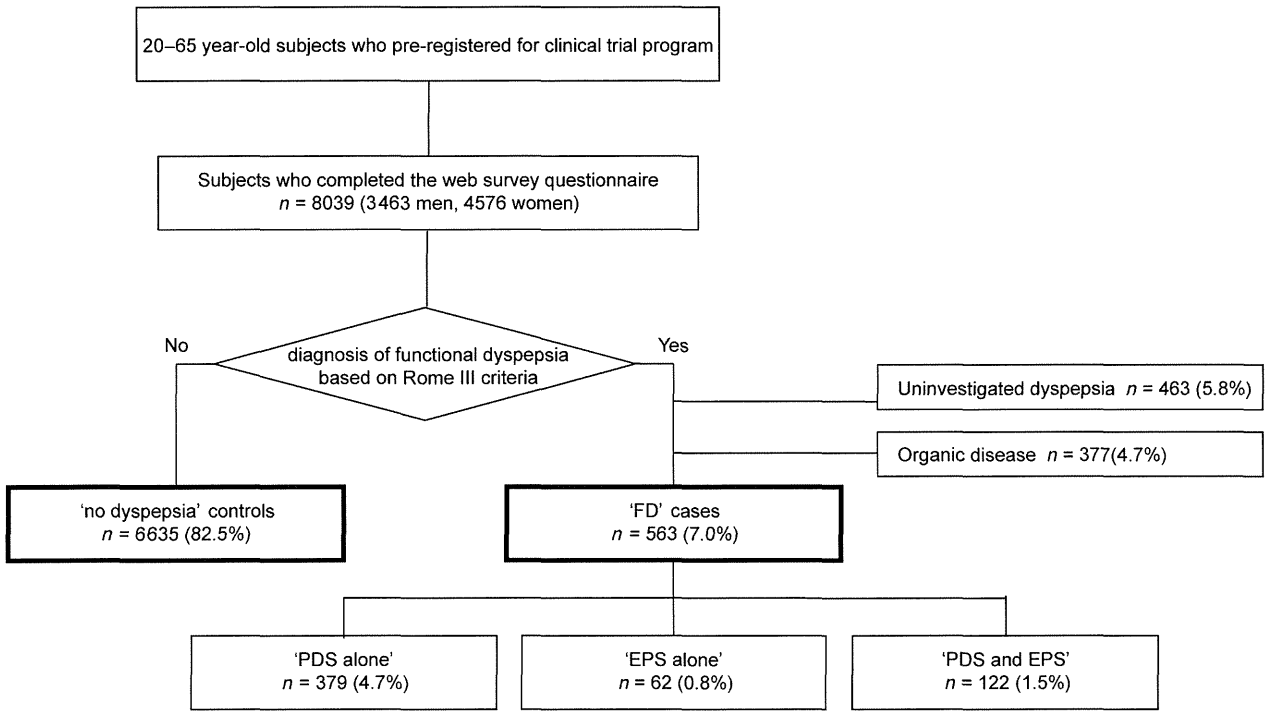

Figure 1 The study population.

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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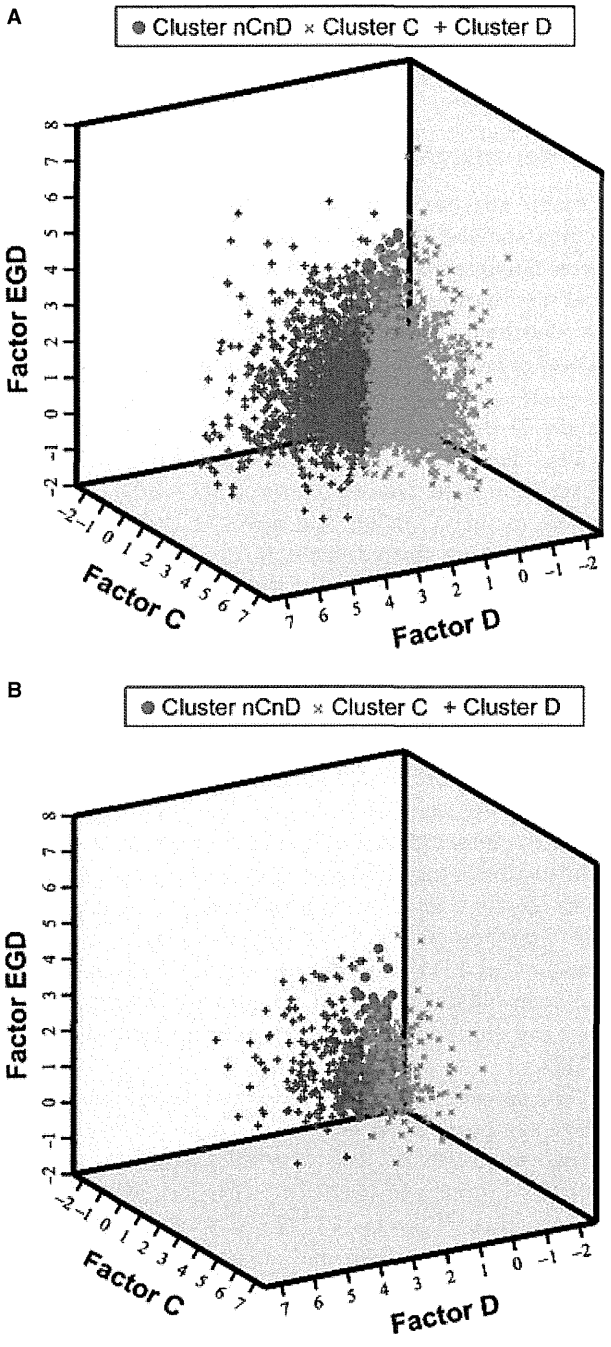

Figure 2 Distribution of cluster nCnD, cluster C, and cluster D. The 3D spatial distribution of overall 8038 participants (A) and 563 functional dyspepsia participants (B) with three symptom factor scores derived from factor analysis showed that the three symptom clusters were well separated.

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.

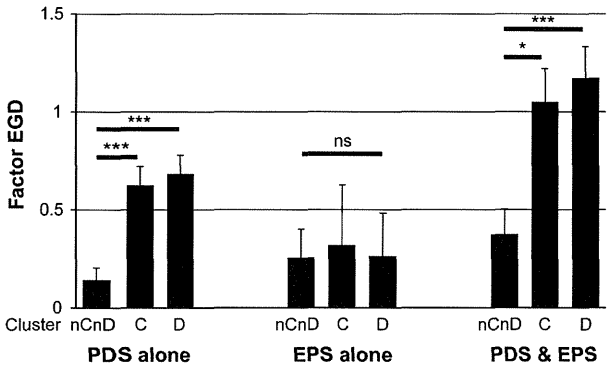

Figure 3 Associations between upper gastrointestinal symptoms and the three symptom clusters in each subgroup of functional dyspepsia. ***P < 0.001, *P < 0.05 significant difference using one-way ANOVA and Tukey's *post hoc* analysis. EPS, epigastric pain syndrome; ns, not significant; PDS, postprandial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

REFERENCES

1 Tack J, Talley NJ, Camilleri M *et al.* Functional gastroduodenal disorders. *Gastroenterology* 2006; **130**: 1466–79.
2 Okumura T, Tanno S, Ohhira M. Prevalence of functional dyspepsia in an outpatient clinic with primary care physicians in Japan. *J Gastroenterol* 2010; **45**: 187–94.
3 Geeraerts B, Tack J. Functional dyspepsia: past, present, and future. *J Gastroenterol* 2008; **43**: 251–5.
4 Tack J, Talley NJ. Gastroduodenal disorders. *Am J Gastroenterol* 2010; **105**: 757–63.
5 Talley NJ, Dennis EH, Schettler-Duncan VA, Lacy BE, Olden KW, Crowell MD. Overlapping upper and lower gastrointestinal symptoms in irritable bowel syndrome patients with constipation or diarrhea. *Am J Gastroenterol* 2003; **98**: 2454–9.
6 Ford AC, Marwaha A, Lim A, Moayyedi P. Systematic review and meta-analysis of the prevalence of irritable bowel syndrome in individuals with dyspepsia. *Clin Gastroenterol Hepatol* 2010; **8**: 401–9.
7 Savarino E, Pohl D, Zentilin P *et al.* Functional heartburn has more in common with functional dyspepsia than with non-erosive reflux disease. *Gut* 2009; **58**: 1185–91.
8 Piessevaux H, De Winter B, Louis E *et al.* Dyspeptic symptoms in the general population: a factor and cluster analysis of symptom groupings. *Neurogastroenterol Motil* 2009; **21**: 378–88.
9 Svedlund J, Sjodin I, Dotevall G. GSRS – a clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. *Dig Dis Sci* 1988; **33**: 129–34.
10 Hongo M, Fukuhara S, Green J. Shokaki-ryoiki ni okeru QOL -nihongo ban GSRS niyoru QOL hyouka. *Shindan to Chiryo* 1999; **87**: 731–6.
11 Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480–91.
12 Van Oudenhove L, Holvoet L, Vandenberghe J, Vos R, Tack J. Do we have an alternative for the Rome III gastroduodenal symptom-based subgroups in functional gastroduodenal disorders? A cluster analysis approach. *Neurogastroenterol Motil* 2011; **23**: 730–8.
13 Corsetti M, Caenepeel P, Fischler B, Janssens J, Tack J. Impact of coexisting irritable bowel syndrome on symptoms and pathophysiological mechanisms in functional dyspepsia. *Am J Gastroenterol* 2004; **99**: 1152–9.
14 Kaji M, Fujiwara Y, Shiba M *et al.* Prevalence of overlaps between GERD, FD and IBS and impact on health-related quality of life. *J Gastroenterol Hepatol* 2010; **25**: 1151–6.
15 Lee HJ, Lee SY, Kim JH *et al.* Depressive mood and quality of life in functional gastrointestinal disorders: differences between functional dyspepsia, irritable bowel syndrome and overlap syndrome. *Gen Hosp Psychiatry* 2010; **32**: 499–502.
16 Manabe N, Wong BS, Camilleri M, Burton D, McKinzie S, Zinsmeister AR. Lower functional gastrointestinal disorders: evidence of abnormal colonic transit in a 287 patient cohort. *Neurogastroenterol Motil* 2010; **22**: 293–e82.
17 Caballero-Plasencia AM, Valenzuela-Barranco M, Herrerias-Gutierrez JM, Esteban-Carretero JM. Altered gastric emptying in patients with irritable bowel syndrome. *Eur J Nucl Med* 1999; **26**: 404–9.
18 Stanghellini V, Tosetti C, Barbara G *et al.* Dyspeptic symptoms and gastric emptying in the irritable bowel syndrome. *Am J Gastroenterol* 2002; **97**: 2738–43.
19 Shindo T, Futagami S, Hiratsuka T *et al.* Comparison of gastric emptying and plasma ghrelin levels in patients with functional dyspepsia and non-erosive reflux disease. *Digestion* 2009; **79**: 65–72.
20 Kindt S, Caenepeel P, Bisschops R, Vos R, Tack J. Association of postprandial distress syndrome and epigastric pain syndrome with putative pathophysiological abnormalities in functional dyspepsia. *Gastroenterology* 2007; **132**: A73.
21 Heiervang E, Goodman R. Advantages and limitations of web-based surveys: evidence from a child mental health survey. *Soc Psychiatry Psychiatr Epidemiol* 2011; **46**: 69–76.
22 Drossman DA, Li Z, Andruzzi E *et al.* U.S. householder survey of functional gastrointestinal disorders. Prevalence,

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sociodemography, and health impact. *Dig Dis Sci* 1993; **38**: 1569–80.
23 Tougas G, Chen Y, Hwang P, Liu MM, Eggleston A. Prevalence and impact of upper gastrointestinal symptoms in the Canadian population: findings from the DIGEST study. Domestic/International Gastroenterology Surveillance Study. *Am J Gastroenterol* 1999; **94**: 2845–54.
24 Moayyedi P, Forman D, Braunholtz D *et al.* The proportion of upper gastrointestinal symptoms in the community associated with Helicobacter pylori, lifestyle factors, and nonsteroidal anti-inflammatory drugs. Leeds HELP Study Group. *Am J Gastroenterol* 2000; **95**: 1448–55.
25 Minocha A, Wigington WC, Johnson WD. Detailed characterization of epidemiology of uninvestigated dyspepsia and its impact on quality of life among African Americans as compared to Caucasians. *Am J Gastroenterol* 2006; **101**: 336–42.
26 Suzuki H, Hibi T. Overlap syndrome of functional dyspepsia and irritable bowel syndrome - are both diseases mutually exclusive? *J Neurogastroenterol Motil* 2011; **17**: 360–5.

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

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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.

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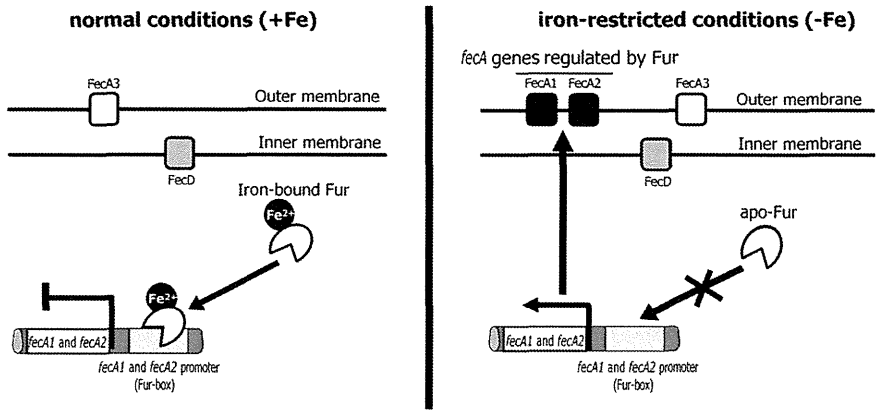

Fig. 1. Regulation of the *fecA* genes for the Fe^{3+}-dicitrate transporter by Fur in *H. pylori*. The transcription of *fecA3* is not regulated by Fur. Iron-bound Fur binds to the promoter of *fecA1* and *fecA2*, leading to transcriptional repression under normal cultivation conditions (iron-replete 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′-GGTGGTATATC-CAGTGATTTTTTTCTCCATATTCATCATGCCCCCTGTTCTTAAG; *cat* region, forward 5′-ACTTAAGAACAGGGGGCATGATGAATATGGAGAAAAAAAT-CACTGGATATACCACC and reverse 5′-CCATACCGCTGTATAGTTGTTAAA-CAGTTACGCCCCGCCCTGCCACTCATCGC; 3′*fecA1* region, forward 5′-GCGATGAGTGGCAGGGCGGGGCGTAACTGTTTAACAACTATACAGCGGTAT GG 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′-GCTTCTTCAATGCTCT-GATTGG; *fecA2* mRNA, forward 5′-AGCTCTCGCACGGTGATTTCCAAC and reverse 5′-CTATGCCCGTTACCGCCCC; *pfr* mRNA, forward 5′-TTGATCATGCGGCTGAAGAATACG and reverse 5′-TGATGTGTTGCT-CATGTTCATAGGC. 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*)

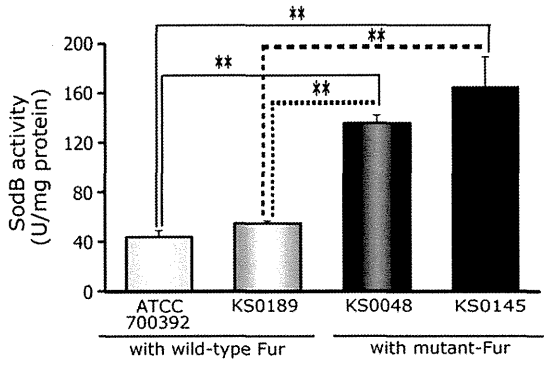

Fig. 2. Enhancement of SodB activity in the Mtz-resistant strains with mutant Fur under normal cultivation conditions. Under normal cultivation conditions, the SodB activity in ATCC700392 and KS0189 (Mtz-susceptible strains with wild-type Fur) and KS0048 and KS0145 (Mtz-resistant strains with mutant 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-

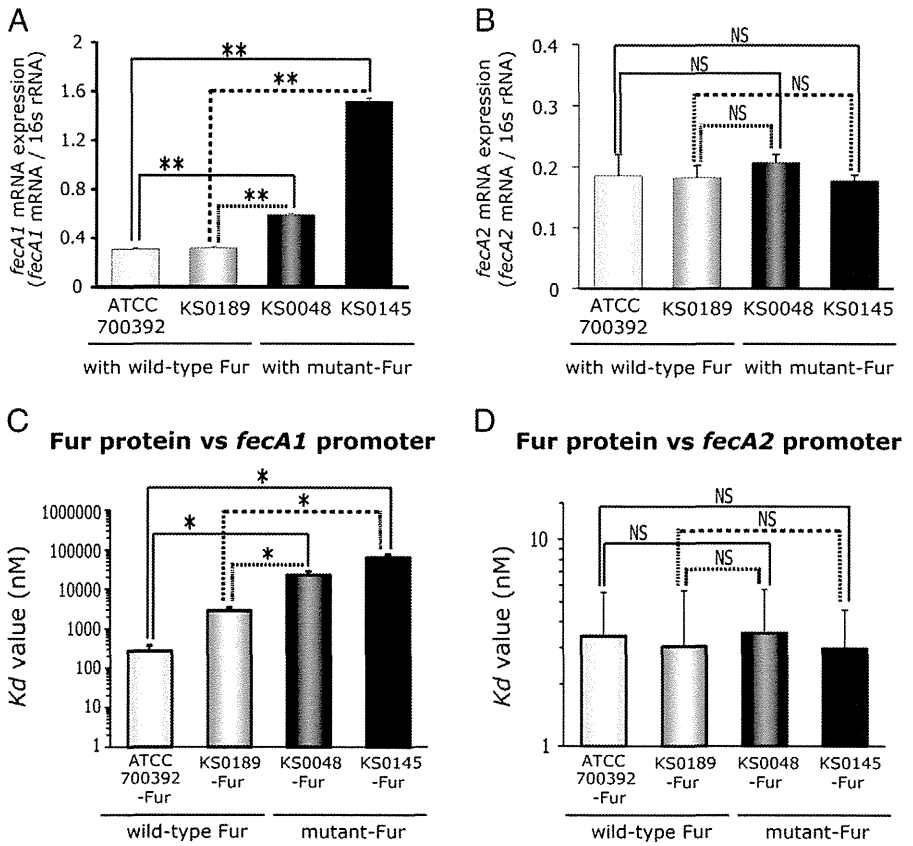

Fig. 3. Derepression of *fecA1* mRNA transcription by decreased affinity of mutant Fur for the *fecA1* promoter. (A) Under normal cultivation conditions, transcription of *fecA1* mRNA in ATCC700392 and KS0189, KS0048, and KS0145 was measured by quantitative RT-PCR. Results are means ± SD of three independent 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

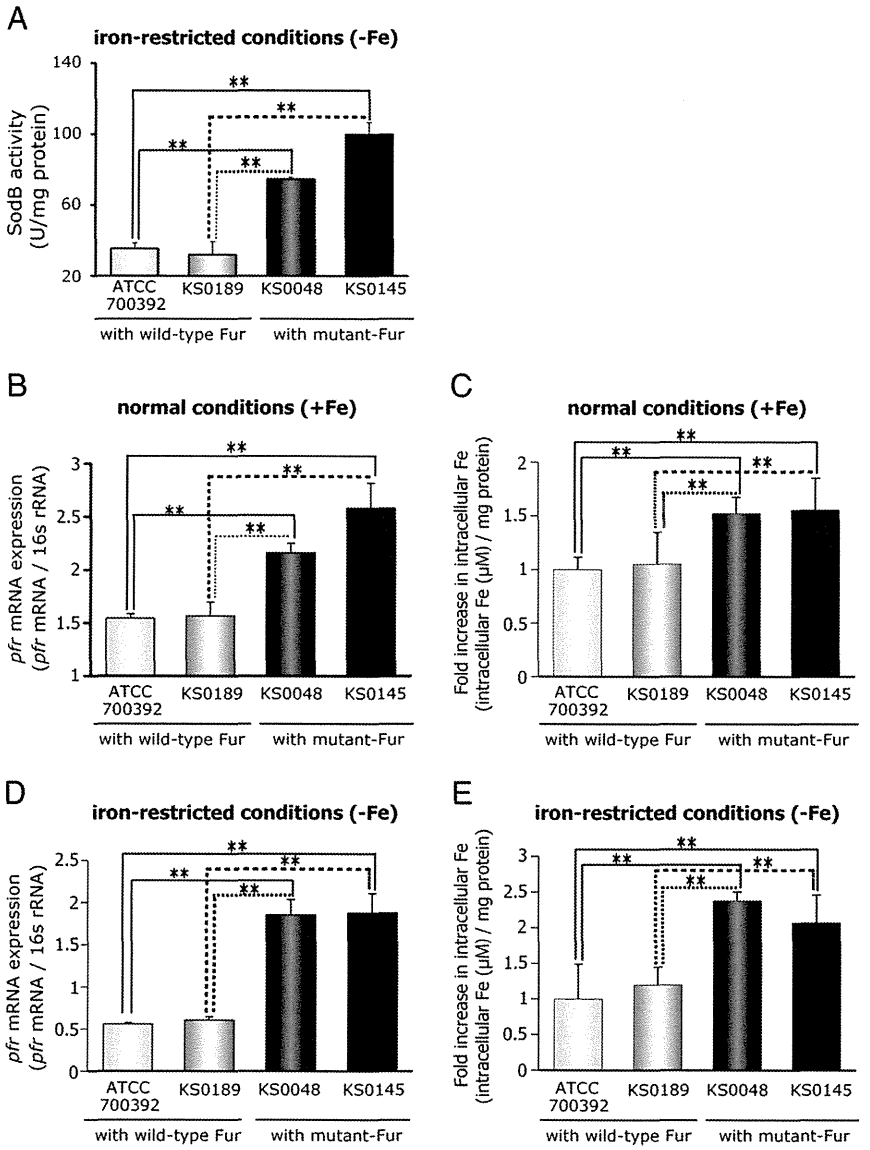

Fig. 4. SodB activity of the Mtz-resistant strains with mutant Fur under iron-restricted conditions was supported by the increase in the iron-storage ability. (A) Under iron-restricted conditions, the SodB activity in ATCC700392, KS0189, KS0048, and KS0145 was measured by the method described under Materials 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].

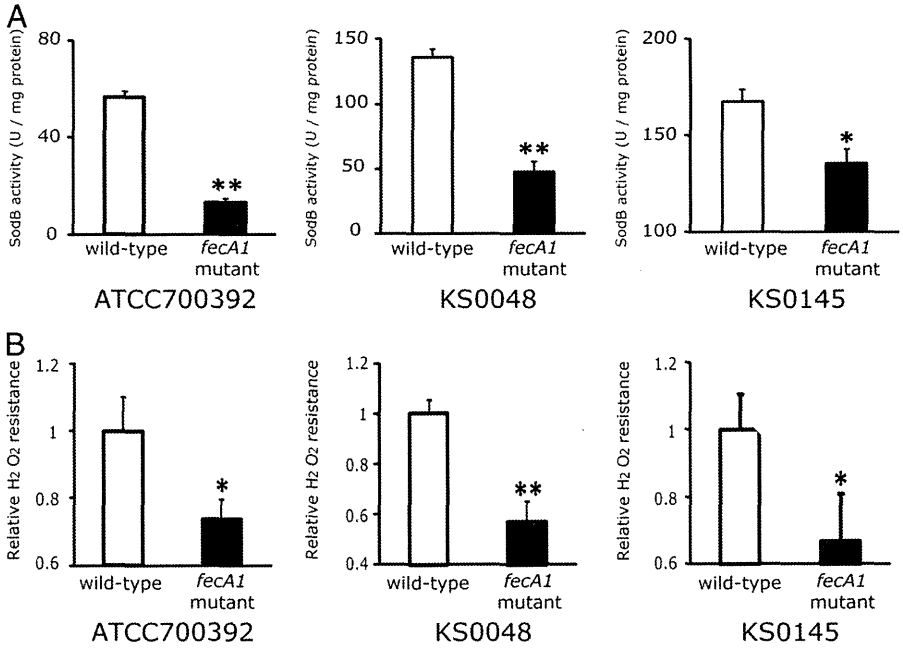

Fig. 5. Deletion of *fecA1* reduces SodB activity and H_2O_2 resistance. (A) The *fecA1*-deletion mutant was constructed as described under Materials and methods. The SodB activity in the wild-type and *fecA1*-deletion mutants for each strain was measured as described under Materials and methods. The 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.

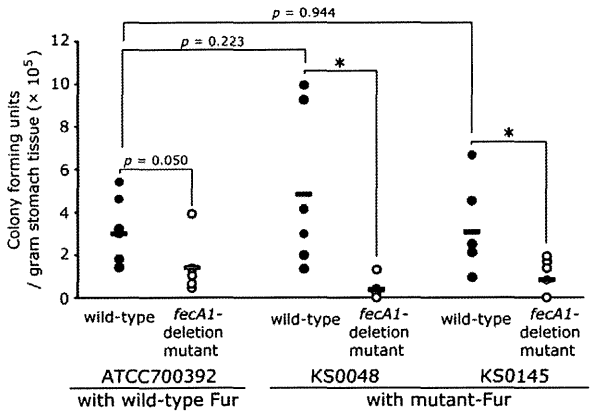

Fig. 6. Deletion of *fecA1* reduces the ability of *H. pylori* to colonize the stomach of Mongolian gerbils. Total colonization of the stomach was determined by sacrificing the animals at 12 weeks, and the results were expressed as the number of CFU/g of stomach tissue. Mongolian gerbils were infected 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.

Acknowledgments

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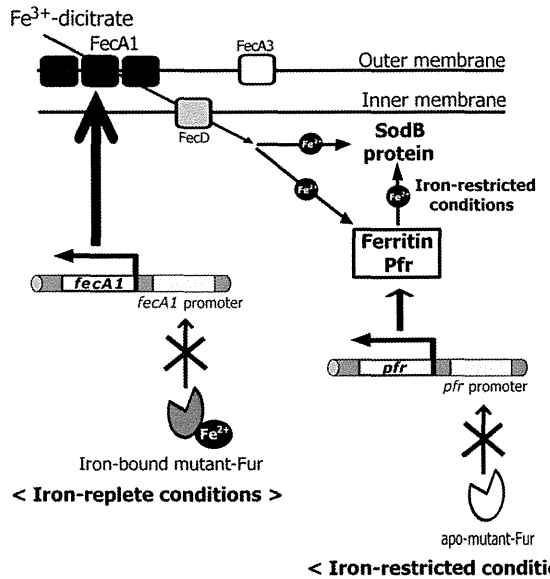

Fig. 7. Schematic representation of the ferrous ion (Fe^{2+})-supply system to the SodB protein in the Mtz-resistant strains with mutant Fur. Under iron-replete conditions, *fecA1* mRNA expression is derepressed by iron-bound mutant Fur, and then Fe^{2+} is supplied to the SodB protein. Under iron-restricted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

[1] Suzuki, H.; Hibi, T.; Marshall, B. J. *Helicobacter pylori*: present status and future prospects in Japan. *J. Gastroenterol.* **42:**1–15; 2007.
[2] Wang, G.; Alamuri, P.; Maier, R. J. The diverse antioxidant systems of *Helicobacter pylori*. *Mol. Microbiol.* **61:**847–860; 2006.
[3] Bereswill, S.; Neuner, O.; Strobel, S.; Kist, M. Identification and molecular analysis of superoxide dismutase isoforms in *Helicobacter pylori*. *FEMS Microbiol. Lett.* **183:** 241–245; 2000.
[4] Spiegelhalder, C.; Gerstenecker, B.; Kersten, A.; Schiltz, E.; Kist, M. Purification of *Helicobacter pylori* superoxide dismutase and cloning and sequencing of the gene. *Infect. Immun.* **61:**5315–5325; 1993.
[5] Ernst, F. D.; Homuth, G.; Stoof, J.; Mader, U.; Waidner, B.; Kuipers, E. J.; Kist, M.; Kusters, J. G.; Bereswill, S.; van Vliet, A. H. Iron-responsive regulation of the *Helicobacter pylori* iron-cofactored superoxide dismutase SodB is mediated by Fur. *J. Bacteriol.* **187:**3687–3692; 2005.
[6] Tsugawa, H.; Suzuki, H.; Satoh, K.; Hirata, K.; Matsuzaki, J.; Saito, Y.; Suematsu, M.; Hibi, T. Two amino acids mutation of ferric uptake regulator determines *Helicobacter pylori* resistance to metronidazole. *Antioxid. Redox Signal.* **14:**15–23; 2011.
[7] Seyler Jr., R. W.; Olson, J. W.; Maier, R. J. Superoxide dismutase-deficient mutants of *Helicobacter pylori* are hypersensitive to oxidative stress and defective in host colonization. *Infect. Immun.* **69:**4034–4040; 2001.
[8] Esposito, L.; Seydel, A.; Aiello, R.; Sorrentino, G.; Cendron, L.; Zanotti, G.; Zagari, A. The crystal structure of the superoxide dismutase from *Helicobacter pylori* reveals a structured C-terminal extension. *Biochim. Biophys. Acta* **1784:**1601–1606; 2008.
[9] Touati, D. Iron and oxidative stress in bacteria. *Arch. Biochem. Biophys.* **373:**1–6; 2000.
[10] van Vliet, A. H.; Stoof, J.; Vlasblom, R.; Wainwright, S. A.; Hughes, N. J.; Kelly, D. J.; Bereswill, S.; Bijlsma, J. J.; Hoogenboezem, T.; Vandenbroucke-Grauls, C. M.; Kist, M.; Kuipers, E. J.; Kusters, J. G. The role of the ferric uptake regulator (Fur) in regulation of *Helicobacter pylori* iron uptake. *Helicobacter* **7:**237–244; 2002.
[11] Ernst, F. D.; Bereswill, S.; Waidner, B.; Stoof, J.; Mader, U.; Kusters, J. G.; Kuipers, E. J.; Kist, M.; van Vliet, A. H.; Homuth, G. Transcriptional profiling of *Helicobacter pylori* Fur- and iron-regulated gene expression. *Microbiology* **151:**533–546; 2005.
[12] Ernst, F. D.; Stoof, J.; Horrevoets, W. M.; Kuipers, E. J.; Kusters, J. G.; van Vliet, A. H. NikR mediates nickel-responsive transcriptional repression of the *Helicobacter pylori* outer membrane proteins FecA3 (HP1400) and FrpB4 (HP1512). *Infect. Immun.* **74:**6821–6828; 2006.
[13] Danielli, A.; Romagnoli, S.; Roncarati, D.; Costantino, L.; Delany, I.; Scarlato, V. Growth phase and metal-dependent transcriptional regulation of the fecA genes in *Helicobacter pylori*. *J. Bacteriol.* **191:**3717–3725; 2009.
[14] Delany, I.; Pacheco, A. B.; Spohn, G.; Rappuoli, R.; Scarlato, V. Iron-dependent transcription of the frpB gene of *Helicobacter pylori* is controlled by the Fur repressor protein. *J. Bacteriol.* **183:**4932–4937; 2001.
[15] Merrell, D. S.; Thompson, L. J.; Kim, C. C.; Mitchell, H.; Tompkins, L. S.; Lee, A.; Falkow, S. Growth phase-dependent response of *Helicobacter pylori* to iron starvation. *Infect. Immun.* **71:**6510–6525; 2003.
[16] Allen, L. A. Phagocytosis and persistence of *Helicobacter pylori*. *Cell. Microbiol.* **9:** 817–828; 2007.
[17] Olczak, A. A.; Olson, J. W.; Maier, R. J. Oxidative-stress resistance mutants of *Helicobacter pylori*. *J. Bacteriol.* **184:**3186–3193; 2002.
[18] Debets-Ossenkopp, Y. J.; Pot, R. G.; van Westerloo, D. J.; Goodwin, A.; Vandenbroucke-Grauls, C. M.; Berg, D. E.; Hoffman, P. S.; Kusters, J. G. Insertion of mini-IS605 and deletion of adjacent sequences in the nitroreductase (rdxA) gene cause metronidazole resistance in *Helicobacter pylori* NCTC11637. *Antimicrob. Agents Chemother.* **43:** 2657–2662; 1999.
[19] Heuermann, D.; Haas, R. A stable shuttle vector system for efficient genetic complementation of *Helicobacter pylori* strains by transformation and conjugation. *Mol. Gen. Genet.* **257:**519–528; 1998.
[20] Tsugawa, H.; Ogawa, A.; Takehara, S.; Kimura, M.; Okawa, Y. Primary structure and function of a cytotoxic outer-membrane protein (ComP) of *Plesiomonas shigelloides*. *FEMS Microbiol. Lett.* **281:**10–16; 2008.
[21] Osaki, T.; Hanawa, T.; Manzoku, T.; Fukuda, M.; Kawakami, H.; Suzuki, H.; Yamaguchi, H.; Yan, X.; Taguchi, H.; Kurata, S.; Kamiya, S. Mutation of luxS affects motility and infectivity of *Helicobacter pylori* in gastric mucosa of a Mongolian gerbil model. *J. Med. Microbiol.* **55:**1477–1485; 2006.
[22] Nagayama, A.; Yamaguchi, K.; Watanabe, K.; Tanaka, M.; Kobayashi, I.; Nagasawa, Z. Final report from the Committee on Antimicrobial Susceptibility Testing, Japanese Society of Chemotherapy, on the agar dilution method (2007). *J. Infect. Chemother.* **14:**383–392; 2008.
[23] Bereswill, S.; Waidner, U.; Odenbreit, S.; Lichte, F.; Fassbinder, F.; Bode, G.; Kist, M. Structural, functional and mutational analysis of the pfr gene encoding a ferritin from *Helicobacter pylori*. *Microbiology* **144:**2505–2516; 1998.
[24] Doig, P.; Austin, J. W.; Trust, T. J. The *Helicobacter pylori* 19.6-kilodalton protein is an iron-containing protein resembling ferritin. *J. Bacteriol.* **175:**557–560; 1993.
[25] Frazier, B. A.; Pfeifer, J. D.; Russell, D. G.; Falk, P.; Olsen, A. N.; Hammar, M.; Westblom, T. U.; Normark, S. J. Paracrystalline inclusions of a novel ferritin containing nonheme iron, produced by the human gastric pathogen *Helicobacter pylori*: evidence for a third class of ferritins. *J. Bacteriol.* **175:**966–972; 1993.
[26] Velayudhan, J.; Hughes, N. J.; McColm, A. A.; Bagshaw, J.; Clayton, C. L.; Andrews, S. C.; Kelly, D. J. Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. *Mol. Microbiol.* **37:**274–286; 2000.
[27] Worst, D. J.; Gerrits, M. M.; Vandenbroucke-Grauls, C. M.; Kusters, J. G. *Helicobacter pylori* ribBA-mediated riboflavin production is involved in iron acquisition. *J. Bacteriol.* **180:**1473–1479; 1998.
[28] Demple, B. Regulation of bacterial oxidative stress genes. *Annu. Rev. Genet.* **25:** 315–337; 1991.
[29] Leclere, V.; Chotteau-Lelievre, A.; Gancel, F.; Imbert, M.; Blondeau, R. Occurrence of two superoxide dismutases in *Aeromonas hydrophila*: molecular cloning and differential expression of the sodA and sodB genes. *Microbiology* **147:** 3105–3111; 2001.
[30] Carpenter, B. M.; Gancz, H.; Benoit, S. L.; Evans, S.; Olsen, C. H.; Michel, S. L.; Maier, R. J.; Merrell, D. S. Mutagenesis of conserved amino acids of *Helicobacter pylori* fur reveals residues important for function. *J. Bacteriol.* **192:**5037–5052; 2011.
[31] Dian, C.; Vitale, S.; Leonard, G. A.; Bahlawane, C.; Fauquant, C.; Leduc, D.; Muller, C.; de Reuse, H.; Michaud-Soret, I.; Terradot, L. The structure of the *Helicobacter pylori* ferric uptake regulator Fur reveals three functional metal binding sites. *Mol. Microbiol.* **79:**1260–1275; 2011.

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

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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 hydrochlor ide; IBS, irritable bowel syndrome; LMW, low-molecular-weight; PBS, phosphate-buffered saline; S.E.M, standard error of the mean.

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bio-defense activity against bacterial, viral, fungal and parasitic challenge, increasing hematopoiesis and radioprotection, stimulating the wound healing response, and decreasing serum lipid levels [17–20]. Interestingly, it was recently reported that β-glucans suppress inflammatory responses in some animal models [21–26], suggesting that β-glucan could be an interesting immunomodulator, causing opposing effects on different aspects of the immune system.

We succeeded in the purification and industrial-scale production of low-molecular-weight β-(1,3–1,6)-D-glucan from *Aureobasidium pullulans* (*A. pullulans*) GM-NH-1A1 strain (LMW β-glucan) [27,28]. The characteristic features of LMW β-glucan are its low molecular weight (about 100 kDa), low viscosity, high water-solubility and high level of β-(1–6) branching (50–80%) [27,28]. We previously reported that LMW β-glucan has various clinically beneficial effects, such as suppression of the allergic response, suppression of restraint stress-induced immunosuppression and anti-tumor and anti-metastatic actions [27–29]. Moreover, we recently reported that LMW β-glucan protects the gastric mucosa against the formation of irritant-induced lesions by increasing levels of defensive factors such as heat shock protein 70 and gastric mucin [30]. In the present study, we use different animal models for IBS to test the hypothesis that LMW β-glucan could be effective in the treatment of this condition. Our results suggest that the oral administration of LMW β-glucan suppresses not only fecal pellet output but also the visceromotor response to CRD (visceral pain response). These findings suggest that LMW β-glucan could be therapeutically effective for the treatment of IBS.

2. Materials and methods

2.1. Chemicals and animals

LMW β-glucan was prepared from the conditioned culture medium of *A. pullulans* GM-NH-1A1, as described previously [27,28]. Analysis of ^{1}H and ^{13}C NMR spectra and gel-filtration chromatography revealed that the LMW β-glucan contains approximately 70% β-(1–6) branches and an average molecular weight of 100 kDa, as described previously [27,28]. Clonidine hydrochloride and castor oil were from WAKO Pure Chemicals (Osaka, Japan). Sodium butyrate, brewer's yeast and carbamyl- β-methylcholine chloride (bethanecol) were obtained from Sigma (St. Louis, MO). Loperamide hydrochloride and 5-hydroxytryptamine hydrochloride (5-HT) were purchased from Nacalai Tesque (Kyoto, Japan). Wild-type mice (C57/BL6, 6–8 weeks of age) and Wistar rats (4–6 weeks of age) were obtained from Charles River (Yokohama, Japan). Wistar-Imamichi rats (4 weeks of age) were purchased from the Institute for Animal Reproduction (Kasumigaura, Japan). The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and were approved by the Animal Care Committees of Keio University and Kumamoto University.

2.2. Analysis of fecal pellet output in mice

Female mice were subjected to restraint stress by being placed individually into a 50 ml Falcon tube (Becton Dickinson, Franklin Lakes, NJ) for 1 h, as described previously [31]. These tubes are small enough to restrain a mouse so that it is able to breathe but unable to move freely. Control mice were left to move freely in the cage. The number of fecal pellets excreted during the 1-h restraint stress period was measured. β-Glucan was dissolved in phosphate-buffered saline (PBS) and administered orally 2 h before animals were subjected to the restraint stress. Control animals were administered PBS.

In a separate experiment, mice were administered one of different drugs that stimulate intestinal motility (bethanecol and 5-HT), cause diarrhea (castor oil) or cause constipation (loperamide and clonidine). Animals were then placed in a cage and the number or wet weight of fecal pellets excreted in the subsequent 1-, 2- or 24-h period determined. Drugs administered subcutaneously were bethanechol (3 mg/kg) and 5-HT (3 mg/kg), while those administered orally were loperamide (10 mg/kg), clonidine (3.5 mg/kg) and castor oil (300 μl/mouse).

β-Glucan was dissolved in PBS and administered orally 2 h before animals were subjected to the restraint stress or drug-treatment. Control animals were administered PBS.

2.3. Electromyography and CRD

Rats were deeply anaesthetized with pentobarbital sodium (40 mg/kg) and then electromyography electrodes (Star Medical, Tokyo, Japan) sutured into the external oblique muscle of the abdomen for electromyogram recording. Electrode leads were tunneled subcutaneously and exteriorized at the nape of the neck for future access. After surgery, rats were housed individually and allowed to recuperate for 6 days before being used for visceromotor response testing.

Repeated CRD was performed as described previously [32]. Rats were restrained in a plastic conical-shape tube (diameter, 6 cm; height, 15 cm), 15 min before electromyography. To reduce confounding effects due to restraint stress, rats were habituated to the tube 30 min per day for 3 days prior to the experiment. A polyethylene bag (length 2 cm) was inserted in the distal colon, positioned 1 cm proximal to rectum, and connected to a balloon catheter which was anchored with tape to the base of the tail. The pressure and volume of the balloon were controlled and monitored by a pressure controller-timing device (Distender Series II; G & J Electronics, Toronto, Canada), connected to the balloon. Rats were subjected to repeated CRD (80 mm Hg, 30 s, 5-min interstimulus interval, 12 times) on day 7. β-Glucan was given orally once daily for 7 days (from day 0 to day 6).

In separate experiments, CRD associated with the use of butyrate enemas was examined as described previously [15]. Rats were instilled with 1 ml sodium butyrate (110 mg/ml, pH 6.9) or saline into the colon twice daily for 3 days (day 1, 2 and 3). Rats were subjected to CRD (10, 20, 40 60 and 80 mm Hg, 20 s, 150-s interstimulus interval) on day 7. β-Glucan was given orally once daily for 7 days (from day 0 to day 6).

Visceromotor responses were monitored by electromyography, as described previously [11,33], 12 h after the last administration of β-glucan. Electromyograph data were collected and analyzed using 8 STAR software (version 6.0–19.2 for Windows; Star Medical, Tokyo, Japan). Responses evoked by contraction of the external oblique musculature were quantified by calculating the area under the curve (AUC) of the voltage alteration graph. The baseline was determined by data collected 20 s (butyrate enema) or 30 s (repeated CRD) before each distention.

2.4. Inflamed paw pressure nociception test

The pain threshold in Wistar-Imamichi rats was measured using a Randall–Sellito test with an analgometer (Ugo basile, Comerio, Italy), as described previously [34]. Brewer's yeast (20%, 1 ml) was injected into one of the hind paws. Seven hours later, an increasing pressure was applied to the underside of the hind limb and the pain threshold was defined as the pressure in grams eliciting a cry from the animal.

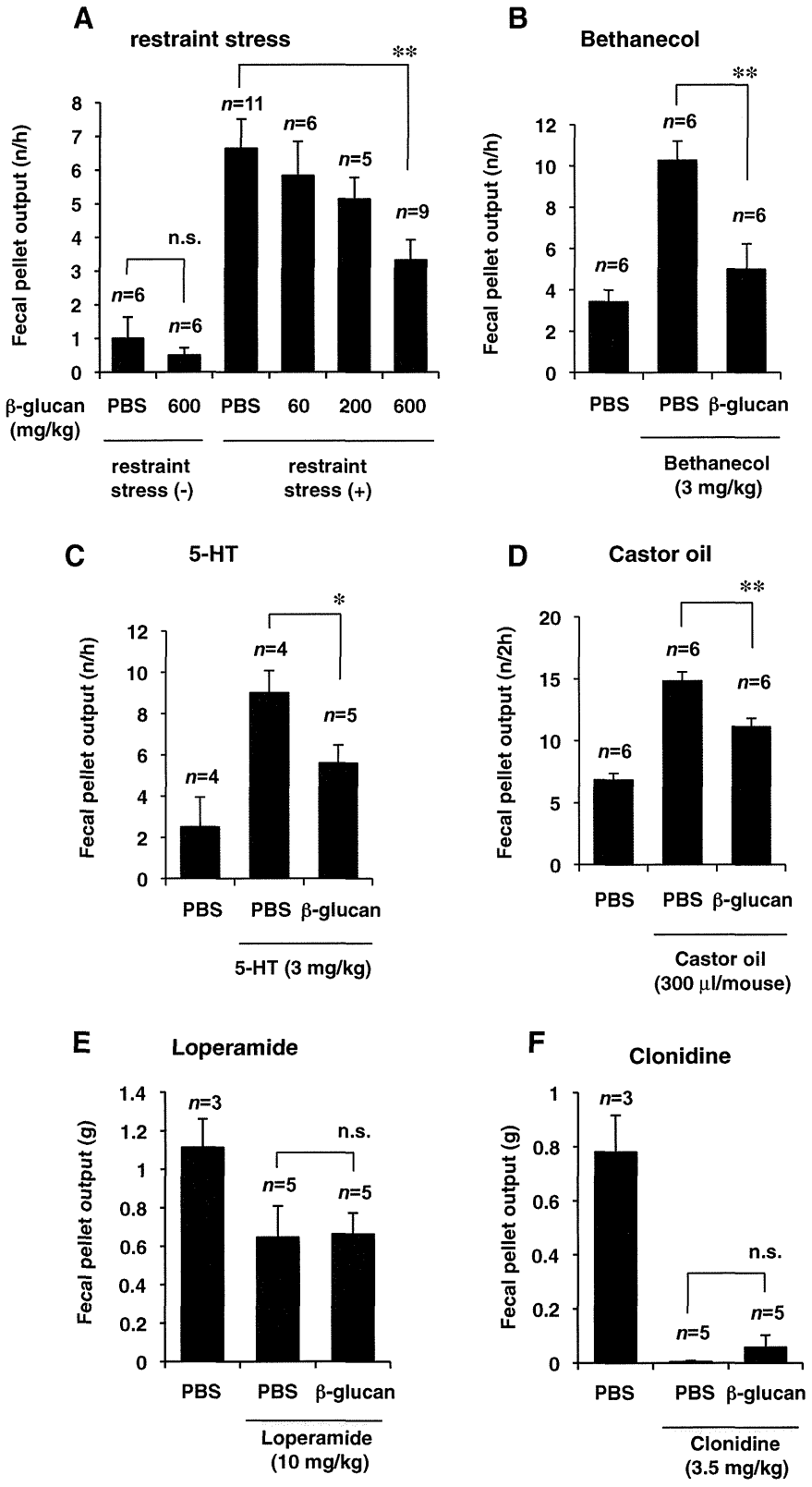

Fig. 1. Effects of LMW β-glucan on restraint stress- or drug-induced alteration of fecal pellet output in mice. Mice were orally administered indicated doses (A) or 600 mg/kg (B–F) of LMW β-glucan or vehicle (PBS). Two hours later, mice were exposed to restraint stress (A) or administered bethanecol (3 mg/kg, *s.c.*) (B), 5-HT (3 mg/kg, *s.c.*) (C), castor oil (300 μl/mouse, *p.o.*) (D), loperamide (10 mg/kg, *p.o.*) or clonidine (3.5 mg/kg, *p.o.*). The number (A–D) or wet weight (E and F) of fecal pellets excreted in the subsequent 0–1 h (A–C), 0–2 h (D) or 0–24 h (E and F) period was determined. Values are mean ± S.E.M $^{*}P < 0.05$; $^{**}P < 0.01$; n.s., not significant.

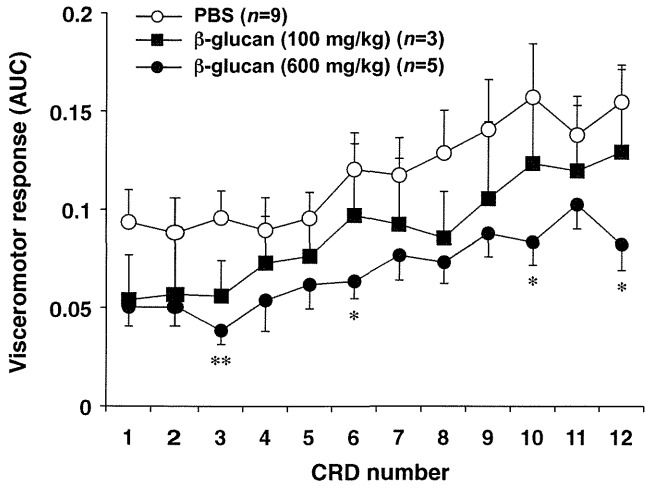

Fig. 2. Effect of LMW β-glucan on the viscermotor response to CRD in rats. The indicated doses (mg/kg) of β-glucan or PBS were orally administered to female Wistar rats once daily for 7 days. Twelve hours after the last administration of LMW β-glucan, rats were subjected to repetitive CRD and the visceromotor response was recorded and analysed as described in Section 2. Values are mean ± S.E.M $^{*}P < 0.05$; $^{**}P < 0.01$.

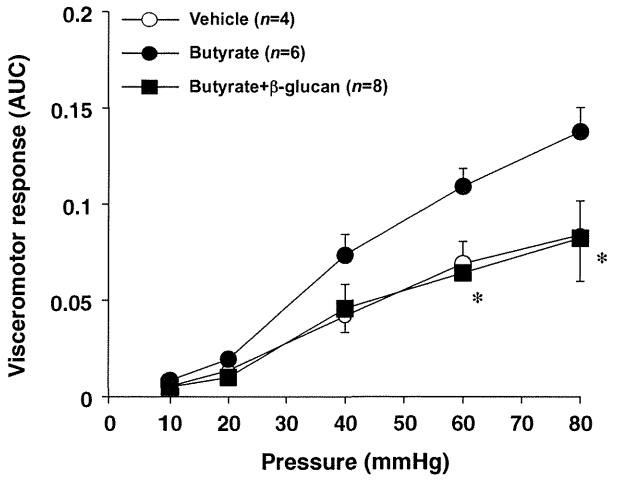

Fig. 3. Effect of LMW β-glucan on butyrate enema-induced colonic hypersensitivity to CRD in rats. Butyrate enemas were administered twice daily on days 1, 2 and 3. Administration of LMW β-glucan (600 mg/kg) (once daily from day 0 to day 6) and monitoring and analysis of the visceromotor response to CRD (on day 7) were performed as described in the legend of Fig. 2. Values are mean ± S.E.M $^{*}P < 0.05$.

2.5. Statistical analysis

All values are expressed as the mean ± S.E.M. Two-way ANOVA followed by the Tukey test or a Student's *t* test for unpaired results was used to evaluate differences between more than two groups or between two groups, respectively. Differences were considered to be significant for values of $P < 0.05$.

3. Results and discussion

3.1. Effect of LMW β-glucan on fecal pellet output in mice

We first examined the effect of a once-only oral administration of LMW β-glucan on restraint stress-induced fecal pellet output in mice. In untreated mice (administered PBS vehicle only), restraint stress (restricted movement by placement of mouse in a 50 ml plastic tube) caused a more than 5-fold increase in fecal pellet output per hour compared to unrestrained mice (Fig. 1A), as described previously [35]. The once-only oral pre-administration of LMW β-glucan suppressed this increase in a dose-dependent manner without affecting the basal level (without restraint stress) of fecal pellet output (Fig. 1A). Similar results were observed in response to a once-daily oral administration of LMW β-glucan for 7 days (data not shown). The LMW β-glucan-dependent suppression of restraint stress-induced fecal pellet output was also confirmed in rats (data not shown).

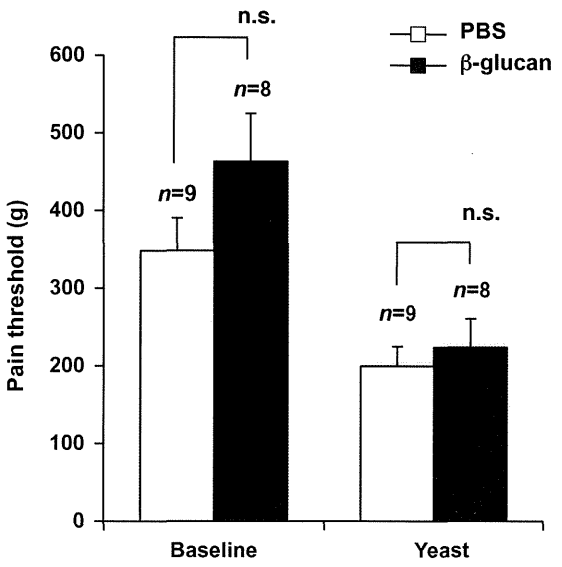

Fig. 4. Effect of LMW β-glucan on the pain response of rats in the inflamed paw pressure test. LMW β-glucan (600 mg/kg) was administered orally as described in the legend of Fig. 2. Twelve hours after the last administration of β-glucan, the inflamed paw pressure test was performed as described in Section 2. The pain threshold before (baseline) and after (yeast) the yeast injection was determined. Values are mean ± S.E.M n.s., not significant.

We also examined the effect of LMW β-glucan on the fecal pellet output induced by drugs that increase intestinal motility (bethanecol and 5-HT) or cause diarrhea (castor oil) [8,36]. As shown in Fig. 1B–D, the oral administration of LMW β-glucan (600 mg/kg) to mice suppressed the fecal pellet output induced by each of these drugs.

We then examined the effect of LMW β-glucan on drug-induced constipation. As shown in Fig. 1E and F, administration of loperamide or clonidine to mice decreased fecal pellet output, as described previously [36]. The oral pre-administration of LMW β-glucan did not alter the fecal pellet output. The results in Fig. 1 thus suggest that orally administered LMW β-glucan suppresses the restraint stress- or drug-induced stimulation of intestinal motility but does not affect the motility in the absence of these stimuli or in presence of constipation-inducing drugs. The mechanism underlying the LMW β-glucan-dependent suppression of intestinal motility is not clear at present.

3.2. Effect of LMW β-glucan on the visceromotor response to CRD in rats

In addition to alterations of fecal pellet output, hypersensitivity to visceral pain is one of the principle pathogenetic pathways for IBS. To study this phenomenon, we examined the effect of LMW β-glucan on visceromotor response to CRD, which has been used as an index of visceral pain response [33]. Rats were used for this analysis since the techniques for measuring the visceromotor response and CRD were established with these animals. As a single oral administration of LMW β-glucan did not significantly affect the visceromotor response to CRD (data not shown), we decided

to determine the effect of LMW β-glucan administered orally once-daily for 7 days. In control rats (PBS-treated), CRD evoked a visceromotor response which increased in amplitude in response to repeated CRDs (Fig. 2), as described previously [32]. Oral pre-administration of LMW β-glucan (600 mg/kg) to animals significantly decreased the visceromotor response to CRD not only after repetitive CRDs but also upon the first CRD (Fig. 2). Pre-administration of LMW β-glucan (100 mg/kg) also showed a tendency to decrease the visceromotor response to CRD, however the effect was not statistically significant (Fig. 2). These results indicate that oral pre-administration of high dose of LMW β-glucan suppresses the visceral pain response to CRD.

Since the visceromotor response to the first CRD was reduced by the pre-administration of LMW β-glucan, the results in Fig. 2 can be interpreted to indicate that LMW β-glucan suppresses the visceral pain response to CRD itself, but does not affect the repeated CRD-induced hypersensitivity to visceral pain. However, although we tried to habituate rats to the tube used for CRD experiment (see Section 2), it is possible that the animals entered into a state of restraint-like stress. Thus, it is also possible that LMW β-glucan suppresses the restraint stress-induced hypersensitivity to visceral pain.

We then examined the effect of LMW β-glucan on the visceral pain response in another animal model, butyrate-induced hypersensitivity to CRD. The butyrate enema is known to reduce the threshold of the visceromotor response to CRD [15,16]. We confirmed that twice-daily butyrate enemas (on days 1, 2 and 3) stimulated the visceromotor response to CRD on day 7 and found that when LMW β-glucan was orally pre-administered once daily from day 0 to day 6, the visceromotor response to CRD was similar to that measured in control rats (not given butyrate enemas) (Fig. 3). This result suggests that LMW β-glucan suppresses butyrate-induced hypersensitivity to CRD.

Finally, we tested whether the inhibitory effect of LMW β-glucan on the pain response is specific for visceral pain. For this purpose, we used the inflamed paw pressure test in which a yeast solution was administered to one of hind paws of rats to induce inflammation and the pressure-induced pain response was subsequently determined. As shown in Fig. 4, oral administration of LMW β-glucan once daily for 7 days did not affect the paw pressure required to elicit a nociception response (pain threshold) in both presence and absence of yeast injection. This finding suggests that LMW β-glucan does not affect the pain response in general but specifically affects the visceral pain response.

In conclusion, we have shown here that the oral administration of LMW β-glucan suppresses not only restraint stress- or drug-induced fecal pellet output, but also suppresses the visceral pain response. The difficulty associated with therapeutic management of IBS can be attributed to the fact that both abdominal pain and bowel habit disorders must be addressed. The results presented in this study thus suggest that LMW β-glucan could prove therapeutically beneficial for the prevention and treatment of IBS, especially in relation to the diarrhea-predominant IBS.

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References

[1] G.F. Longstreth, W.G. Thompson, W.D. Chey, L.A. Houghton, F. Mearin, R.C. Spiller, Functional bowel disorders, Gastroenterology 130 (2006) 1480–1491.
[2] D.A. Drossman, M. Camilleri, E.A. Mayer, W.E. Whitehead, AGA technical review on irritable bowel syndrome, Gastroenterology 123 (2002) 2108–2131.
[3] M. Shinozaki, S. Fukudo, M. Hongo, T. Shimosegawa, D. Sasaki, K. Matsueda, S. Harasawa, S. Miura, T. Mine, H. Kaneko, T. Arakawa, K. Haruma, A. Torii, T. Azuma, H. Miwa, M. Fukunaga, M. Handa, S. Kitamori, T. Miwa, High prevalence of irritable bowel syndrome in medical outpatients in Japan, J. Clin. Gastroenterol. 42 (2008) 1010–1016.
[4] C.W. Hammerle, C.M. Surawicz, Updates on treatment of irritable bowel syndrome, World J. Gastroenterol. 14 (2008) 2639–2649.
[5] D. Hulisz, The burden of illness of irritable bowel syndrome: current challenges and hope for the future, J Manag. Care Pharm. 10 (2004) 299–309.
[6] E.A. Mayer, S.M. Collins, Evolving pathophysiologic models of functional gastrointestinal disorders, Gastroenterology 122 (2002) 2032–2048.
[7] S. Okano, H. Nagaya, Y. Ikeura, H. Natsugari, N. Inatomi, Effects of TAK-637, a novel neurokinin-1 receptor antagonist, on colonic function in vivo, J. Pharmacol. Exp. Ther. 298 (2001) 559–564.
[8] S. Kobayashi, K. Ikeda, M. Suzuki, T. Yamada, K. Miyata, Effects of YM905, a novel muscarinic M3-receptor antagonist, on experimental models of bowel dysfunction in vivo, Jpn. J. Pharmacol. 86 (2001) 281–288.
[9] R. Moriya, T. Shirakura, H. Hirose, T. Kanno, J. Suzuki, A. Kanatani, NPY Y2 receptor agonist PYY(3–36) inhibits diarrhea by reducing intestinal fluid secretion and slowing colonic transit in mice, Peptides 31 (2010) 671–675.
[10] J. Munakata, B. Naliboff, F. Harraf, A. Kodner, T. Lembo, L. Chang, D.H. Silverman, E.A. Mayer, Repetitive sigmoid stimulation induces rectal hyperalgesia in patients with irritable bowel syndrome, Gastroenterology 112 (1997) 55–63.
[11] J.A. Christianson, G.F. Gebhart, Assessment of colon sensitivity by luminal distension in mice, Nat. Protoc. 2 (2007) 2624–2631.
[12] M. Larsson, S. Arvidsson, C. Ekman, A. Bayati, A model for chronic quantitative studies of colorectal sensitivity using balloon distension in conscious mice – effects of opioid receptor agonists, Neurogastroenterol. Motil. 15 (2003) 371–381.
[13] W.R. Treem, N. Ahsan, G. Kastoff, J.S. Hyams, Fecal short-chain fatty acids in patients with diarrhea-predominant irritable bowel syndrome: in vitro studies of carbohydrate fermentation, J. Pediatr. Gastroenterol. Nutr. 23 (1996) 280–286.
[14] C. Tana, Y. Umesaki, A. Imaoka, T. Handa, M. Kanazawa, S. Fukudo, Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome, Neurogastroenterol. Motil. 22 (2010) 512–519, e114–515.
[15] S. Bourdu, M. Dapoigny, E. Chapuy, F. Artigue, M.P. Vasson, P. Dechelotte, G. Bommelaer, A. Eschalier, D. Ardid, Rectal instillation of butyrate provides a novel clinically relevant model of noninflammatory colonic hypersensitivity in rats, Gastroenterology 128 (2005) 1996–2008.
[16] C. Rousseaux, X. Thuru, A. Gelot, N. Barnich, C. Neut, L. Dubuquoy, C. Dubuquoy, E. Merour, K. Geboes, M. Chamaillard, A. Ouwehand, G. Leyer, D. Carcano, J.F. Colombel, D. Ardid, P. Desreumaux, *Lactobacillus acidophilus* modulates intestinal pain and induces opioid and cannabinoid receptors, Nat. Med. 13 (2007) 35–37.
[17] J. Chen, R. Seviour, Medicinal importance of fungal beta-(1→3), (1→6)-glucans, Mycol. Res. 111 (2007) 635–652.
[18] S.V. Tsoni, G.D. Brown, Beta-Glucans and dectin-1, Ann. NY Acad. Sci. 1143 (2008) 45–60.
[19] M. Berdal, H.I. Appelbom, J.H. Eikrem, A. Lund, S. Zykova, L.T. Busund, R. Seljelid, T. Jenssen, Aminated beta-1,3-D-glucan improves wound healing in diabetic db/db mice, Wound Repair Regen. 15 (2007) 825–832.
[20] S. Bell, V.M. Goldman, B.R. Bistrian, A.H. Arnold, G. Ostroff, R.A. Forse, Effect of beta-glucan from oats and yeast on serum lipids, Crit. Rev. Food Sci. Nutr. 39 (1999) 189–202.
[21] G. Sener, E. Eksioglu-Demiralp, M. Cetiner, F. Ercan, B.C. Yegen, Beta-glucan ameliorates methotrexate-induced oxidative organ injury via its antioxidant and immunomodulatory effects, Eur. J. Pharmacol. 542 (2006) 170–178.
[22] A. Bedirli, M. Kerem, H. Pasaoglu, N. Akyurek, T. Tezcaner, S. Elbeg, L. Memis, O. Sakrak, Beta-glucan attenuates inflammatory cytokine release and prevents acute lung injury in an experimental model of sepsis, Shock 27 (2007) 397–401.
[23] O.I. Lyuksutova, E.D. Murphey, T.E. Toliver-Kinsky, C.Y. Lin, W. Cui, D.L. Williams, E.R. Sherwood, Glucan phosphate treatment attenuates burn-induced inflammation and improves resistance to *Pseudomonas aeruginosa* burn wound infection, Shock 23 (2005) 224–232.
[24] J. Soltys, M.T. Quinn, Modulation of endotoxin- and enterotoxin-induced cytokine release by in vivo treatment with beta-(1,6)-branched beta-(1,3)-glucan, Infect. Immun. 67 (1999) 244–252.
[25] H.Z. Toklu, A.O. Sehirli, A. Velioglu-Ogunc, S. Cetinel, G. Sener, Acetaminophen-induced toxicity is prevented by beta-D-glucan treatment in mice, Eur. J. Pharmacol. 543 (2006) 133–140.
[26] V.B. Shah, D.L. Williams, L. Keshvara, Beta-glucan attenuates TLR2- and TLR4-mediated cytokine production by microglia, Neurosci. Lett. 458 (2009) 111–115.
[27] Y. Kimura, M. Sumiyoshi, T. Suzuki, M. Sakanaka, Effects of water-soluble low-molecular-weight beta-1, 3-D-glucan (branch beta-1, 6) isolated from *Aureobasidium pullulans* 1A1 strain black yeast on restraint stress in mice, J. Pharm. Pharmacol. 59 (2007) 1137–1144.
[28] Y. Kimura, M. Sumiyoshi, T. Suzuki, M. Sakanaka, Antitumor and antimetastatic activity of a novel water-soluble low molecular weight beta-1, 3-D-glucan (branch beta-1,6) isolated from *Aureobasidium pullulans* 1A1 strain black yeast, Anticancer Res. 26 (2006) 4131–4141.

[29] Y. Kimura, M. Sumiyoshi, T. Suzuki, M. Sakanaka, Inhibitory effects of water-soluble low-molecular-weight beta-(1,3–1,6) D-glucan purified from *Aureobasidium pullulans* GM-NH-1A1 strain on food allergic reactions in mice, Int Immunopharmacol 7 (2007) 963–972.
[30] K. Tanaka, Y. Tanaka, T. Suzuki, T. Mizushima, Protective effect of beta-(1,3 → 1,6)-D-glucan against irritant-induced gastric lesions, Br. J. Nutr. 106 (2011) 475–485.
[31] T.L. Bale, R. Picetti, A. Contarino, G.F. Koob, W.W. Vale, K.F. Lee, Mice deficient for both corticotropin-releasing factor receptor 1 (CRFR1) and CRFR2 have an impaired stress response and display sexually dichotomous anxiety-like behavior, J. Neurosci. 22 (2002) 193–199.
[32] A. Ravnefjord, M. Brusberg, H. Larsson, E. Lindstrom, V. Martinez, Effects of pregabalin on visceral pain responses and colonic compliance in rats, Br. J. Pharmacol. 155 (2008) 407–416.
[33] K. Saito-Nakaya, R. Hasegawa, Y. Nagura, H. Ito, S. Fukudo, Corticotropin-releasing hormone receptor 1 antagonist blocks colonic hypersensitivity induced by a combination of inflammation and repetitive colorectal distension, Neurogastroenterol. Motil. 20 (2008) 1147–1156.
[34] L.O. Randall, J.J. Selitto, A method for measurement of analgesic activity on inflamed tissue, Arch. Int. Pharmacodyn. Ther. 111 (1957) 409–419.
[35] E. Mazzon, S. Cuzzocrea, Role of TNF-alpha in ileum tight junction alteration in mouse model of restraint stress, Am. J. Physiol. Gastrointest. Liver Physiol. 294 (2008) G1268–G1280.
[36] T. Saito, F. Mizutani, Y. Iwanaga, K. Morikawa, H. Kato, Laxative and anti-diarrheal activity of polycarbophil in mice and rats, Jpn. J. Pharmacol. 89 (2002) 133–141.