Investigation of the effects of the chromosomal regions of mouse chromosome 2 on susceptibility to dental caries using congenic strains

コンジェニック系統を用いた齲蝕感受性に関する
マウス第 2 番染色体の染色体領域の影響の研究

日本大学大学院　松戸歯学研究科　歯学専攻

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Title: Investigation of the effects of the chromosomal regions of mouse chromosome 2 on susceptibility to dental caries using congenic strains

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ABSTRACT

Background: Dental caries is a widespread infectious disease influenced by environmental and genetic factors. Thus far, studies have identified several environmental factors influencing dental caries; however, little remains known about the underlying genetic factors. Recent studies using mice have reported the major genes responsible for dental caries to be located on mouse chromosome 2. Using congenic mice, this study aimed to clarify if the chromosomal region on mouse chromosome 2 influenced dental caries.

Materials and methods: We examined the dental caries scores obtained from caries induction, salivary secretion volume, and enamel hardness in the strains C57BL/6Slc, C3H/HeSlc, B6-Chr.2^{C3H}, and three types of congenic mouse strains that we generated.

Results: We successfully generated three types of congenic mouse strains. The caries scores of congenic mice, which had the C3H/HeSlc-derived interval between D2Mit126 (84 Mega base pair; Mbp) and D2Mit226 (163 Mbp), were significantly lower than that of any other mouse strain studied herein ($p < 0.05$). Moreover, the salivary secretion volume of the congenic mice described above tended to be more than that of any other congenic strain. However, enamel hardness was not significantly different among the strains.

Conclusion: Several genes associated with caries resistance could be located between D2Mit126 and D2Mit226. Salivary secretion volume was one of the most important factors related to dental caries, and the genes influencing the rate of salivary secretion might be located in the same region.

Keywords

Dental caries susceptibility, genetic factors, congenic mice

1. Introduction

Dental caries is a common chronic infectious disease globally. Susceptibility to dental caries is influenced by various factors, such as host factors, oral flora, and the environment. Environmental and bacterial factors have been studied as the causes of dental caries, but not much is known about host genetic factors.

Studies on twins have indicated that dental caries has a significant genetic contribution, of about 40–60% [1, 2]. In other studies on twins raised in different environments, significant genetic variance (45–67%) for dental caries was proved by monozygotic twins and was supported by the findings in dizygotic twins [1, 3]. The results of these studies reinforced the fact that dental caries is determined by a genetic component as well [1].

Animal models have also demonstrated genetic contribution to dental caries susceptibility: Kurihara et al [4] identified two inbred strains of mice—C57BL/6NJcl, which is highly susceptible to dental caries, and C3H/HeNJcl, which is highly resistant to dental caries. Quantitative trait locus (QTL) analysis of these strains showed that chromosome 2 had a high likelihood of being related to dental caries susceptibility [5]. To prove the effect of this QTL located on chromosome 2, Nomi et al [6] produced a consomic mouse strain, termed B6-Chr.2^{C3H}, in which a C57BL/6Slc-derived interval of chromosome 2 was replaced with that from the caries-resistant strain C3H/HeSlc. B6-Chr.2^{C3H} mice had a six times lower dental caries susceptibility and higher salivary secretion volume compared to C57BL/6Slc [7, 8]. They suggested that this QTL (the C3H/HeSlc interval) on chromosome 2 played a role in susceptibility to dental caries and salivary secretion.

In this study, to elucidate the genetic component of dental caries susceptibility on chromosome 2, we induced dental caries using several types of congenic mice containing different C3H/HeSlc-derived intervals on chromosome 2 in a C57BL/6Slc genetic background. Considering the results of the caries score obtained from caries induction, the volume of stimulated saliva secretion, and enamel hardness, we also aimed to narrow down the chromosomal region influencing dental caries susceptibility using congenic mice.

2. Materials and methods

2.1. Mice and breeding conditions

C57BL/6Slc and C3H/HeSlc mice were purchased from Sankyo Lab Service Co. (Tokyo, Japan). B6-Chr.2^{C3H} mice were established in our laboratory and maintained at Sankyo Lab Service Co. All

mice were maintained at room temperature (25 °C ± 1 °C), a relative humidity of 55% ± 5%, and a 12 h light/dark cycle. All the animal use protocols of this study were reviewed and approved by the Nihon University Institutional Review Board (Chiba, Japan; AP14MD005-1, AP17MD010).

2.2. Polymerase chain reaction (PCR) conditions

PCR was performed by the usual methods using Ex Taq® (TaKaRa, Shiga, Japan). The amplification conditions were as follows: DNA denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; and final extension at 72 °C for 10 min. After the PCR products were electrophoresed in 4% agarose gels, the gels were stained with ethidium bromide and examined under ultraviolet light.

2.3. Generation of congenic mouse strains

The congenic strains were produced as described previously [9, 10, 11]. DNA was extracted from the tails of mice using the DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany) following the instruction manual, and the genotypes were confirmed by PCR using Massachusetts Institute of Technology primers, D2Mit237 (40 megabase pairs; Mbp), D2Mit90 (65 Mbp), D2Mit126 (84 Mbp), D2Mit100 (106 Mbp), D2Mit107 (133 Mbp), D2Mit226 (163 Mbp), D2Mit200 (179 Mbp) (http://www.ensembl.org) [6]. Based on the genotyping, we selected the mice and crossed them to the mice of the subsequent generations. Finally, the male and female mice that were checked for homozygous genotypes on chromosome 2 were intercrossed to establish congenic mouse strains.

2.4. Bacterial strains and culture conditions

Streptococcus mutans JC-2 (serotype *c*), which is resistant to streptomycin (200 μg/mL), was cultured in brain-heart infusion (BHI; Difco, Detroit, MI, USA) under an atmosphere of 95% N_2 and 5% CO_2 at 37 °C for 18 h. The bacterial cells were collected by centrifugation and suspended in 5 mL of BHI broth. [12]

2.5. Dental caries induction

The mice were weaned at 21 days and fed on Diet#2000 including 56% sucrose (CLEA Japan Inc., Tokyo, Japan). Each mouse was infected for 7 days with *S. mutans* by inoculating a bacterial solution of 10^9 colony-forming units into the oral cavity. Bacterial colonization on the dental surface was confirmed on the 28th day by placing a sterilized swab into the mouth and culturing the oral swab material on Mitis Salivarius agar plates containing streptomycin (200 μg/mL). At 49 days of

age, the mice were euthanized under CO_2. Their mandible bones were removed, and the soft tissue was dissolved at 42 °C for 24 h in 2% KOH. [13]

2.6. Calculation of the caries score

Both sides of the molars on the mandible were examined by microscopy and micro-computed tomography (micro-CT) (Rigaku, Tokyo, Japan). The micro-CT images were taken at 90 kV and 200 μA for 3 min. The depth and extent of dental caries were examined using three parts of the images (buccal, central, and lingual). The caries score was evaluated according to the modified Keyes method applicable to mice [13, 14]. The maximum scores were as follows: 4 points at the first and second fissures in the mandibular first molar (M1), 2 points at the first fissure in the mandibular second molar (M2), and 1 point at the third fissure in M1 and first fissure in mandibular third molar (M3). The caries scores were calculated by adding the scores of M1, M2, and M3 on the left and right mandibules.

2.7. Measurement of the volume of stimulated saliva secretion

The volume of stimulated saliva secretion was measured as reported earlier [8]. Mice of all strains studied herein (C57BL/6Slc, C3H/HeSlc, B6-Chr.2^{C3H}, and 3 congenic mouse strains) were intraperitoneally injected with pilocarpine (0.05 μg/100 g body weight) at 49 days of age. After injection, saliva was collected from the oral cavity of each mouse for 30 min using a pipette. The total saliva was weighed and statistically tested for significant differences by ANOVA.

2.8. Measurement of enamel hardness

Enamel hardness was measured as previously reported [8]. The mandibles were extracted from the mice of each strain, and the soft tissue was manually removed. Mandibules were mounted on slide glass with composite resin. The enamel hardness of the lingual side of the first molar on the left mandible was measured with a Dynamic Ultra Micro Hardness Tester (Shimadzu, Kyoto, Japan). Enamel hardness was measured at three sites on the left first molar in one mouse of each strain, and the average was calculated.

3. Results

3.1. Generation of congenic mouse strains

We generated three types of congenic mouse strains: one with a C3H/HeSlc-derived interval

between D2Mit226 and D2Mit200 in a C57BL/6Slc genetic background (congenic 1); the second, with a C3H/HeSlc-derived interval between D2Mit126 and D2Mit226 (congenic 2); and the third, with a C3H/HeSlc-derived interval between D2Mit126 and D2Mit100 (congenic 3) (Fig. 1).

3.2. Calculation of the caries score in each strain

The mandibles of each mouse strain were observed by microscopy and micro-CT (Fig. 2). The means of the caries scores were 3.6 ± 4.9 in B6-Chr.2^{C3H}, 23.4 ± 1.1 in congenic 1, 14.8 ± 1.8 in congenic 2, and 27.2 ± 4.3 in congenic 3. They differed significantly between B6-Chr.2^{C3H} and the congenic strains, and between each congenic mouse strain (Fig. 3). As control groups, C57BL/6Slc and C3H/HeSlc were also infected with *S. mutans* under the same conditions. The means of the caries scores were 47.6 ± 7.9 in C57BL/6Slc and 0.2 ± 0.4 in C3H/HeSlc. These results corresponded with those of earlier studies [4, 7], and the difference was significant at $p < 0.05$, as determined by Tukey's test.

3.3. Measurement of stimulated saliva secretion volume

The salivary secretion volumes were 0.05 ± 0.03 g in C57BL/6Slc, 0.24 ± 0.05 g in C3H/HeSlc, 0.14 ± 0.05 g in B6-Chr.2^{C3H}, 0.15 ± 0.02 g in congenic 1, 0.19 ± 0.03 g in congenic 2, and 0.15 ± 0.02 g in congenic 3. The salivary secretion volume between congenic 2 and C3H/HeSlc were not significantly different (Fig. 4).

3.4. Measurement of enamel hardness

Enamel hardness of the first molar on the left side is shown in Fig. 5. Enamel strength values were 221.57 ± 12.37 in C3H/HeSlc, 231.87 ± 40.94 in C57BL/6Slc, 240.77 ± 25.49 in B6-Chr.2^{C3H}, 202.74 ± 31.04 in congenic 1, 215.53 ± 60.04 in congenic 2, and 228.81 ± 34.76 in congenic 3. The means of the strains did not differ significantly from one another (Fig. 5).

4. Discussion

The occurrence of dental caries is known to be affected by a complex interplay of environmental and host factors. For many years, the etiology of dental caries has been studied in terms of both environmental factors, such as diet, bacterial flora, oral hygiene, and fluoride exposure, as well as host factors, such as salivary flow, salivary component, and tooth structure. However, the genetic factors influencing dental caries susceptibility remain poorly understood. The identification of the

factors influencing susceptibility to dental caries will enable the prediction of the risk of dental caries occurrence in childhood.

Genetic variation in several candidate genes has been shown to be involved in dental caries susceptibility. For example, variations in enamel-formation genes [15], such as *AMELX* [16], *ENAM* [17], *AMBN* [18], and *TUFT1* [19]; amelogenesis genes, such as *ACTN2* [20]; genes involved in the immune response, such as *DEFB1* [21] and *TRAV4* [22]; and genes affecting taste preference, such as *TAS2R38* and *TAS1R2* [23], have been associated with caries susceptibility in human studies.

The importance of genetic factors in dental caries has also been demonstrated in animal model studies. Nariyama et al [5] reported that the genes with major effects on susceptibility to dental caries were on chromosomes 1, 2, 7, and 8 by QTL analysis, with a significant QTL located on chromosome 2. Based on these results, Nomi et al [6] established a consomic strain, termed B6-Chr.2^{C3H}, in which the C3H/HeSlc-derived interval of chromosome 2 was replaced in a C57BL/6Slc genetic background. Orino et al [7] reported that B6-Chr.2^{C3H} was significantly less predisposed to dental caries than C57BL/6Slc and was not different from C3H/HeSlc in this aspect. In addition, Hiraki et al [8] reported that the stimulated saliva secretion volume of B6-Chr.2^{C3H} was higher than that of C57BL/6Slc but was not different from that of C3H/HeSlc. Thus, they suggested that the major genetic factor of dental caries susceptibility was located on chromosome 2, and the genes influencing salivary secretion influenced dental caries susceptibility.

Congenic mouse strains are produced by the repeated crossing of susceptible and resistant mice to introduce donor-derived chromosomal intervals, including resistance or susceptible genes, into a recipient genetic background. Previous studies have successfully identified the location of the causative genes using congenic mouse strains [10, 24, 25]. Therefore, congenic mouse strains are very useful to localize the region of candidate genes for disease. In this study, we successfully generated and bred three types of congenic mouse strains.

The risk of dental caries to the enamel occurs because of the formation of a surface layer on the tooth. Previous studies have reported enamel hardness to be associated with a high risk of dental caries susceptibility, and that genetic variation in enamel-formation genes, such as *AMELX*, *AMBN*, and *ENAM*, contributes to high dental caries susceptibility in humans [15, 26]. This study showed that enamel hardness was not significantly different among the studied strains. We found that enamel hardness did not affect dental caries susceptibility in congenic mice we generated, suggesting that the gene(s) responsible might not be localized on mouse chromosome 2 [27, 28, 29].

The caries score of congenic 2 mice was markedly lower than those of both congenic 1 and congenic 3 mice. This implied that the genes associated with caries resistance might be located

between D2Mit126 and D2Mit226 loci. Furthermore, the caries scores of congenic 1 and congenic 3 mice were lower than that of C57BL/6Slc mice. It is possible to locate the genes associated with caries resistance between D2Mit226 and D2Mit200 loci and between D2Mit126 and D2Mit100 loci. Therefore, owing to the overlap of the C3H/HeSlc-derived intervals of congenic 1 and congenic 3, congenic 2 had the lowest caries score. These results agreed with those of a previous study [5] and could demonstrate *in vivo* the identification of the effects of mouse chromosome 2 on dental caries susceptibility, using congenic mouse strains.

In humans, stimulating the flow of saliva by chewing sugar-free gum has been shown to reduce the development of dental caries [30]. Saliva is the most important caries-preventive factor; it not only aids the clearance of oral debris by a flushing effect but also protects teeth by a neutralizing effect [31]. Our study showed that the salivary secretion volume of congenic 2 was not significantly different from that of C3H/HeSlc. We suggested that the major gene(s) related to salivary secretion might be located between D2Mit126 and D2Mit226, and that salivary secretion might be associated with dental caries susceptibility.

Hiraki et al [8] reported that *Slc24a3*, located on mouse chromosome 2, is in myoepithelial and ductal cells of salivary grounds and might be concerned with sodium reabsorption and calcium and potassium secretion during salivary secretion. *Slc24a3* is located in the C3H/HeSlc-derived interval of congenic 2, which is D2Mit126 to D2Mit226. Therefore, *Slc24a3* might be involved in dental caries susceptibility [8]. However, the caries score and salivary secretion volume of congenic 2 mice did not decrease similarly to that of B6-Chr.2^{C3H} mice. It appears likely that dental caries was caused by multiple genetic factors rather than a single genetic factor.

In this study, we were unable to breed congenic mice in which a C3H/HeSlc-derived interval including D2Mit237 and D2Mit90 was present in a C57BL/6Slc genetic background, although we could obtain male and female congenic mice as described above. Thus, congenic mice having the C3H/HeSlc-derived interval including D2Mit237 and D2Mit90 might have low reproduction ability.

In conclusion, several genes associated with caries resistance could be localized between D2Mit126 and D2Mit226 loci. Salivary secretion volume was one of the most important factors associated with dental caries susceptibility, and the candidate genes for salivary secretion might be located in the same region. To localize the locus responsible for dental caries susceptibility more precisely, we will need to generate and test congenic mouse strains with smaller C3H/HeSlc-derived intervals. We propose that the identification of the factors influencing susceptibility to dental caries will enable the prediction of the risk of dental caries occurrence in childhood and the development of

prophylactic measures against dental caries.

Conflicts of Interest

The authors declare that no conflict of interest exists.

Acknowledgments

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Figure captions

Figure 1 Diagram of mouse chromosome 2 in the three congenic mouse strains we generated. Marker positions on the map were obtained from Ensembl (http://www.ensembl.org). C3H/HeSlc-derived intervals are shown as black squares and C57BL/6Slc, as white squares.

Figure 2 Images observed by microscopy and micro-CT in each strain. The micro-CT images show buccal, central, and lingual slices. Arrowheads indicate dental caries.

Figure 3 Comparison of caries scores among C57BL/6Slc, C3H/HeSlc, B6-Chr.2^{C3H}, and three types of congenic strains. The number of each strains is five. Bars indicate the caries score in each strain. The caries score of B6-Chr.2^{C3H} was significantly lower than that of all the congenic strains; furthermore, the caries score of congenic 2 mice was significantly lower than those of congenic 1 and congenic 3 mice. The caries score of C57BL/6Slc showed a significant difference among that of all the mouse strains we used in this study, and the caries score of C3H/HeSlc showed a significant difference among those of C57BL/6Slc and all the congenic strains. * Significant differences at $p < 0.05$, as determined by ANOVA.

Figure 4 Comparison of the volumes of stimulated salivary secretion for 30 min among C57BL/6Slc, C3H/HeSlc, B6-Chr.2^{C3H}, and three types of congenic strains. The number of each strains is five. Bars indicate stimulated salivary secretion volumes in each strain.

Figure 5 Comparison of enamel hardness among C57BL/6Slc, C3H/HeSlc, B6-Chr.2^{C3H}, and three types of congenic strains. The number of each strains is three. Bars indicate enamel hardness by dynamic hardness in each strain.

Figure 1

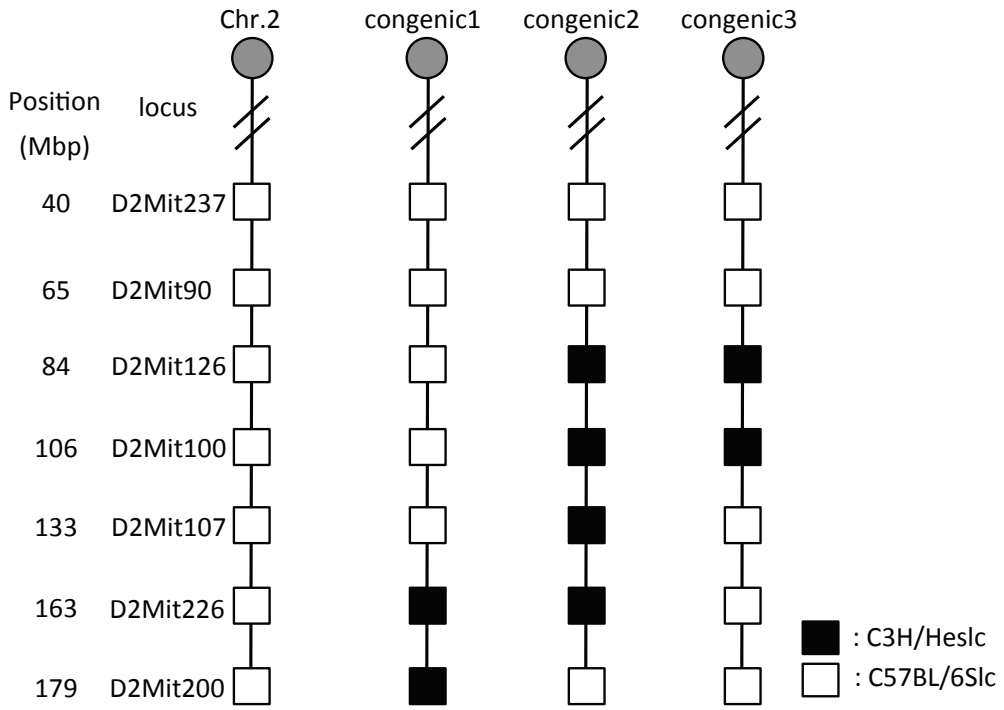

Chr.2
congenic1
congenic2
congenic3
Position
(Mbp)
locus
40
D2Mit237
65
D2Mit90
84
D2Mit126
106
D2Mit100
133
D2Mit107
163
D2Mit226
179
D2Mit200
: C3H/Heslc
: C57BL/6Slc

Figure 2

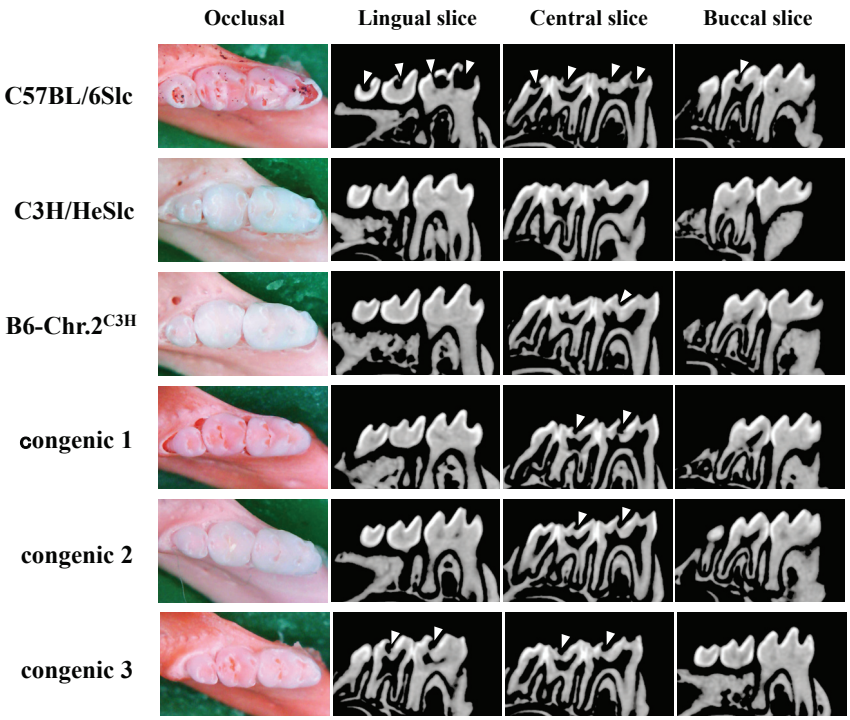

Occlusal
Lingual slice
Central slice
Buccal slice
C57BL/6Slc
C3H/HeSlc
B6-Chr.2C3H
congenic 1
congenic 2
congenic 3

Figure 3

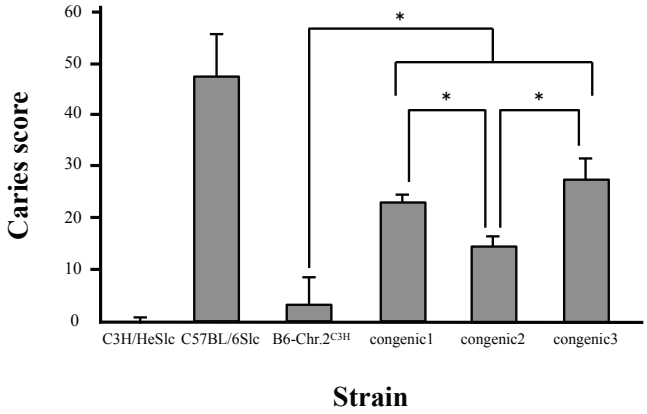

Caries score
60
50
40
30
20
10
0
*
*
*
C3H/HeSlc
C57BL/6Slc
B6-Chr.2C3H
congenic1
congenic2
congenic3
Strain

Figure 4

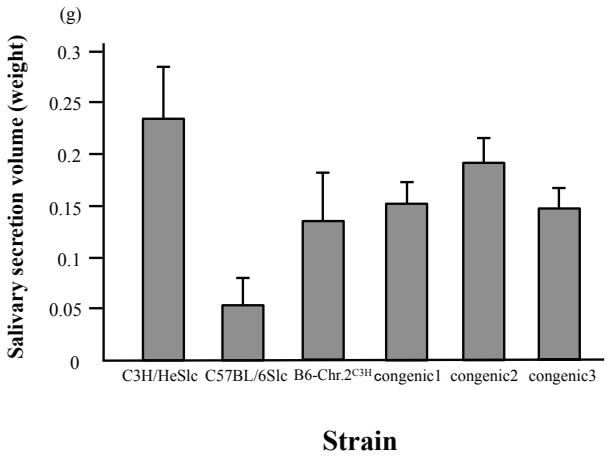
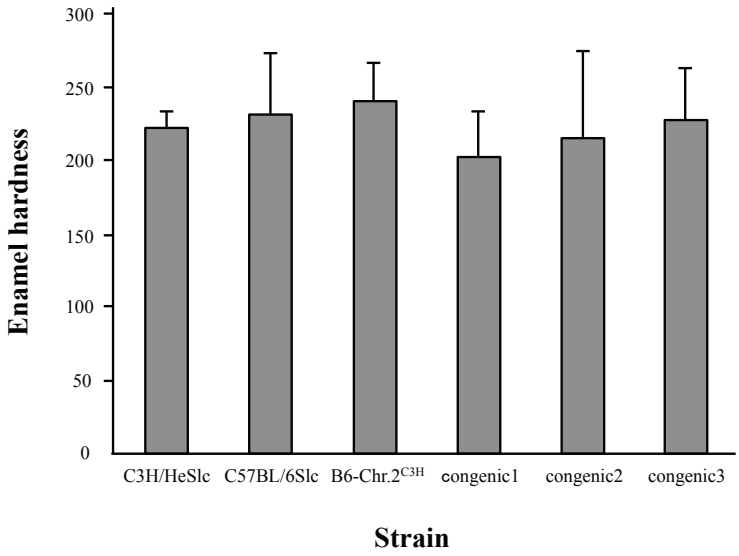

(g)
Salivary secretion volume (weight)
0.3
0.25
0.2
0.15
0.1
0.05
0
C3H/HeSlc
C57BL/6Slc
B6-Chr.2C3H congenic1
congenic2
congenic3
Strain

Figure 5


Enamel hardness
300
250
200
150
100
50
0
C3H/HeSlc
C57BL/6Slc
B6-Chr.2C3H
congenic1
congenic2
congenic3
Strain