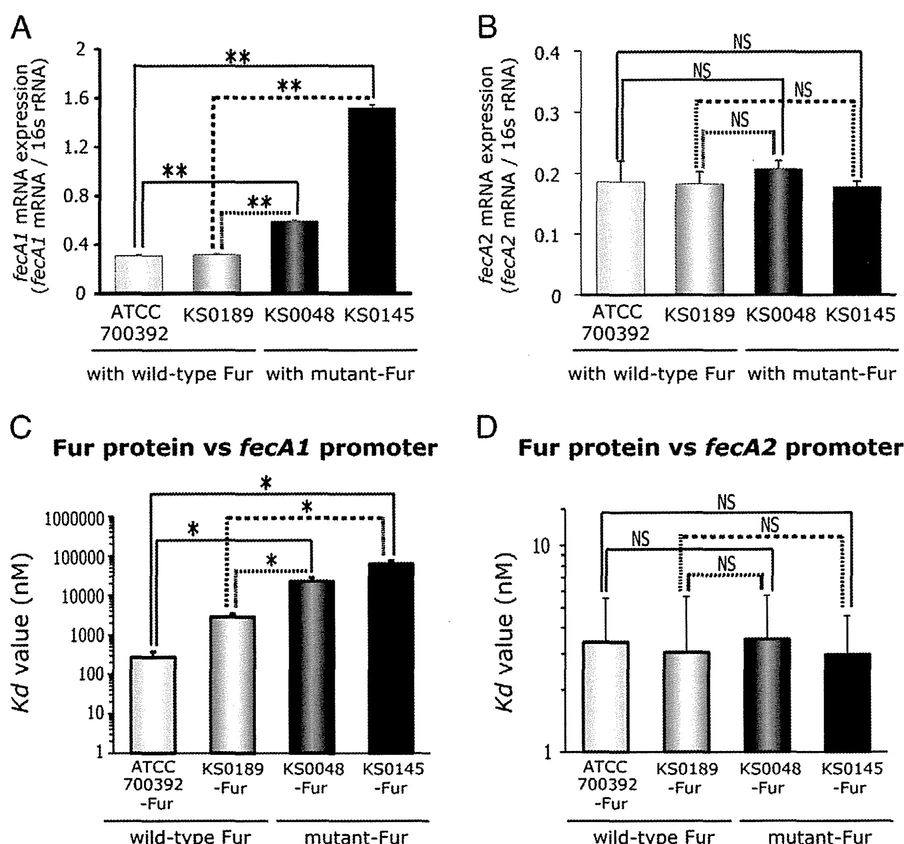

Fig. 3. Derepression of *fecA1* mRNA transcription by decreased affinity of mutant Fur for the *fecA1* promoter. (A) Under normal cultivation conditions, transcription of *fecA1* mRNA in ATCC700392 and KS0189, KS0048, and KS0145 was measured by quantitative RT-PCR. Results are means ± SD of three independent assays. ** $P < 0.01$, statistically significant difference compared with the *fecA1* mRNA expression in ATCC700392 and KS0189. (B) Under normal cultivation conditions, transcription of *fecA2* mRNA in ATCC700392 and KS0189, KS0048, and KS0145 was measured by quantitative RT-PCR. Results are means ± SD of three independent assays. NS, not significant. (C) The K_d value for binding of each Fur protein to the *fecA1* promoter was calculated as a reference in the non-*fecA1* promoter-immobilized flow cells using BIAevaluation software. The white bar indicates the affinity of wild-type Fur for the *fecA1* promoter, and the black bar indicates the affinity of mutant Fur for the *fecA1* promoter. Results are means ± SD of three independent assays. * $P < 0.05$, statistically significant difference compared with ATCC700392-Fur and KS0189-Fur. (D) The K_d value for binding of each Fur protein to the *fecA2* promoter was calculated as a reference in the non-*fecA2* promoter-immobilized flow cells using BIAevaluation software. The white bar indicates the affinity of wild-type Fur for the *fecA2* promoter, and the black bar indicates the affinity of mutant Fur for the *fecA2* promoter. Results are means ± SD of three independent assays. NS, not significant.

Table 1
H_2O_2 and Mtz resistance of Mtz-resistant strains carrying mutant Fur under iron-restricted conditions.

Strain	Substitutions in Fur [6]	Iron-replete (normal cultivation) condition		Iron-restricted condition (20 μM deferoxamine mesylate)		
		Mean inhibition zone (mm)	*P* value	Mean inhibition zone (mm)	*P* value	MIC (μg/ml)
ATCC700392	Wild type	2.8 ± 0.29		4.5 ± 0.89		<0.5
KS0189	N118H	3.4 ± 0.52	0.18	4.6 ± 0.40	0.91	<0.5
KS0048	P114S, N118H	1.9 ± 0.10	0.006**	2.2 ± 0.25	0.013*	16
KS0145	C78Y, N118H	1.6 ± 0.47	0.020**	2.1 ± 0.12	0.042*	32
ATCC700392, pHel3::sodB	Wild type	1.6 ± 0.45	0.019**	4.3 ± 0.58	0.78	4
ATCC700392, pHel3 control	Wild type	3.0 ± 0.45	0.69	4.5 ± 0.30	1.0	2

Results are means ± SD of three independent assays.MIC, minimum inhibitory concentration (μg/mL).
**P*<0.05 compared with ATCC700392.

restricted conditions (Fig. 4A). From this result, we expected that KS0048 and KS0145 might show enhanced iron (Fe^{2+} and Fe^{3+})-storage ability under normal cultivation conditions and then may make efficient reuse of the ferrous ion under iron-restricted conditions. Therefore, to examine the Fe^{2+}-storage ability of KS0048 and KS0145, we evaluated the mRNA expression of ferritin *pfr*, which is the major Fe^{2+}-storage protein regulated by Fur in *H. pylori* under normal cultivation conditions [23-25]. The expression levels of *pfr* mRNA in KS0048 and KS0145 were significantly increased compared with those in ATCC700392 and KS0189 under normal cultivation conditions (Fig. 4B). Actually, the levels of intracellular iron (Fe^{2+} and Fe^{3+}) in KS0048 and KS0145 were significantly higher than those in ATCC700392 and KS0189 under normal cultivation conditions (Fig. 4C). Additionally, under iron-restricted conditions, the *pfr* mRNA expression in the KS0048 and KS0145 strains was significantly derepressed compared with that in ATCC700392 and KS0189 (Fig. 4D), and the levels of intracellular iron in the KS0048 and KS0145 strains were also increased (Fig. 4E). These results suggested that KS0048 and KS0145 have an enhanced capability for ferrous ion storage by derepression of *pfr* under both normal cultivation and iron-restricted conditions.

Contribution of FecA1 to SodB activity, H_2O_2 sensitivity, and Mtz resistance

To characterize the contribution of FecA1 to the SodB activity, H_2O_2 sensitivity, and Mtz resistance of *H. pylori*, we constructed a *fecA1*-deletion mutant strain of each *H. pylori* strain (ATCC700392 *fecA1*-deletion mutant, KS0048 *fecA1*-deletion mutant, and KS0145 *fecA1*-deletion mutant). Deletion of the *fecA1* gene hardly influenced the bacterial growth in this study (data not shown). One reason for this may be that there was no decrease in the uptake of Fe ions (both $^{55}Fe^{2+}$ and $^{55}Fe^{3+}$) into the bacterial cells of the *fecA1*-deletion mutant strains [26]. The SodB activity of all *fecA1*-deletion mutant strains was significantly decreased (Fig. 5A). The SodB activity of ATCC700392 was the most significantly decreased with *fecA1* deletion, suggesting that Fe^{2+} is supplied to SodB through FecA1 in *H. pylori*, regardless of the presence/absence of amino acid mutations in Fur. Similarly, the H_2O_2 resistance of each *fecA1*-deletion mutant was significantly decreased by 30–60% (Fig. 5B). In addition, the MICs of Mtz for KS0048 and KS0145 decreased dramatically from 32 to 4 and from 128 to 32 μg/ml, respectively. Especially, the Mtz resistance of KS0048 was completely reversed by *fecA1* deletion (MIC<8 μg/ml). To assess whether derepression of *fecA1* mRNA expression was dependent on mutant Fur, we measured the MIC of Mtz in a *fecA1*-deletion mutant of ATCC43504. Development of Mtz resistance in ATCC43504 was caused by the deletion of the *rdxA* gene [18]. Alignment of the predicted amino acid sequences of ATCC43504-Fur showed that ATCC43504-Fur was the wild type. This sequence showed a 100% homology with KS0189-Fur (data not shown). The MIC of Mtz for ATCC43504 decreased slightly (from 128 to 64 μg/ml) after *fecA1* deletion. This finding demonstrated that development of Mtz resistance by FecA1 depended on the mutant Fur.

Colonization of Mongolian gerbils by the fecA1-deletion mutant

To assess the role of FecA1 in the host-colonization ability of *H. pylori*, we measured the colonization of the gastric mucosa by wild-type and *fecA1*-deletion mutant strains at 12 weeks after inoculation into Mongolian gerbils. The *fecA1*-deletion mutant of ATCC700392 tended to show reduced host colonization compared with the wild-type ATCC700392 ($P = 0.050$; Fig. 6). The *fecA1*-deletion mutants KS0048 and KS0145 showed a significantly reduced capability for host colonization compared with the wild type of each strain ($P = 0.014$ and $P = 0.016$, respectively; Fig. 6). Our finding did not indicate whether the host-colonization abilities of KS0048 and KS0145 were significantly increased compared with that of the ATCC700392 (Fig. 6). This result suggested that derepression of *fecA1* by mutant Fur alone did not lead to enhanced host colonization.

Discussion

H. pylori encodes only one single iron (Fe^{2+})-cofactored SOD (SodB). Therefore, ferrous ion is indispensable for activation of SOD in *H. pylori* [8]. Our findings indicate that the enhanced Fe^{2+}-supply system associated with SodB activation in the KS0048 and KS0145 strains can be explained as follows: under iron-replete conditions, Fe^{3+}-dicitrate transport was enhanced by derepression of *fecA1* mRNA expression by iron-bound mutant Fur. Intracellular ferric ion (Fe^{3+}) was reduced to Fe^{2+} by Fe^{3+}-reductase (ribBA) [27], providing Fe^{2+} to SodB (Fig. 7). Under iron-restricted conditions, Fe^{2+} storage in KS0048 and KS0145 was enhanced through derepression of *pfr* mRNA expression by apo-mutant Fur, supplying Fe^{2+} to SodB (Fig. 7). In addition, our results demonstrated, for the first time, that FecA1 may play an indispensable role in the bacterial survival in the stomach and in the development of Mtz resistance of *H. pylori* through Fe^{2+} supply to SodB.

Because *H. pylori* is a highly genetically diverse organism, different strains may show great variations in phenotype. However, in this study, all *fecA1*-deletion mutant strains of *H. pylori* (ATCC700392, KS0048, and KS0145) showed reduced SodB activity and reduced gastric mucosal colonization ability. Therefore, it is thought that Fe^{3+}-dicitrate transport by FecA1 is associated with the activation of SodB, regardless of the genetic diversity of the strains.

The *fecA1* and *fecA2* genes, encoded in *H. pylori* as a Fe^{3+}-dicitrate transporter, are both regulated by Fur [10,11]. In this study, interestingly, in KS0048 and KS0145, only *fecA1* expression was derepressed by mutant Fur under normal cultivation conditions (Fig. 3A), whereas the expression of *fecA2* was repressed (Fig. 3B). The underlying reason was the high affinity of iron-bound wild-type Fur for the *fecA2* promoter compared with that for the *fecA1* promoter; the K_d value of iron-bound wild-type Fur binding to the *fecA2* promoter ($K_d = 3.4$ nM) was low compared with that to the *fecA1* promoter ($K_d = 273$ nM) (Figs. 3C and D). This result suggested that the expression of *fecA1*, but not of *fecA2*, was more influenced by amino acid mutations in Fur, and then only the expression of *fecA1* was derepressed by mutant Fur. Recently, Ernst et al. [5] reported that the K_d value for the *sodB* promoter of apo-wild-type Fur was also low ($K_d = 270$ nM, from the data of [5]), similar to the K_d value for the *fecA1* promoter of iron-bound wild-type Fur. Because *H. pylori* is continuously exposed to superoxides generated by its own respiration and metabolism and the

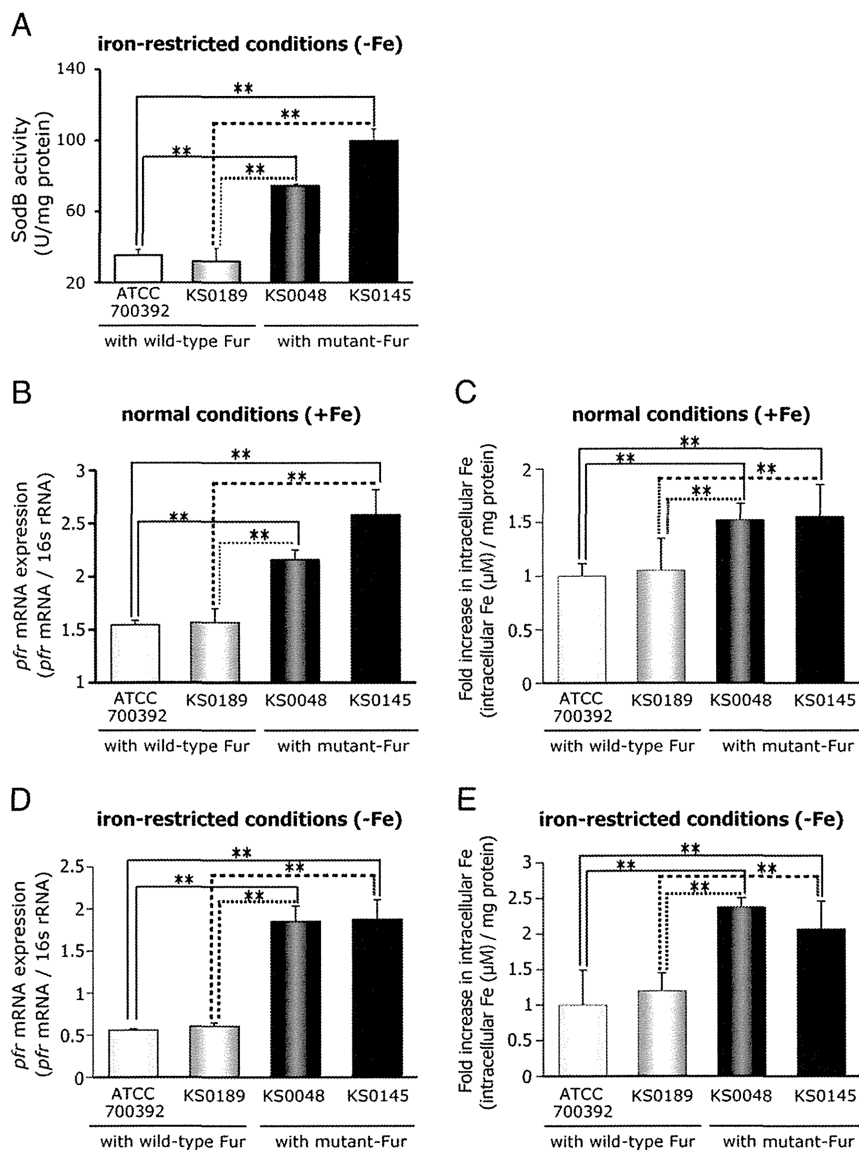

Fig. 4. SodB activity of the Mtz-resistant strains with mutant Fur under iron-restricted conditions was supported by the increase in the iron-storage ability. (A) Under iron-restricted conditions, the SodB activity in ATCC700392, KS0189, KS0048, and KS0145 was measured by the method described under Materials and methods. Results are means ± SD of three independent assays. $^{**}P<0.01$, statistically significant difference compared with the SodB activity in ATCC700392 and KS0189. (B) Under normal cultivation conditions, expression of *pfr* mRNA in ATCC700392, KS0189, KS0048, and KS0145 was measured by quantitative RT-PCR. Results are means ± SD of three independent assays. $^{**}P<0.01$, statistically significant difference compared with *pfr* mRNA expression in ATCC700392 and KS0189. (C) Under normal cultivation conditions, intracellular iron (Fe^{2+} and Fe^{3+}) concentration was measured by the method described under Materials and methods. The data for the intracellular iron concentration were corrected by total cellular protein. Results are means ± SD of three independent assays. $^{**}P<0.01$, statistically significant difference compared with the intracellular iron concentration in ATCC700392 and KS0189. (D) Under iron-restricted conditions, expression of *pfr* mRNA in ATCC700392, KS0189, KS0048, and KS0145 was measured by quantitative RT-PCR. Results are means ± SD of three independent assays. $^{**}P<0.01$, statistically significant difference compared with *pfr* mRNA expression in ATCC700392 and KS0189. (E) Under iron-restricted conditions, intracellular iron (Fe^{2+} and Fe^{3+}) concentration was measured by the method described under Materials and methods. Results are means ± SD of three independent assays. $^{**}P<0.01$, statistically significant difference compared with the intracellular iron concentration in ATCC700392 and KS0189.

host immune response, sustained expression of SodB activity is required for the dismutation of such superoxides [28,29]. Hence, it is thought that a low affinity of apo-Fur and iron-bound Fur for the *sodB* and *fecA1* promoters, respectively, is required for efficient and persistent activation of SodB.

In *H. pylori,* Fur regulates the gene expression of both iron-bound and apo-Fur [11]. *sodB* mRNA expression is repressed by apo-Fur; on the other hand, *fecA1* mRNA expression is repressed by iron-bound Fur [5,14,15]. In fact, despite the difference in the binding patterns of Fur to the *sodB* promoter and *fecA1* promoter, the mRNA expression of both *sodB* and *fecA1* was co-derepressed by mutant Fur (Fig. 3) [6], suggesting that the amino acid mutations (C78Y and P114S) in Fur alter its binding to promoter DNA, but not to Fe^{2+}. *H. pylori* Fur monomer contains two domains, the N-terminal DNA-binding domain and the C-terminal dimerization domain with metal-binding sites, and after dimerization, the Fur protein binds to the target promoter DNA [6,30]. We showed, using homology modeling, that the mutation C78Y was localized in the DNA-binding domain, whereas P114S was localized in the oligomerization domain [6]. Changes in the target-DNA binding of Fur by amino acid mutation have been categorized into the following two groups: (i) effects on the binding ability of Fe^{2+} and (ii) effects on dimerization [30]. Dian et al. identified the S2 functional domain, which was essential for dimerization in *H. pylori* Fur [31]. According to that report, replacement of Cys 78 with tyrosine is predicted to interfere with the formation of the S2 site [31].

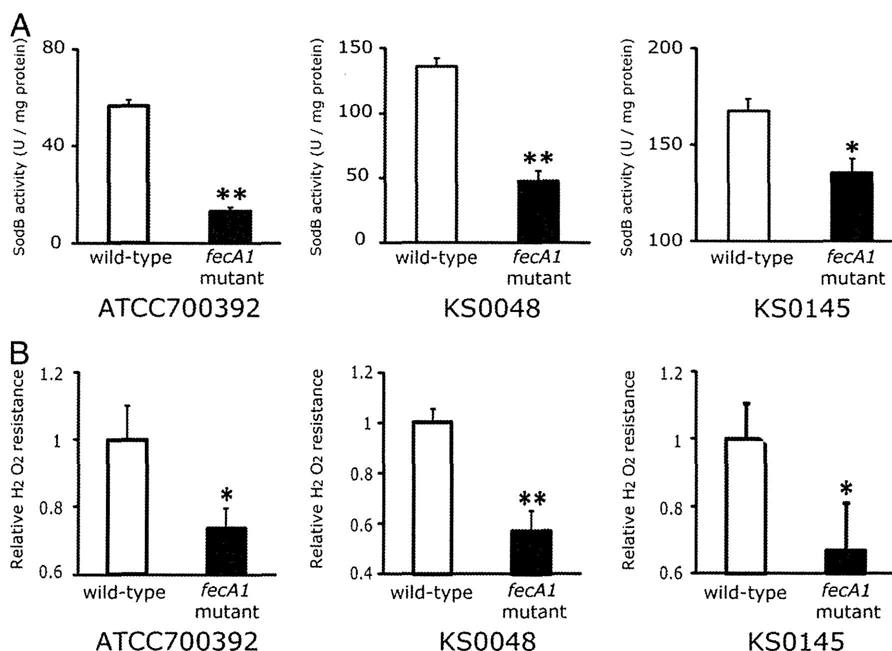

Fig. 5. Deletion of *fecA1* reduces SodB activity and H_2O_2 resistance. (A) The *fecA1*-deletion mutant was constructed as described under Materials and methods. The SodB activity in the wild-type and *fecA1*-deletion mutants for each strain was measured as described under Materials and methods. The results are expressed as means ± SD of three independent assays. *$P<0.05$, **$P<0.01$, statistically significant difference compared with the wild-type for each strain. (B) H_2O_2 resistance was measured by the inhibition zone assay described under Materials and methods. The results are means ± SD of three independent assays. *$P<0.05$, **$P<0.01$, statistically significant difference compared with the wild-type for each strain.

Our in vivo studies demonstrated that the colonization ability of *H. pylori* in Mongolian gerbils was greatly impaired by *fecA1* deletion, regardless of the presence of Fur mutation. From this result, it is thought that the SodB activation in *H. pylori* is supported by Fe^{2+} supply through FecA1 to combat the oxidative stress evoked by the host immune response. Because recently there has been a gradual increase in reports of multiple-drug-resistant *H. pylori*, the development of a novel bactericidal therapy, different from antibiotics, is required. FecA1 is one possible target for the development of a novel bactericidal therapy as well as possibly a preventive therapy against *H. pylori* infection.

In conclusion, Fe^{3+}-dicitrate transport by FecA1 is an essential process in the activation of SodB, which determines the gastric mucosal colonization ability of *H. pylori* in Mongolian gerbils and also the development of Mtz resistance.

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2011.12.011.

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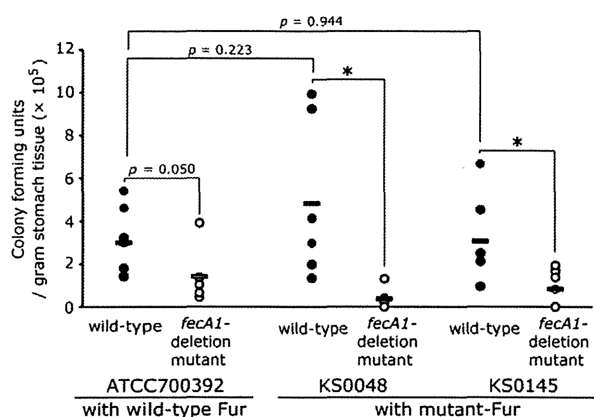

Fig. 6. Deletion of *fecA1* reduces the ability of *H. pylori* to colonize the stomach of Mongolian gerbils. Total colonization of the stomach was determined by sacrificing the animals at 12 weeks, and the results were expressed as the number of CFU/g of stomach tissue. Mongolian gerbils were infected with either a wild-type *H. pylori* strain (filled circle) or a *fecA1*-deletion mutant *H. pylori* strain (open circle). Each circle indicates the results for a single animal. The geometric means are indicated by bars. *$P<0.05$, statistically significant difference compared with the wild type.

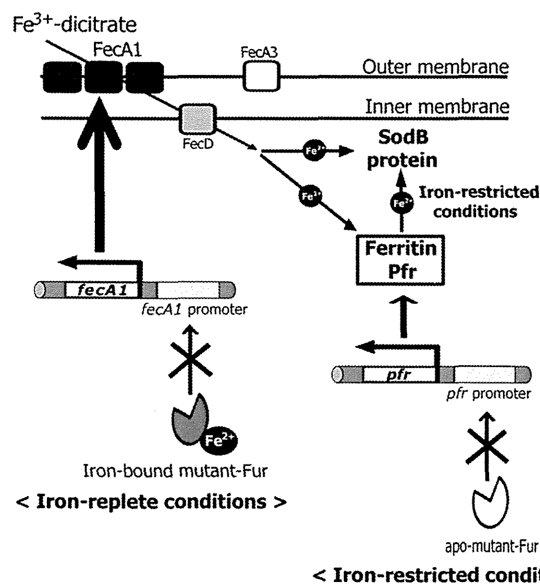

Fig. 7. Schematic representation of the ferrous ion (Fe^{2+})-supply system to the SodB protein in the Mtz-resistant strains with mutant Fur. Under iron-replete conditions, *fecA1* mRNA expression is derepressed by iron-bound mutant Fur, and then Fe^{2+} is supplied to the SodB protein. Under iron-restricted conditions, the capability of Fe^{2+} storage in the KS0048 and KS0145 strains is enhanced by derepression of *pfr* mRNA expression by apo-mutant Fur, and then Fe^{2+} is supplied to SodB from Pfr.

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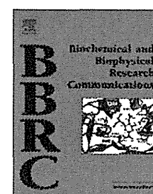

Effects of β-(1,3–1,6)-D-glucan on irritable bowel syndrome-related colonic hypersensitivity

Teita Asano [a], Ken-ichiro Tanaka [b], Shintaro Suemasu [a], Tomoaki Ishihara [a], Kayoko Tahara [a], Toshio Suzuki [c], Hidekazu Suzuki [d], Shin Fukudo [e], Tohru Mizushima [a,b,*]

[a] Department of Analytical Chemistry, Faculty of Pharmacy, Keio University, Tokyo 105-8512, Japan
[b] Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan
[c] Research and Development, Daiso Co., Ltd., Amagasaki 660-0842, Japan
[d] Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan
[e] Department of Behavioral Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan

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ABSTRACT

Irritable bowel syndrome (IBS) is a gastrointestinal disorder characterized by chronic abdominal pain associated with altered bowel habits. Since the prevalence of IBS is very high and thus, involves elevated health-care costs, treatment of this condition by methods other than prescribed medicines could be beneficial. β-(1,3)-D-glucan with β-(1,6) branches (β-glucan) has been used as a nutritional supplement for many years. In this study, we examined the effect of β-glucan on fecal pellet output and visceral pain response in animal models of IBS. Oral administration of β-glucan suppressed the restraint stress- or drug-induced fecal pellet output. β-Glucan also suppressed the visceral pain response to colorectal distension. These results suggest that β-glucan could be beneficial for the treatment and prevention of IBS.
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1. Introduction

IBS is a functional gastrointestinal disorder characterized by chronic and recurrent abdominal pain and discomfort (colonic hypersensitivity) that are associated with altered bowel habits but not with any detectable structural or biochemical abnormality [1,2]. IBS is categorized into subtypes according to the predominant bowel habit: diarrhea-predominant IBS, constipation -predominant IBS, and mixed pattern IBS [1]. In spite of the significant impact that IBS has on patient quality-of-life, currently available clinical treatments for IBS have proved unsatisfactory, mainly due to the difficulty in suppressing the visceral pain associated with IBS.

IBS is one of the most common gastrointestinal disorders, estimated to affect 7–15% of the general population in the USA and 6–12% in Asian countries [2,3]. Considering the health-care costs associated with treating the condition, the identification of effective therapies (such as the taking of supplements) that do not involve prescription drugs is beneficial [4,5].

Although the pathogenesis of IBS is not completely understood, studies have suggested that genetic factors, previous inflammation, mental stressors and microbiota play important roles [6]. A number of animal models for IBS has been established and used to evaluate clinical protocols designed to treat the condition. Mental stressor- or drug-induced alterations in defecation have been used as a model for defecation disorders related to IBS in animals [7–9]. Since hypersensitivity to colorectal distension (CRD) was observed in IBS patients [10], monitoring the electrical activity of the abdominal muscles (visceromotor response) in response to CRD is a standard procedure to detect IBS-related abdominal pain (visceral pain) in animals [11,12]. Furthermore, based on the increased colonic level of butyrate in IBS patients [13,14], butyrate enema-induced hypersensitivity to CRD is also considered as a useful animal model for IBS [15,16].

β-Glucans are naturally-occurring polysaccharides found in the cell walls of yeast, fungi, cereal plants and certain bacteria [17,18]. As suggested by the fact that various foods contain β-glucans, they are known to have few toxic and adverse effects [18]. β-Glucans from mushrooms have been used in Japan as anti-tumor drugs due to their immunostimulating activities [17]. In addition, β-(1,3)-D-glucans with β-(1,6) branches have been reported to have various clinically beneficial effects, such as enhancing the

Abbreviations: AUC, area under the curve; β-glucan, β-(1,3)-D-glucan with β-(1,6) branches; CRD, colorectal distention; 5-HT, 5-hydroxytryptamine hydrochloride; IBS, irritable bowel syndrome; LMW, low-molecular-weight; PBS, phosphate-buffered saline; S.E.M, standard error of the mean.

* Corresponding author at: Department of Analytical Chemistry, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan. Fax: +81 3 5400 2628.

E-mail address: mizushima-th@pha.keio.ac.jp (T. Mizushima).

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