FULL PAPER *Laboratory Animal Science*

Piebald mutation on a C57BL/6J background

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ABSTRACT. The classic piebald mutation in the endothelin receptor type B (*Ednrb*) gene was found on *rolling* Nagoya genetic background (PROD-*s*/*s*) mice with white coat spotting. To examine whether genetic background influenced the phenotype in the piebald mutant mice, we generated a congenic strain (B6.PROD-*s*/*s*), produced by repeated backcrosses to the C57BL/6J (B6) strain. Although B6.PROD-*s*/*s* mice showed white coat spotting, 7% of B6.PROD-*s*/*s* mice died between 2 and 5 weeks after birth due to megacolon. The PROD-*s*/*s*, *s*/*s* and Japanese fancy mouse 1 (JF1) strains, which also have piebald mutations on different genetic backgrounds with B6, showed only pigmentation defects without megacolon. In expression analyses, rectums of B6.PROD-*s*/*s* with megacolon mice showed ~5% of the level of *Ednrb* gene expression versus B6 mice. In histological analyses, aganglionosis was detected in the rectum of megacolon animals. The aganglionic rectum was thought to lead to severe constipation and intestinal blockage, resulting in megacolon. We also observed an abnormal intestinal flora, including a marked increase in Bacteroidaceae and Erysipelotrichaceae and a marked decrease in *Lactobacillus* and Clostridiales, likely inducing endotoxin production and a failure of the mucosal barrier system, leading ultimately to death. These results indicate that the genetic background plays a key role in the development of enteric ganglion neurons, controlled by the *Ednrb* gene, and that B6 has modifier gene (s) regarding aganglionosis.

KEY WORDS: aganglionic rectum, *Ednrb* gene, genetic background, piebald mutation

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The autosomal recessive ataxic *rolling* Nagoya strain (PROD-*rol*/*rol*) mice, which exhibit white spots on an agouti coat, were found among descendants of a cross between the SIII and C57BL/6Nga strains and have been maintained by intercross mating [16]. In our previous study, PROD-*rol*/*rol* was shown to be a double-mutant strain with an amino acid change at R1262G in the *Cacna1a* gene on chromosome 8, causing neuronal $Ca_v2.1$ channel dysfunction, and the piebald mutation in the endothelin receptor type B (*Ednrb*) gene on chromosome 14, causing the coat pigment defect [23]. The PROD-*rol*/*rol* has a mutation in the voltage-sensing S4 segment of the third repeat in the $Ca_v2.1\alpha_1$ [14]. The Ca^{2+} current amplitude exhibits a 40% reduction in PROD-*rol*/*rol* compared to wild-type *rolling* Nagoya (PROD-*s*/*s*) [14]. The PROD-*s*/*s* mice have no apparent abnormal behaviors [20, 21] and two silent nucleotide substitutions in the coding region and insertion of a retroposon-like element in intron 1 of the *Ednrb* gene [23]. The same mutation in the *Ednrb* gene was detected in the Japanese fancy mouse 1 (JF1) mice [4, 12, 13], derived from a Japanese wild strain, and in the *s*/*s* mice [7, 22], derived from laboratory strain with SSLE/Le background. They have a spotting defect without megacolon [4, 22].

In addition to the coat spotting, null mutations of the *Ednrb* gene in rats ($Edbrb^{sl/sl}$ rats) [19] and mice (s^l/s^l mice) [9] cause megacolon owing to aganglionosis. Interestingly, genetic backgrounds strongly affect the penetrance and severity of aganglionosis in $Ednrb^{sl/sl}$ rats. Thus, 90% of $Edbrb^{sl/sl}$ rats with the LEH/Hkv genetic background (LEH-$Edbrb^{sl/sl}$ rats) showed aganglionosis [6]. However, about 40% of $Ednrb^{sl/sl}$ rats with the F344 genetic background (F344-$Ednrb^{sl/sl}$ rats) showed aganglionosis [6].

The $Edbrb^{sl/sl}$ rats and s^l/s^l mice are known as an animal model for human Hirschsprung's (HSCR) disease [6, 9]. HSCR disease shows aganglionosis and causes severe constipation and intestinal blockage, resulting in megacolon [1, 2, 8, 10]. Incomplete penetrance and inter-familial variation are commonly observed in HSCR disease [1, 17], suggesting that genetic background is an important factor in the development of HSCR disease.

However, it remains unknown whether megacolon phenotype is influenced by the genetic background of the piebald mutant mice. Thus, in this study, we examined whether genetic background change can lead to the occurrence of megacolon in the piebald mutant mice.

MATERIALS AND METHODS

Animals: The research was approved by the Animal Experiments Committee of RIKEN (Approved ID: No. H24-2–206). To generate a congenic strain having classic piebald mutation with C57BL/6J (B6) mice (Charles River Japan, Yokohama, Japan), PROD-*s*/*s* strain [16, 23], backcrossed to B6 for 12 generations, produced B6.PROD-*s*/*s*. The mice were allowed *ad libitum* access to water and food pellets (CRF-1; Oriental Yeast, Tokyo, Japan) and kept at room temperature (23 ± 1°C) and 55 ± 5% humidity under a 12/12-hr light/dark cycle (light from 8:00 am to 8:00 pm).

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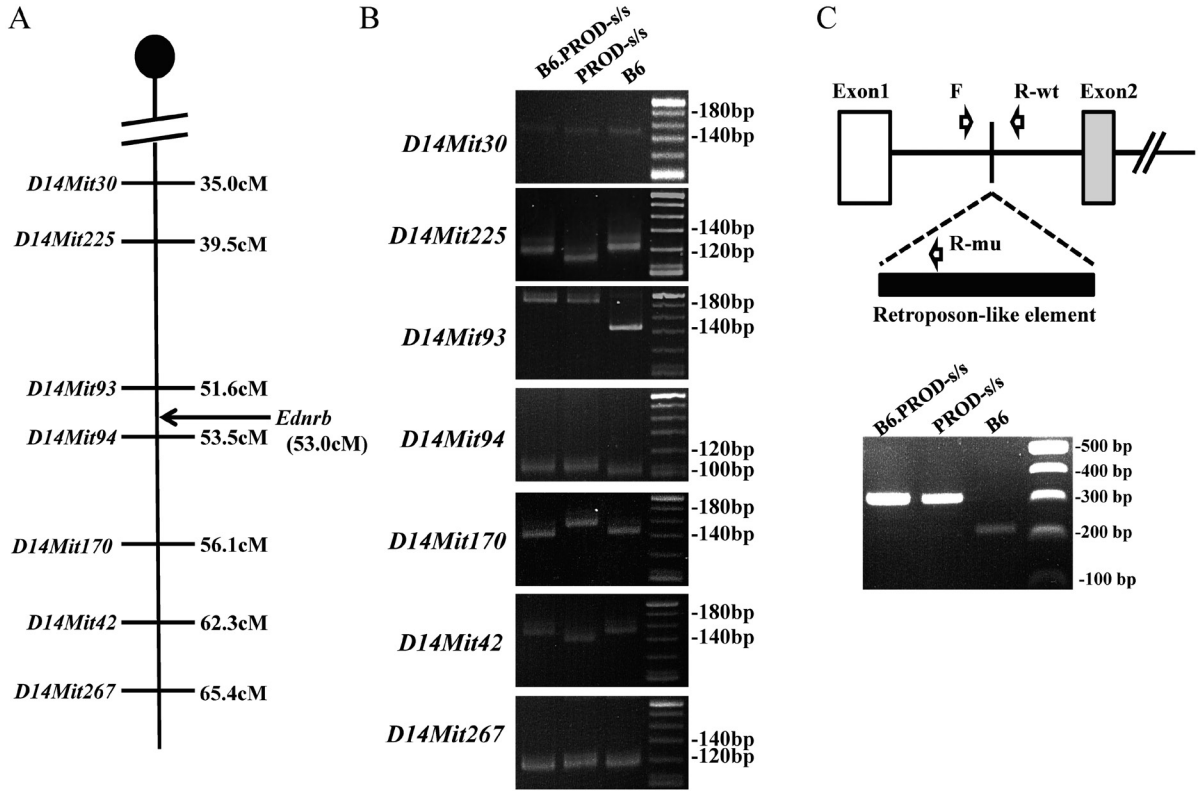

Fig. 1. Genotyping of microsatellite makers and mutant alleles. (A) A linkage map of mouse chromosome 14, including the *Ednrb* and microsatellite loci used in this study, is presented. (B) Genotyping results of the microsatellite markers are shown. (C) In the *Ednrb* gene of piebald mutant mice, exon 1 and exon 2 are shown as white boxes, and retroposon-like region is shown as a black box (upper). White arrows represent the PCR primers used to amplify the normal and mutant fragments. Genotyping results of the wild-type and mutant type are shown (lower).

Microsatellite genotyping: To confirm the establishment of the congenic strain with a B6 background, microsatellite markers located on chromosome 14 (*D14Mit30*, *D14Mit225*, *D14Mit93*, *D14Mit94*, *D14Mit170*, *D14Mit42* and *D14Mit267*) were typed using genomic DNA obtained from the tails of B6.PROD-*s/s* (male, female: n=8, 6), PROD-*s/s* (male, female: n=8, 6) and B6 (male, female: n=8, 7) mice. The polymerase chain reaction (PCR) protocols and primers of microsatellite markers were reported in the Mouse Microsatellite Data Base of Japan (MMDBJ, http://www.shigen.nig.ac.jp/mouse/mmdbj/).

Genomic and gene structure analysis: To distinguish normal alleles from those with the insertion of a retroposon-like element in intron 1 of the *Ednrb* gene (GenBank ID: AB242436.1), the PCR products were amplified with genomic DNA from the tails of B6.PROD-*s/s* (male, female: n=8, 6), PROD-*s/s* (male, female: n=8, 6) and B6 (male, female: n=8, 7) mice, and allele-specific primers designed according to a previous report [4]. The F and R-wt primers correspond to intron 1 of the *Ednrb* gene (Fig. 1C) and amplify a 225-base pair (bp) fragment as the wild type. The F and R-mu primers amplify a 318-bp fragment containing part of intron 1 of the *Ednrb* gene and the retroposon-like element of the mutant type.

Total RNAs from the brains of B6.PROD-*s/s* (n=8), PROD-*s/s* (n=8) and B6 (n=8) male mice were isolated using the TRIzol reagent (Invitrogen, Burlington, Canada). According to a previous study [23], the amplified fragments of *Ednrb* cDNA were sequenced.

Real-time quantitative RT-PCR (real-time qRT-PCR): The levels of *Ednrb* mRNA in the colon and rectum of B6.PROD-*s/s* with megacolon (n=8), B6.PROD-*s/s* (n=8) and B6 (n=8) male mice were measured using Applied Biosystems TaqMan Gene Expression Assays (*Ednrb*, Assay ID: Mm01224433_m1) and normalized relative to the 18S ribosomal RNA (Assay ID: Hs99999901_s1) as reported previously [23].

Histochemistry: The large intestine of B6.PROD-*s/s* with megacolon (n=5), B6.PROD-*s/s* (n=6) and B6 (n=5) male mice was dissected after perfusion with saline followed by 4% paraformaldehyde, fixed with tissue fixative (Gonostaff, Co., Ltd., Tokyo, Japan), embedded in paraffin wax and cut into sections of 6 μm for hematoxylin and eosin (H&E) staining and *in situ* hybridization (ISH). The protocol, including the probe sequence for the *Cacna1a* gene, was reported previously [18, 23].

Bacteriological analysis: The dilated region of the intestines from anesthetized B6.PROD-*s/s* with megacolon

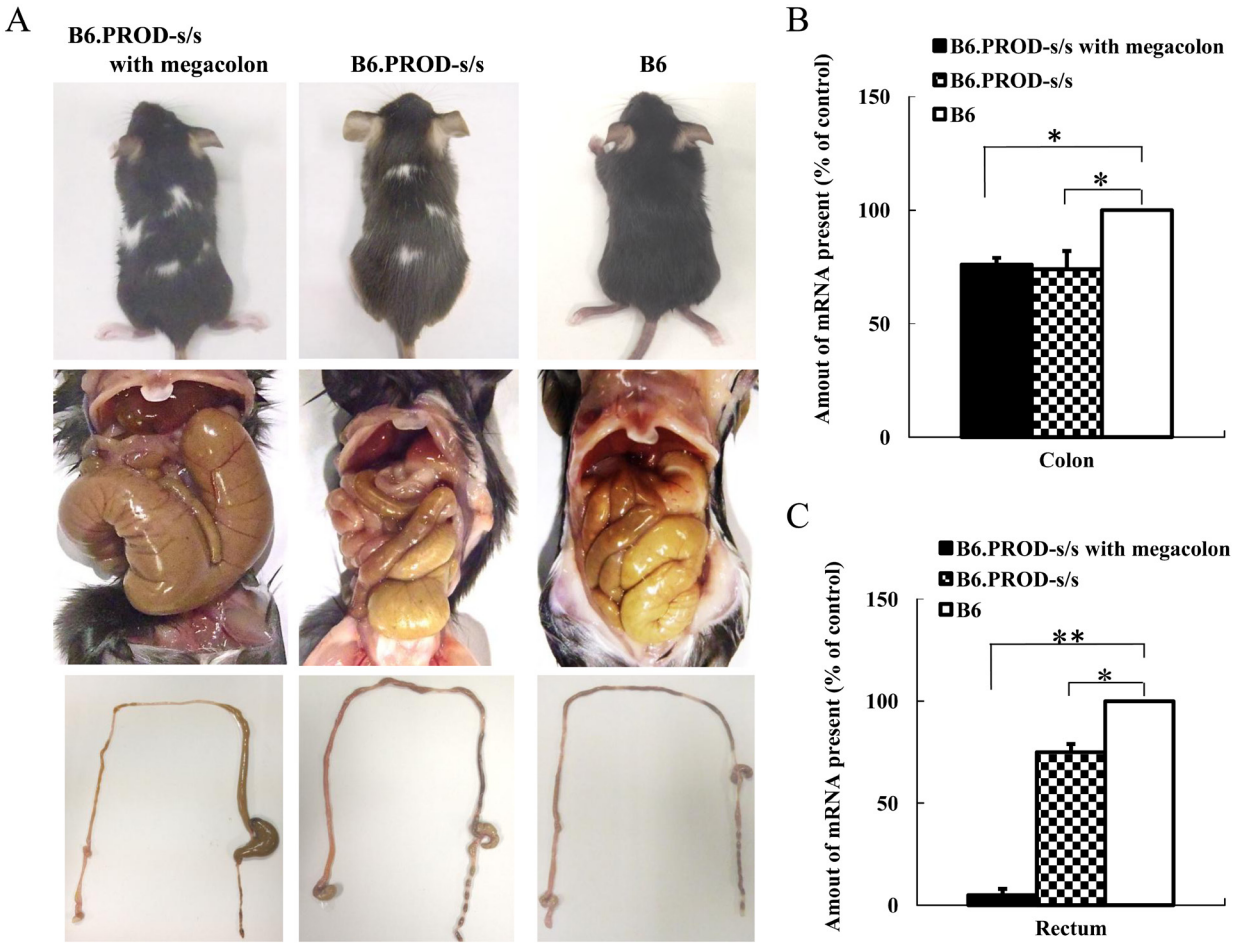

Fig. 2. Phenotypes of B6.PROD-*s/s* with megacolon and B6.PROD-*s/s* mice. (A) Representative photographs showing white spots on agouti coat of B6.PROD-*s/s* with megacolon (upper, left) and B6.PROD-*s/s*(upper, center) mice are presented. Representative photographs of the open abdomen of mice are shown (middle). Representative photographs of the symptoms of aganglionosis of PROD-*s/s* with megacolon mice (lower, left). (B) The expression levels of *Ednrb* mRNA in the colon determined by real-time qRT-PCR. (C) The expression levels of *Ednrb* mRNA in the rectum determined by real-time qRT-PCR. The *Ednrb* mRNA expression level for each strain was calculated relative to that in B6 mice. $^{**}P<0.01$, $^{*}P<0.05$, vs. the appropriate control (Dunnett's test).

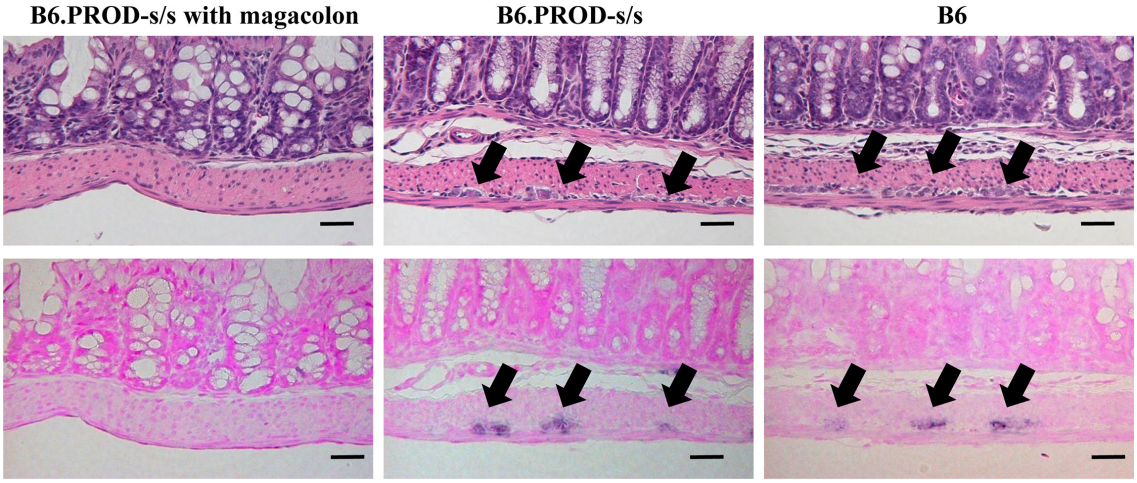

Fig. 3. H&E staining and *in situ* hybridization. Representative photographs of H&E staining (upper) and *in situ* hybridization (lower) in the rectum are shown. Arrows indicate enteric nerve plexus (upper) and localization of $Ca_v2.1\alpha_1$ mRNA (lower). The scale bar is 50 μm.

(n=7) and B6.PROD-*s/s* (n=14) male mice was removed aseptically, and the contents of the intestines were isolated. We used terminal restriction fragment length polymorphism (T-RFLP) analysis as reported previously [15] to examine the intestinal microflora.

Statistical analysis: The data are presented as the means ± standard error of the mean (SEM). Statistical analyses were conducted using Excel Statistics 2006 (SSRI, Tokyo, Japan). The data were analyzed using Dunnett's test between groups where appropriate. In all analyses, $P<0.05$ was taken to indicate statistical significance.

RESULTS

Generation of congenic strain: A congenic strain (B6.PROD-*s*/*s*) was produced by repeated backcrosses to B6 for 12 generations with selection for white spotting as a marker from the PROD-*s*/*s*. Because the *Ednrb* gene is located on chromosome 14 in the mice, we typed microsatellite loci on chromosome 14 to confirm the changed genetic background. The location order and distances among the loci are presented in Fig. 1A. As shown in Fig. 1B, fragments were amplified from B6.PROD-*s*/*s*, PROD-*s/s* and B6 mice. According to MMDBJ, MGI and our previous report [23], different sizes between PROD and B6 strains are produced in *D14Mit30* (PROD, B6: size=154 bp, 154 bp), *D14Mit225* (PROD, B6: size=101 bp, 118 bp), *D14Mit93* (PROD, B6: size=189 bp, 147 bp), *D14Mit94* (PROD, B6: size=108 bp, 104 bp), *D14Mit170* (PROD, B6: size=163 bp, 146 bp), *D14Mit42* (PROD, B6: size=142 bp, 152 bp) and *D14Mit267* (PROD, B6: size=114 bp, 114 bp) loci. The amplified fragment lengths were similar between B6.PROD-*s/s* and B6 mice in the *D14Mit30*, *D14Mit225*, *D14Mit170*, *D14Mit42* and *D14Mit267* (Fig. 1B) loci.

PCR analyses showed the insertion of a retroposon-like element in intron 1 of the *Ednrb* gene of B6.PROD-*s/s* and PROD-*s/s* mice (wild type, mutant type: size=225 bp, 318 bp; Fig. 1C) and two silent nucleotide substitutions in the coding region (data not shown).

Influence of genetic background on intestinal malformation: The PROD-*s/s* and B6 showed no megacolon symptoms until 12 months after birth. However, ~7% (male: n=12, female: n=10) of B6.PROD-*s/s* (male: n=153, female: n=131) showed megacolon symptoms (B6.PROD-*s/s* with megacolon; Table 1) and died between 2 and 5 weeks after birth. The symptoms in B6.PROD-*s/s* with megacolon were detected primarily from the ileum to the colon (Fig. 2A).

Ednrb gene expression levels in the colon and rectum: Real-time qRT-PCR analysis was performed to determine the levels of *Ednrb* mRNA in enteric neurons of B6.PROD-*s/s* with megacolon, B6.PROD-*s/s* and B6 mice. In the colon, B6.PROD-*s/s* with megacolon and B6.PROD-*s/s* showed about 75% of the level of *Ednrb* expression compared to control B6 mice (Fig. 2B). In the rectum, although the B6.PROD-*s/s* showed ~75% of the level of *Ednrb* expression versus the control B6 mice, B6.PROD-*s/s* with megacolon mice showed ~5% of the level of *Ednrb* gene expression versus the B6 mice (Fig. 2C).

Table 1. Incidence of aganglionosis in PROD-s/s and B6.PROD-s/s strains

Phenotype	PROD-s/s		B6.PROD-s/s	
	Males	Femals	Males	Femals
Normal (n)	128	132	141	121
Megacolon (n)	0	0	12	10
Incidence (%)	0	0	7	7

Total number of mice: PROD-s/s, n=260; B6.PROD-s/s, n=262.

Histological analyses: In H&E staining of the large intestine of B6.PROD-*s/s* with megacolon, B6.PROD-*s/s* and control B6 mice at 3 weeks of age (Fig. 3), B6.PROD-*s/s* with megacolon showed the absence of ganglion cells in the rectum. Although ISH showed similar localization of neuronal *Cacna1a* mRNA of B6.PROD-*s/s* and B6 mice, *Cacna1a* mRNA expression was not detected in the rectum of B6.PROD-*s/s* with megacolon (Fig. 3). No signal was seen in the intestine using the control sense probes in any of these strains (data not shown). However, the results of the *in situ* hybridization analysis indicated similar patterns of *Cacna1a* mRNA expression in the ileum and colon between B6.PROD-*s/s* with megacolon and B6.PROD-*s/s* mice (data not shown). The aganglionosis resulted in severe constipation and intestinal blockage, resulting in megacolon. However, there was no apparent alteration in the thickness or occurrence of inflammation in the mucosal or muscular layers of the rectum in these strains (data not shown).

Intestinal microflora: The bacterial flora in the intestine of B6.PROD-*s/s* with megacolon and B6.PROD-*s/s* mice at 3 weeks of age was analyzed by T-RFLP (Table 2). The relative abundance (occupancy) of Bacteroidales and Erysipelotrichaceae was significantly higher, and *Lactobacillus* and Clostridiales were significantly lower in B6.PROD-*s/s* with megacolon than the control B6 animals.

DISCUSSION

Hirschsprung's (HSCR) disease is a congenital intestinal disease, characterized by the loss of ganglion cells in the intestinal tract [1, 2, 8, 10]. This disorder occurs in about 1/5,000 live births. Due to the lack of ganglia, the stool cannot be passed through the colon, resulting in megacolon. Several susceptibility genes have been identified for HSCR disease: the RET proto-oncogene, endothelin-3 (EDN3) gene, endothelin receptor B (EDNRB) gene, glial cell line derived neurotrophic factor (GDNF) gene and SRY-related HMG-box 10 (SOX10) gene [17]. These genes encode ligands, receptors and transcription factors and play important roles in the formation of the enteric nervous system [1, 2, 8, 10]. There are both incomplete penetrance and inter-familial variation in HSCR disease [1, 17], suggesting that genetic background or multiple molecular interactions are important in the development of HSCR disease.

The EDNRB is a G-protein-coupled seven-transmembrane receptor that interacts with a family of ligands known as the endothelins [11]. The piebald mutation has an insertion of a

Table 2. Composition of intestinal flora (%)

Bacterial Groups	B6.PROD-s/s with megacolon	B6.PROD-s/s
Bacteroidales	49.4 ± 2.16*	34.1 ± 1.67
Lactobacillus	5.2 ± 0.91*	12.9 ± 1.07
Clostridiales	20.3 ± 1.08*	36.6 ± 3.25
Erysipelotrichaceae	20.2 ± 0.88**	6.8 ± 0.54
Akkermansia	0 ± 0.05	0.1 ± 0.05
Anaeroplasma	0	0
Coriobacteriales	0.7 ± 0.07	0.8 ± 0.07
Mucispirillum	0.3 ± 0.07	0.4 ± 0.07
Parasutterella	1.3 ± 0.30	0.9 ± 0.30
Others	3.1 ± 0.53	3.7 ± 0.37

Total number of mice: B6.PROD-s/s with megacolon, n=14; B6.PROD-s/s, n=7. *P<0.05, **P<0.01 compared to the appropriate control (Dunnett's test).

retroposon-like element in intron 1 of the *Ednrb* gene [22]. This mutation induces a reduced level of the normal *Ednrb* transcript [22, 23]. It has been reported that homozygous piebald mutated mice, including PROD-*s*/*s* mice with a SIII × C57BL/6N background [16], JF1 mice with a Japanese wild mouse background [12] and *s*/*s* mice with a SSLE/Le background [22], all show pigmented defects without megacolon. In this study, we produced a congenic strain, B6.PROD-*s*/*s*, by backcrossing the PROD-*s*/*s* strain to the C57BL/6J strain for 12 generations and then evaluated the impact of genetic background on aganglionosis symptoms. The PROD-*s*/*s* did not show megacolon symptoms, but interestingly, some B6.PROD-*s*/*s* did show megacolon and died between 2 and 5 weeks after birth. The symptoms in B6.PROD-*s*/*s* with megacolon were detected primarily from the ileum to the colon, suggesting that a dysfunctional enteric nervous system was present in the rectum. In real-time qRT-PCR studies, B6.PROD-*s*/*s* mice showed ~25% lower levels of *Ednrb* gene expression in the colon and rectum than control B6 mice. This expression level in B6.PROD-*s*/*s* mice was similar to that of PROD-*s*/*s* mice reported previously [23]. However, although the colon of B6.PROD-*s*/*s* mice with megacolon showed ~25% lower levels of *Ednrb* gene expression, the rectum showed ~95% lower levels of *Ednrb* gene expression than the B6 mice. In the *in situ* hybridization analysis in the rectum, although the results indicated similar patterns of *Cacna1a* mRNA localization in the enteric neurons of B6.PROD-*s*/*s* and B6 mice, no signal was seen in the B6.PROD-*s*/*s* mice with megacolon. Apparently, ~75% of the wild-type level of *Ednrb* gene expression would be sufficient to develop a functional enteric nervous system. Our results also suggest that an as-yet-undiscovered rectum-specific *Ednrb* gene expression dysfunction mechanism resulted in the serious aganglionosis. In previous studies using rat models, although almost 60% of F344-*Ednrb*$^{sl/sl}$ rats did not show any symptoms of aganglionosis, 90% of LEH-*Ednrb*$^{sl/sl}$ rats showed the aganglionosis phenotype [6]. These results indicate that the genetic background can affect the development of the enteric nervous system in the *Ednrb* gene mutants. In humans, although the *EDNRB* gene has been implicated in the development of HSCR disease, the inheritance shows incomplete penetrance [17]. A further study of the modifier loci using the animal model may provide useful information for understanding the human disease with incomplete penetrance.

In a previous study [4], JF1 mice do not show any megacolon symptoms, despite the decrease in the expression level of the *Ednrb* gene and the occurrence of hypoganglionosis. Interestingly, approximately 3% strain of the F2 (JF1 × B6) mice exhibited severe megacolon. These results and our study suggest that B6 mice have modifier gene (s) to aganglionosis.

Mutations in the gene encoding the EDNRB cause three symptoms: aganglionosis, pigmented disorder and hearing loss [1, 2, 17]. In addition, the *Ednrb* null mice and rats show abnormal splenic microarchitecture with lymphopenia [3, 5]. In this study, we have not examined the phenotypes of hearing ability and lymphopenia in the B6.PROD-*s*/*s* mice. We will study them in the next study.

In considering the cause of death in the B6.PROD-*s*/*s* with megacolon, it is interesting to examine the condition of the intestinal microflora after severe intestinal blockage, because a defense system in the intestine is believed to affect the balance of intestinal bacteria. In B6.PROD-*s*/*s* with megacolon, we observed an abnormal composition of the intestinal bacteria, suggesting that they might induce excess harmful products (e.g., endotoxins) and/or cause failure of the mucosal barrier system, ultimately leading to death.

In conclusion, the present study has shown that genetic background contributes importantly to the phenotypes of piebald *Ednrb* gene mutation and that use of different strains may facilitate understanding of a complex disease, such as HSCR disease.

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