Development of a High-Throughput Diagnosis Method for Detecting the *ALDH2* Gene Using Fingernail DNA

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Abstract: A clinical method for effective genetic screening of the aldehyde dehydrogenase 2 (ALDH2) gene was developed, using the fingernail as a source of DNA material. A highly effective protease that could solubilize fingernail keratin and inactivate any DNase co-existing in the tissue was obtained by cloning and sequencing the gene for alkaline protease from *Bacillus alcalophilus*, followed by expression of the gene in *Bacillus subtilis.* The amino acid sequence of MIB029 protease contained common regions found in four other subtilisin-like proteases. In the fingernails of 113 female university students (average age 20.8 ± 0.7 years; body mass index, 20.4 ±1.6), ALDH2 frequency was 0.66 for the typical Glu homozygote, 0.32 for the heterozygote (Glu487Lys), and 0.020 for the atypical Lys homozygote. Through a questionnaire, it was found that the subjects had not previously received information regarding the relationship between their genetic background and consumption of alcoholic beverages. We found that the genetic single nucleotide polymorphism (SNP) background to alcoholism can be easily detected by collecting fingernails, which is convenient for subjects or patients.

Keywords: ALDH2 gene detection, genetic diagnosis, keratinolytic, *Bacillus,* alkaline protease.

1. INTRODUCTION

The symptoms of lifestyle-related disease are generally caused by interactions between hereditary predisposition and environmental factors [1, 2]. Although hereditary predisposition toward diseases such as alcoholism cannot be altered, individuals should be educated about their hereditary predisposition [3]. Aldehyde dehydrogenase 2 (ALDH2; EC 1.2.1.10) is a key enzyme involved in alcohol metabolism. A single-nucleotide polymorphism (SNP), Glu487Lys in the *ALDH2* gene (rs671: 135 bp), is commonly found in Orientals, and is responsible for alcohol intolerance [4-7]. The individual acceptable alcohol consumption level is affected by the individual capacity for metabolizing acetaldehyde, a harmful product of alcohol metabolism [4, 5, 7]. In ALDH2 deficiency, one codon on exon 12 of the *ALDH2* gene (the 487th codon in the whole gene) is GAA in individuals with the typical allele associated with high tolerance to alcohol (*ALDH2*1*) and AAA in those with the atypical allele associated with less tolerance to alcohol (*ALDH2*2*), with the corresponding amino acid substitution of lysine for glutamate. The single amino acid substitution by this single base substitution results in a dramatic loss of ALDH activity. Individuals heterozygous for the typical and atypical alleles (*ALDH2*1/ALDH2**2) possess inherently reduced ALDH2 activity [4, 5].

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An ethanol patch test [8] and questionnaire method are currently available as screening methods for alcohol sensitivity in minors. Although the ethanol patch test provides rapid results, it has disadvantages, including skin irritation potential and variability in assessment results depending on the subjects' physical conditions. In contrast, although the questionnaire method is performed using the Tokyo University ALDH2 Phenotype Screening Test (TAST), which is developed by modifying the international standard method the Aldolescent Alcohol Involvement Scale [9] for use in Japanese people [10], it is thought that it cannot be applied to minors or nondrinkers because it contains many questions that cannot be answered by individuals who have never drunk alcohol.

Fingernail sampling can be easily carried out at any time, in any location, and from any person, and handling of samples is simple. Since samples can be kept at room temperature, sampling can be done by family members and delivered through the postal service.

However, fingernails are composed of hard and insoluble keratin, making it difficult to extract DNA from the nail tissues. Moreover, the tissues contain many DNases that decrease the DNA content during preparation [11].

In the present study, we used alkaline serine protease from *Bacillus alcalophilus* subsp. *halodurans* MIB029 because it is highly active at pH 11.0 and 70°C [12]. Additionally, the enzyme functions efficiently in the presence of EDTA, which effectively eliminates

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contaminating DNases in the sample. To determine the current state of alcoholic beverage consumption in minors, fingernail sampling and ethanol patch test were conducted, and any subjects with a history of alcohol consumption were also asked about their intake and frequency of alcoholic beverage consumption to investigate potential associations with ALDH2 hereditary predisposition.

Our goal was to develop a gene expression system for obtaining large amounts of the alkaline protease. We propose a simple diagnostic method using nail clippings that can be used to determine various genotypes associated with lifestyle-related diseases.

2. MATERIALS AND METHODS

2.1. Bacterial Strains and Cell Culture

Bacillus alcalophilus subsp. *halodurans* MIB029 (formerly designated as KP1239) was cultured in medium I, which contained 0.3% KH_2PO_4, 0.5% yeast extract, 0.5% peptone, and 0.5% $NaHCO_3$ (pH 8.3), as described previously [13]. *Bacillus subtilis* DB104 cells bearing each expression plasmid were cultivated at 30°C on LB medium plates containing kanamycin (10 μg/mL).

2.2. Primers and Sequence Analysis

Genomic DNA for strain MIB029 was prepared using the method of Saito and Miura [13]. To amplify the alkaline protease gene, two primers were synthesized: forward primer, 5′-aggacatatgGCGCAA TCAGTGCCATGGGGAATTAG-3′ and reverse primer, 5′-aggaaagcTTAGCGTGTTGCCGCTTCTGCATTGAC AAGGCGACACTTGAGCA AGCTGTT-3′.

PCR amplification was performed using a PCR kit (TaKaRa PCR Thermal Cycler TP600, Shiga, Japan) using 86.8 ng of MIB029 genomic DNA as template, 10 μL of 5×reaction buffer (Mg^{2+} plus), 4 μL each of 2.5 mM dNTP, 10 pmol of each primer, and 1.25 U of PrimeSTAR® HS DNA polymerase in a total volume of 50 μL. The mixture was subjected to 30 cycles of amplification: 98°C for 10 sec, 55°C for 5 sec, and 72°C for 45 sec, then 72°C for 5 min (1 cycle). The PCR product was purified using the TaKaRa Agarose Gel DNA Purification Kit Ver.2.0, and subsequently digested with both 15 U of *Pst*I and *Hind*III restriction enzymes.

The pNCM02 Vector (100 ng: TaKaRa) was digested at 37°C for 4 h with 15 U of *Pst*I and *Hind*III and purified using the Purification Kit Ver.2.0 (TaKaRa). Insert DNA (100 ng) was ligated to vector DNA (50 ng) at 16°C for 4 h. Next, 10 μL of the ligated mixture transformed into 100 μL *Escherichia coli* competent cells JM109 (TaKaRa Dalian Code No. D9052). A PCR fragment of the MIB029 gene was transferred into the host *Bacillus subtilis* DB104 and selected for on LB medium containing 10 μg/mL kanamycin. Expression was conducted in the same medium.

2.3. Stage 1 – Testing Human Nail Clippings as Useful Genetic Tools

Nail tissues collected from 20 volunteers were cut into pieces (1.0 × 2.0 mm), rinsed twice with 100 mL of deionized water, and washed with 30 mL of ethanol. Samples were dried and stored at room temperature until use. The specimen was found to be stable during storage for at least 6 months.

2.4. Enzyme Assays and Protein Measurement

A mixture (1.0 mL) of 100 mM glycine-NaOH buffer (pH 11), 5 mg nail clippings, and 100 mM EDTA was incubated at 70°C for 10–30 min, mixed with 1 mL of trichloroacetic acid, and boiled for 3 min prior to centrifugation at 7500 x*g* for 2 min at 25°C [12]. The A_{275} (1-cm light path) of the supernatant was measured and converted to an equivalent amount of tyrosine. One unit (U) of enzyme activity was defined as the amount of enzyme releasing 1 μmol of tyrosine equivalent/min. Protein concentration was assessed using the Bradford method with bovine serum albumin as the standard [12].

Table 1: Physical Characterisitics of Participants

Numer of subjects	113
Sex	females
Age, years	20.8 ± 0.7
Height (cm)	160.5 ± 6.7
Body weight (kg)	53.3 ± 7.6
Body mass index, kg/m^2	20.4 ± 1.6
Body fat percentage (%)	28.8 ± 5.6

Values are mean±standard deviation.

2.5. Stage 2 Diagnosis Studies Using Nail Clippings as Sources for the ALDH2 Gene

All of the following procedures were conducted with the approval of the research ethics committee of this

University. Informed consent was obtained from each of the 113 healthy female students (average age 20.8 ± 0.7 years; body mass index, 20.4 ± 1.6)) who stated that they had never suffered from alcoholism or alcohol abuse (Table **1**).

2.5.1. DNA Extraction from Fingernails

Each specimen prepared from nail clippings (5.0 mg) from the 113 participants was transferred into a 1.5-mL microcentrifuge tube containing 200 µL of 100 mM glycine-NaOH buffer (pH 11) and 100 mM EDTA and incubated for 30 min at 70°C. The DNA was dissolved in 10µl of TE buffer (pH 8.0).

2.5.2. Ethanol Patch Test

A piece of gauze soaked with 70% ethanol was applied directly to the participants' medial upper arm and removed after 7 minutes, and the color of the skin area in contact with the gauze was observed within 5 seconds. At 10 minutes after the removal of the gauze, the skin color was again observed [8]. If the skin was red immediately after the removal of the gauze, the participant was considered as having no ALDH2 activity, indicating alcohol intolerance. If the skin was not red immediately after the removal of the gauze but was red at 10 minutes, the participant was considered as having reduced ALDH2 activity, indicating low alcohol tolerance. If the skin color remained unchanged, the participant was considered as having normal ALDH2 activity, indicating high alcohol tolerance [6, 8].

2.5.3. Questionares to the Subjects About Alcohol Drinking

In accordance to the ethanol patch test, four questions were given to the participants who has drunken, as shown in Table **2**.

2.5.3. PCR Amplification of the ALDH2 Gene

The allele-specific ALDH2 gene was amplified using forward primer (F) and reverse primer R1 or reverse primer R2 [5]. R1 and R2 represent Glu at 487 and Lys at 487, respectively [4-7].

F: 5′-CAATTACAGGGTCAACTGCT-3′

R1: 5′-CCACACACTCACAGTTTTCTCTTC-3′

R2 : 5′-CCACACTCACAGTTTTCTCTTT-3′

A total of 50 µL PCR solution containing 1× PCR buffer, template DNA, 0.5 mM MgSO4, 0.2 mM dNTP, 0.4 mM forward primer, 0.4 mM reverse primer R1 or R2, and 1.25 KOD plus enzyme was pre-heated at 98°C for 1 min, and then subjected to 35 cycles of amplification by PCR as follows: 98°C for 20 sec, 60°C for 20 sec, and 72°C for 45 sec. After the cycle, final extension was carried out for 5 min at 72°C and the sample was stored at 4°C.

2.5.4. Agarose Gel Electrophoresis

A 5-µL sample of the PCR product was analyzed on a 3% agarose 21 gel [8]. Electrophoresis was carried out at 50 V for 30 min. DNA was visualized by staining with ethidium bromide [11].

3. RESULTS

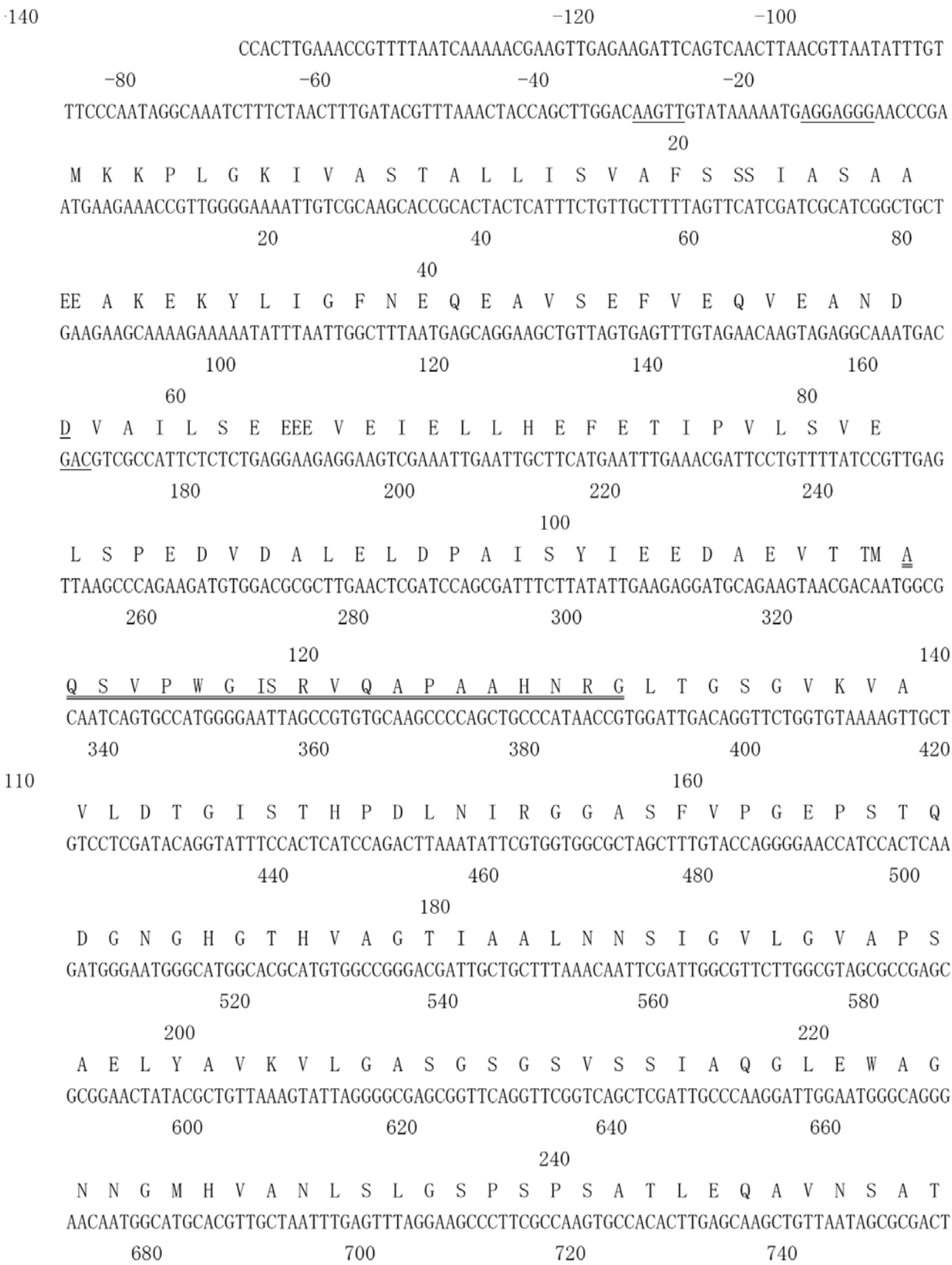
3.1. Gene Structure of MIB029 Protease

The gene encoding serine alkaline protease from *B. alcalophilus* subsp. *halodurans* MIB 029 is depicted in Figure **1**. The open reading frame was 1140 bases. The deduced amino acid sequence was a pre-pro-peptide of 111 residues followed by the mature protease consisting of 269 residues. The protein showed a molecular weight of 29,000 d and a sedimentation coefficient in water at 20°C of 3.3 *S*, as

Table 2: Questions of Habitual Drinking

Q1	How often do you have a drink containing alcohol?
	(0) Never (1) Monthly or less (2) Two to four times a month (3)Two to three times a week (4)Four times a week
Q2	How many drinks containing alcohol do you have on a typical day when you are drinking?
	(0) 1 or 2 (1) 3 or4 (2) 5 or 6 (3) 7 or 8 (4)10 or more
Q3	How often do you have six or more drinks on one occasion?
	(0) Never (1) Less than monthly (2) Monthly (3) Weekly (4) Daily or almost daily
Q4	How often during the last year have found that you were not able to stop drinking once you have started?
	(0) Never (1) Less than monthly (2) Monthly (3) Weekly (4) Daily or almost daily

In determining the response categories it has been assumed that one drink contains about 6 g alcohol.
Sum of individual item scores were recorded to determin types of drinking.


-140
-120
-100
CCACTTGAAACCGTTTTAATCAAAAACGAAGTTGAGAAGATTCAGTCAACTTAACGTTAATATTTGT
-80
-60
-40
-20
TTCCCAATAGGCAAATCTTTCTAACTTTGATACGTTTAAACTACCAGCTTGGACAAGTTGTATAAAAATGAGGAGGGAACCCGA
20
M K K P L G K I V A S T A L L I S V A F S SS I A S A A
ATGAAGAAACCGTTGGGGAAAATTGTCGCAAGCACCGCACTACTCATTTCTGTTGCTTTTAGTTCATCGATCGCATCGGCTGCT
20
40
60
80
40
EE A K E K Y L I G F N E Q E A V S E F V E Q V E A N D
GAAGAAGCAAAAGAAAAATATTTAATTGGCTTTAATGAGCAGGAAGCTGTTAGTGAGTTTGTAGAACAAGTAGAGGCAAATGAC
100
120
140
160
60
80
D V A I L S E EEE V E I E L L H E F E T I P V L S V E
GACGTCGCCATTCTCTCTGAGGAAGAGGAAGTCGAAATTGAATTGCTTCATGAATTTGAAACGATTCCTGTTTTATCCGTTGAG
180
200
220
240
100
L S P E D V D A L E L D P A I S Y I E E D A E V T TM A
TTAAGCCCAGAAGATGTGGACGCGCTTGAACTCGATCCAGCGATTTCTTATATTGAAGAGGATGCAGAAGTAACGACAATGGCG
260
280
300
320
120
140
Q S V P W G IS R V Q A P A A H N R G L T G S G V K V A
CAATCAGTGCCATGGGGAATTAGCCGTGTGCAAGCCCCAGCTGCCCATAACCGTGGATTGACAGGTTCTGGTGTAAAAGTTGCT
340
360
380
400
420
110
160
V L D T G I S T H P D L N I R G G A S F V P G E P S T Q
GTCCTCGATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTTGTACCAGGGGAACCATCCACTCAA
440
460
480
500
180
D G N G H G T H V A G T I A A L N N S I G V L G V A P S
GATGGGAATGGGCATGGCACGCATGTGGCCGGGACGATTGCTGCTTTAAACAATTCGATTGGCGTTCTTGGCGTAGCGCCGAGC
520
540
560
580
200
220
A E L Y A V K V L G A S G S G S V S S I A Q G L E W A G
GCGGAACTATACGCTGTTAAAGTATTAGGGGCGAGCGGTTCAGGTTCGGTCAGCTCGATTGCCCAAGGATTGGAATGGGCAGGG
600
620
640
660
240
N N G M H V A N L S L G S P S P S A T L E Q A V N S A T
AACAATGGCATGCACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCAAGTGCCACACTTGAGCAAGCTGTTAATAGCGCGACT
680
700
720
740

(Figure 1). Continued.

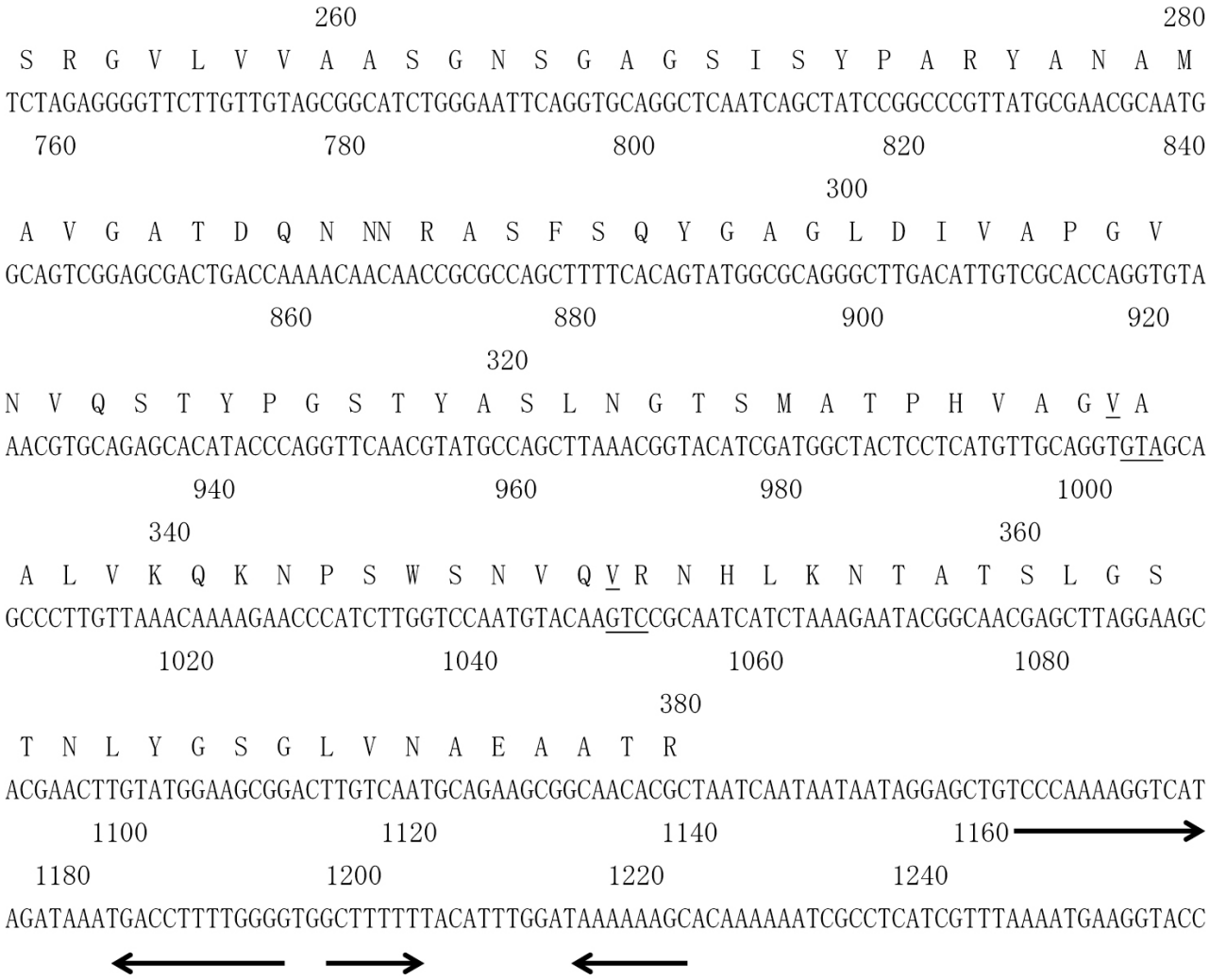


Figure 1: Nucleotide sequence of the gene for MIB029 protease and deduced amino acid sequences.

Both stands were sequenced from several independent overlapping fragments. The putative SD sequence are dotted underlined. The putative transcriptional terminator is shown by the arrows (→ ←). The putative transcriptional terminator is shown by the arrows at Ala334. The N-terminal sequence of 20 residues of mature enzyme deduced from DNA sequence is double underlined. The amino acids Glu32, Valine62 and Valine351 as shown in boxes are different with those of *B*. sp 221 protease (15).

demonstrated for the strain MIB029 [12]. The amino acid sequence of the mature MIB029 protease shared moderate homology with that of subtilisin-like protease (Figure **2**).

MIB029 protease showed 99.8% identity with *B. alcaophillus* sp. 221 protease [14], 62.0% identity with Carlsberg [16], 99.8% with *B*. sp. KSM-K16 protease [16], and 61.5% with subtilisin BPN' [17]. The regions containing the catalytic triad composed of Asp32, His62, and Ser215 of subtilisin BPN' were conserved in MIB029 protease as well as in other subtilisin-type enzymes (Figure **2**). The cloned MIB029 enzyme serologically cross-reacted with the protease either from the mother strain MIB029 or *B*. *alcaophilus* sp. 221 [15], but not with subtilisin Carlsberg [16] or BPN' [17] (Figure **3**).

3.2. Properties of Cloned Protease

Protease activity in the supernatant of *B. subtilus* DB104 bearing the alkaline protease gene reached a maximum level after 134 h of culture, producing 1.21 U/mL of protein (82.2 U/mg). The protease was most active at 70°C in the presence of 100 mM EDTA, whereas melon protease and proteinase K showed 80% and 65% activity compared with MIB029 enzyme (Figure **4**). DNA extraction efficiency at 70°C was estimated to be 75%, which was higher than that for melon protease (69.5%) and proteinase K (60.0%) [11].

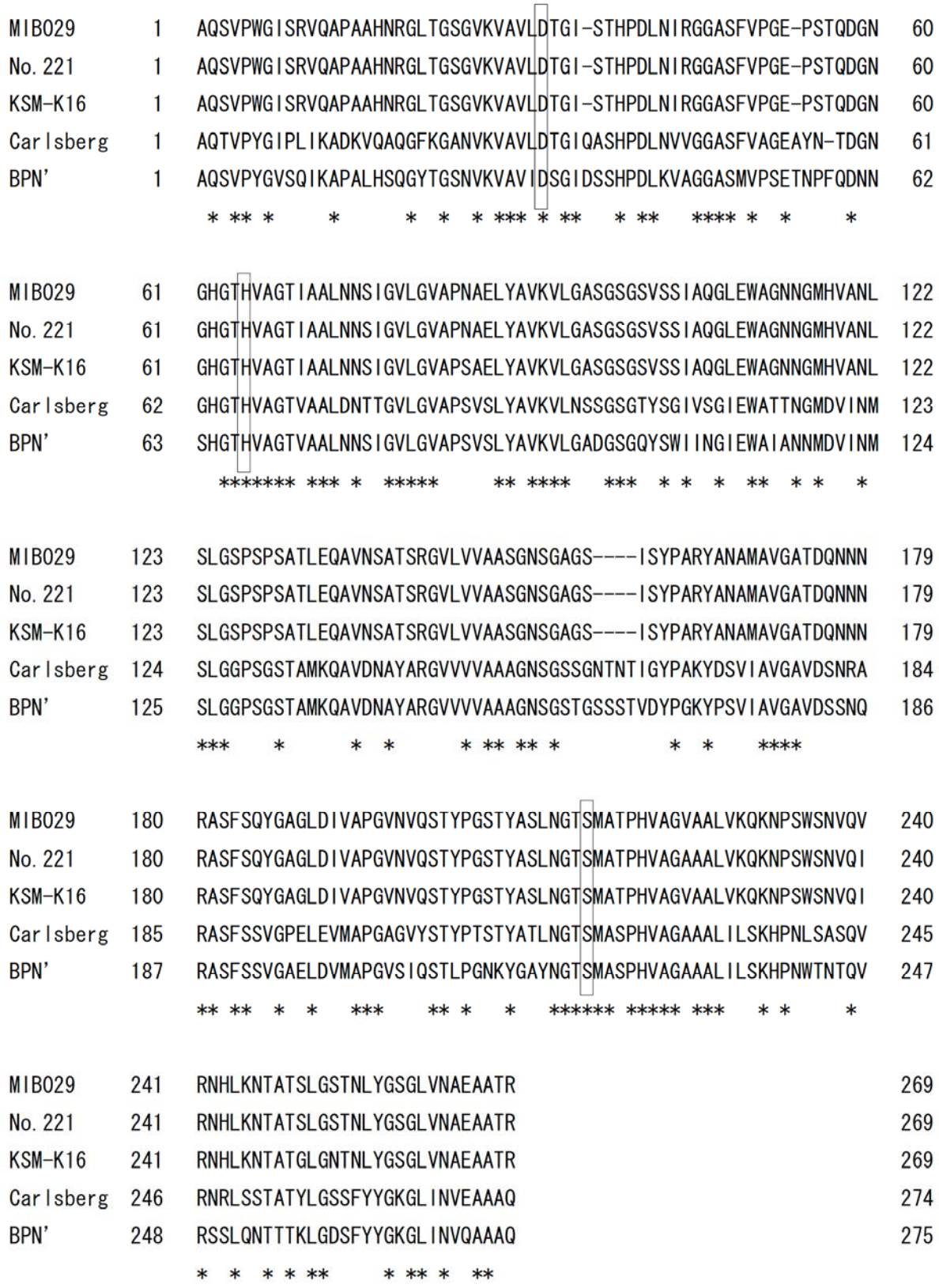


Figure 2: Multiple sequence alignment of MIB029 protease and four subtilisin-like enzymes.

Each amino acid sequence is numbered from the N-terminal residues of the mature enzyme and is indicated by the single-letter codes. A common catalytic trial of the three amino acids, Asp32, His62 and Ser215 is enclosed in the box. Identical amino acid residues for all five proteases are marked with *asterisks* under the sequences. MIB029, MIB029 protease; 221 *Bacillus* sp.21 protease [14]; KSM- K16 protease, *Bacillus* sp. KSM- K16 [15]; BPN', Carlsberg, subtilisin Carlsberg [16]; BPN', subtilisin BPN' [17].

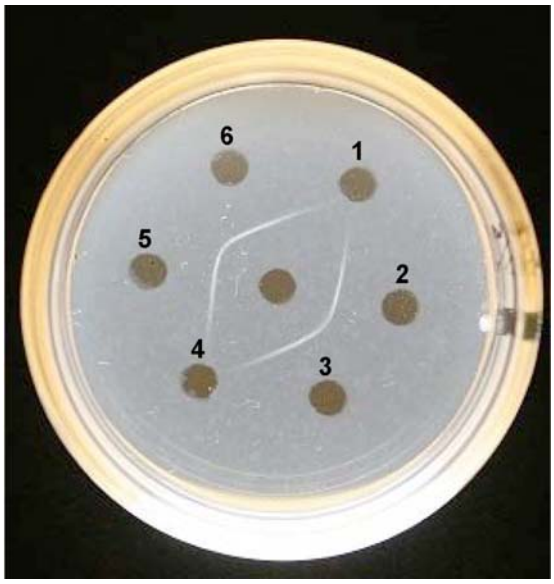


Figure 3: Double immunodiffusion.

Rabbit antiserum (5µl) prepared against *B. alcalophilus* sp. *halodurans* MIB029 (formerly designated as KP1239: reference 13) was added to center well (3 mm diameter) in plate consisting of 1.5% agarose and 25 mM borate/0.1% NaN_3/1 mM PMSF. Wells 2 and 5 contained pure preparation of MIB029 protease (1.0 U); wells 3 and 6 contained the cloned protease (1.0 U); well 1 and 4 contained 10-fold concentrated culture supernatant of subtilisin BPN' (1.0 U) or subtilisin Carlberg (1.3 U). Each enzyme sample used was treated at 4°C for 1 h with 1 mM PMSF before application on the plates. Immunodiffusion on plate was carried out at at 4°C for 24 h. The plate was soaked in 0.85% NaCl/0.1% NaN_3 at 25°C overnight and photographed. One microliter of the antiserum completely precipitated 0.12 U of KP1239 enzyme.

DNA purity was estimated to be 1.790 as $A_{260/280}$, that is identical to the value observed for *Cucumis melo* (data not shown).

3.3. Application of MIB Protease for Detecting the ALDH2 Gene in Fingernail Tissues from Female Students and Alcohol Use Disorder Identification Test

The distribution of the ALDH2 genotype in the subjects (n = 113) was 0.66 for the typical Glu homozygote ALDH2*1/*1, 0.32 for the heterozygote (Glu487Lys) ALDH2*1/*2, and 0.020 for the atypical Lys homozygote ALDH2*2/*2 (Figure **5**). ALDH2 composed of 499 amino acid residues and its gene showed that the Glu489Lys polymorphism [4-7]. ALDH2*1/*1homozyte is highly tolerant to alcohol, the ALDH2*1/*2 (Glu 489Lys) heterozygote is less tolerant to alcohol, and the ALDH2*2/*2 homozygote is intolerant to alcohol.

The alcohol disorder identification test [22] was carried out for all subjects using a health and lifestyle questionnaire, if they have drunk alcohol bebarages (Table **3**). The questionnaire was adjusted to the levels of Japanese drinking habits and lifestyles."Chance drinkers" and "past drinkers" were found to have ALDH2*1/*1 and ALDH2*1/*2, but not ALDH2*2/*2. "Non-drinkers" showed the genotype ALDH2*2/*2, indicating intolerance to alcohol. Equivocal evidence of associations with genotypes was found, although this

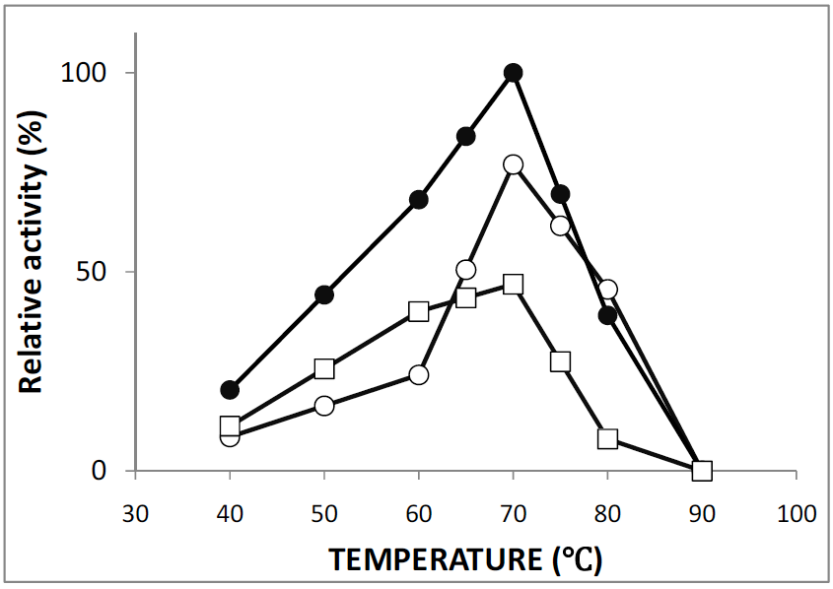


Figure 4: Effect of temperature on the activity of MIB029 protease.

The activity of MIB029 protease (●) was measured as in Enzyme assay, Materilas and methods, except that the incubation temperature was changed, as shown in the figure. The activity was compared with melon protease (○) and proteinase K (□), respectively. The activity observed at 4°C was defied as 100%.

Data are presented as an average of 3 data points, with each value within ±5% of the average value.

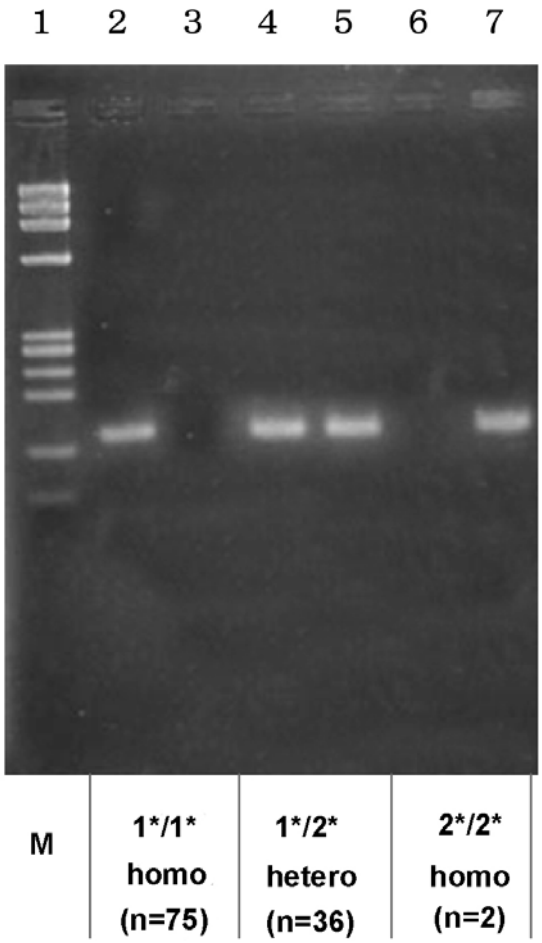


Figure 5: Electrophoresis pattern of the PCR product of a 135 bp DNA fragment containing ALDH2 exon 12 from human nail clippings.

Lane 1, DNA fragment markers: *Hae*III digest of φX174 DNA, consisting of 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 base pairs; lanes 2 and 3, 135 bp DNA fragments for homozyte 1* and 1*, lanes 4 and 5, 135 bp DNA fragments for heterozyte 1* and 2*; lane 6 and 7, 135 bp DNA fragments for homozyte, 2* and 2*. Lane 2, using FR1; Lane 3, using RF2; lane 4, using FR1; lane 5, using FR2; lane 6, using FR1 and lane 7, using FR2.

result was based only on responses from the participants with a history of alcohol consumption (Table **3**). Participants with the typical homozygous genotype (*1/*1), who were highly tolerant to alcohol, demonstrated essentially unchanged skin color after the ethanol patch test, indicating a clear positive correlation with the genotype (93%; Table **4**). A positive reaction with skin flushing was observed in 43 (93%) of 46 participants having the intermediate heterozygous genotype with weak enzymatic activity (*1/*2) and 3 (100%) of 3 participants having the mutant homozygous genotype (*2/*2), who were intolerant to alcohol. These results were consistent with the genotypes.

Table 3: Behavior Characterisitics of Participants to Alcohol Bevarages

Subjects (n = 113)	Number	Number of ALDH2 genotype	
Non-drinker*	2	2*2* = 2	
Chance drinker**	88	1*1* = 55	1*2* = 33
Past drinker***	23	1*1* = 15	1*2* = 8

*Non-drinker: 0 times a week.
**Chance drinker: less than 4 times a week.
***Past drinker: non-drinker but has drunk in the past.

Table 4: The Subjects' Ethanol Patch Test Results According to Genotype

Skin reaction	Genotype		
	*1/*1	*1/*2	*2/*2
Negative	88	3	0
Positive	7	43	3
Total	95	46	3

$\chi^2 = 104.065$, $p<0.00001$.
Negative: not changed.
Positive: changed to red.

DISCUSSION

Subtilisin-like proteases are generally bacterial in origin and secreted extracellularly for the purpose of scavenging nutrients [18-21]. They are highly sensitive towards phenyl methyl sulfonyl fluoride and diisopropyl-fluorophosphate, but not to chelating agents such as EDTA. Thus, we employed MIB029 protease to extract DNA from nail clippings containing strong DNases. pNCMO2 used in the present study is a shuttle vector for *B. subtilis* and *E. coli* . Therefore, we constructed the plasmid in *E. coli* and then introduced the plasmid into *B. subtilis* for protein expression. Using standard molecular cloning techniques, keratiolytic activity was successively elevated by 5-fold compared with the mother strain MIB029.

Fingernails, which are composed of the strong protein keratin, are produced in living skin cells in the fingers. The free edge of the nail extends past the finger and beyond the nail plate. There are no nerve endings in the nail. The growing part of the nail remains under the skin at the proximal end. Thus, we selected nail clippings as a tool for extracting genetic material. No subjects refused to provide nail clippings as DNA specimens. Person with ALDH2*1 homozygote have a higher acceptable alcohol consumption level, they are more predisposed to alcoholism or many other alcohol-related diseases. The ALDH2*2 gene, which almost

half of the Oriental population carry, appears to provide a significant advantage in that it leads to the prevention of the development of such diseases due to excessive alcohol consumption.

Our results demonstrate that cloned protease works more actively at pH 11.0 for extracting DNA from nail clippings than the protease of either *Cucumis melo* or proteinase K. The purity of DNA materials obtained using MIB029 protease was the same as that obtained for *Cucumis melo* protease [11]. Moreover, MIB029 protease has several advantages over than the fruit enzyme: for one, there is no seasonal limitation for obtaining the fruit; in addition, the samples show higher consistency in activity levels.

When our genetic diagnostic method using nail clippings was compared with the alcohol patch test and TAST, we encountered no disadvantages such as skin irritation or allergic symptoms among our subjects, unlike in the alcohol patch test. In addition, no variability was observed in the assessment results depending on the subjects' physical conditions at examination, as seen in the adolescent female subjects. The TAST contains many questions that are assumed to be answered by individuals with a history of alcohol consumption and thus are often inappropriate for minors in terms of its content [3, 4]. Although behavioral characteristics related to alcohol consumption were investigated only in the participants with a history of alcohol consumption, no results consistent with genotypes were obtained (Table **4**), probably owing to the influence of the participants' daily lifestyles rather than their constitutional predisposition to drinking habits.

Our diagnostic method, which uses easily collected fingernail samples as a source of DNA and enables assessment on the basis of resultant genotypes, may be considered as one of the most appropriate tools for screening alcohol sensitivity in minors, as it does not rely on the examiner's subjective assessment and is less affected by the subject's mental status.

In summary, the public should be educated as early as possible during the junior/high school years [3, 22-25]. The ratio of three genotypes for ALDH2 shown in Figure **5** indicates the typical ratio to Mongoloids concerning to alcohol tolerance [1, 4, 5]. Racial differences in alcohol metabolism are known to exist, which appear to be substantially influenced by genetic factors. The proportion of people with low alcohol-metabolizing activity is greater in Asians than in Caucasians [1]. We are currently examining the ALDH2 genotype both in university students in North America and in residents of Mwanza, Tanzania.

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REFERENCES

[1] Yanagisawa Y, Munkhtulga L, Nakayama K, *et al.* Diversity in genes responsible for lifestyle-related diseases in Asia-Pacific region. Asia Pac J Public Health 2008; 20(Suppl): 257-61.

[2] Enoch MA. The influence of gene-environment interactions on the development of alcoholism and drug dependence. Curr Psychiatry Rep 2012; 14(2): 150-8. http://dx.doi.org/10.1007/s11920-011-0252-9

[3] Tarter RE, Kirisci L, Clark DB. Alcohol use disorder among adolescents: impact of paternal alcoholism on drinking behavior, drinking motivation, and consequences. Alcohol Clin Exp Res 1997; 21(1): 171-8. DOI:10.1111/j.1530-0277.1997.tb03745.x

[4] Hashimoto Y, Nakayama T, Futamura A, *et al*. Relationship between genetic polymorphisms of alcohol-metabolizing enzymes and changes in risk factors for coronary heart disease associated with alcohol consumption. Clin Chem 2002; 48(7): 1043-8.

[5] Harada S, Agarwal DP, Goedde HW. Aldehyde dehydrogenase deficiency as cause of facial flushing reaction to alcohol in Japanese. Lancet 1981; 2(8253): 982. http://dx.doi.org/10.1016/S0140-6736(81)91172-7

[6] Yoshida A. Genetic polymorphisms of alcohol metabolizing enzymes related to alcohol sensitivity and alcoholic diseases. Alcohol Alcoholism 1994; 29(6): 693-6.

[7] Higuchi S, Matsushita S, Masaki T, *et al*. Influence of genetic variations of ethanol-metabolizing enzymes on phenotypes of alcohol-related disorders. Ann NY Acad Sci 2004; 1025: 472-80. http://dx.doi.org/10.1196/annals.1316.058

[8] Higuchi S. Significance of ethanol patch test. Nippon Rinsho (Japanese J Clin Med) 1997; 55(Suppl): 582-587.

[9] Mayer J, Filstead WJ. The Aldolescent Alcohol Involvement Scale: An instrument for measuring aldolescents' use and misuse of alcohol. J Stud Alcohol 1979; 40: 291-300.

[10] Yamauchi M, Kimura T, Takeda K, *et al*. Ethanol patch test: a simple method for identifying the effectiveness of cyanamide in alcoholics. Alcohol Clin Exp Res. 2000; 24(4 Suppl): 39S-42S.

[11] Yoshida-Yamamoto S, Nishimura S, Okuno T, Rakuman M, Takii Y. Efficient DNA extraction from nail clipping using the protease solution from *Cucumis melo*. Mol Biotechnol 2010; 46(1): 41-8. http://dx.doi.org/10.1007/s12033-010-9273-6

[12] Takii Y, Kuriyama S, Suzuki Y. Alkaline serine protease produced from citric acid by *Bacillus alcalophilus* subsp. *halodurans* KP1239. Appl Microbiol Biotech 1990; 34(1): 57-62. http://dx.doi.org/10.1007/BF00170924

[13] Saito H, Miura T. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim Biophys Acta 1963; 72: 619-29. http://dx.doi.org/10.1016/0926-6550(63)90386-4

[14] Takami H, Kobayashi T, Kobayashi M, *et al.* Molecular cloning, nucleotide sequence, and expression of the structural gene for alkaline serine protease from alkaliphilic *Bacillus* sp 221. Biosci Biotechnol Biochem 1992; 56(9): 1455-60. http://dx.doi.org/10.1271/bbb.56.1455

[15] Smith EL, Delange RJ, Evans WH, Landon M, Markland FS. Subtilisin Carlsberg V: the complete sequence; comparison with subtilisin BPN';evolutionary relationships. J Biol Chem 1968; 243(9): 2184-91.

[16] Wells JA, Ferrari E, Henner DJ, Estell DA, Chen EY. Cloning, sequencing, and secretion of *Bacillus amyloliquefaciens* subtilisin in *Bacillus subtilis*. Nucl Acids Res 1983; 11(22): 7911-25. http://dx.doi.org/10.1093/nar/11.22.7911

[17] Kobayashi T, Hakamada Y, Adachi S, *et al*. Purification and properties of an alkaline protease from alkalophilic *Bacillus* sp. KSM-K16. Appl Microbiol Biotechnol 1995; 43(3): 473-81. http://dx.doi.org/10.1007/BF00218452

[18] Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biottechnol 2002; 59(1): 15-32. http://dx.doi.org/10.1007/s00253-002-0975-y

[19] Gupta R, Ramnami P. Microbial keratinases and their prospective applications: an overview. Appl Microbiol Biotechnol 2006; 70(1): 21-23. http://dx.doi.org/10.1007/s00253-005-0239-8

[20] Mitsuiki S, Sakai M, Moriyama Y, Goto M, Furukawa K. Purification and some properties of a keratinolytic enzyme from an alkaliphilic *Nocardiopsis* sp. TOA-1. Biosci Biotechnol Biochem 2002; 66(1): 164-7. http://dx.doi.org/10.1271/bbb.66.164

[21] Bach E, Daroit DJ, Corrêa AP, Brandelli A. Production and properties of keratinolytic proteases from three novel Gram-negative feather-degrading bacteria isolated from Brazilian soils. Biodegradation 2011; 22(6): 1191-201. http://dx.doi.org/10.1007/s10532-011-9474-0

[22] Allen JP, Litten RZ, Fertig JB, Babor T. A review of research on the Alcohol Use Disorders Identification Test (AUDIT). Alcohol Clin Exp Res 1997; 21(4): 613-9. http://dx.doi.org/10.1111/j.1530-0277.1997.tb03811.x

[23] Holman CDJ, English DR, Milne E, Winter MG. Meta-analysis of alcohol and all-cause mortality: a validation of NHMRC recommendations. Med J Aust 1996; 164: 141-5.

[24] Zarkin GA, Bra JW, Babor TF, Higgins-Biddle JC. Alcohol drinking patterns and health care utilization in a managed care organization. Health Serv Res 2004; 39(3): 553-70. http://dx.doi.org/10.1111/j.1475-6773.2004.00244.x

[25] Goedde HW, Agarwal DP, Fritze G, *et al.* Distribution of ALDH2 and ALDH2 genotypes in different populations. Hum Genet 1992; 88(3): 677-81. http://dx.doi.org/10.1007/BF00197271

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