Full Paper

Identification of the sulphate ion as one of the key components of yeast spoilage of a sports drink through genome-wide expression analysis

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Because of the growing market for sports drinks, prevention of yeast contamination of these beverages is of significant concern. This research was performed to achieve insight into the physiology of yeast growing in sports drinks through a genome-wide approach to prevent microbial spoilage of sports drinks. The genome-wide gene expression profile of *Saccharomyces cerevisiae* growing in the representative sports drink was investigated. Genes that were relevant to sulphate ion starvation response were upregulated in the yeast cells growing in the drink. These results suggest that yeast cells are suffering from deficiency of extracellular sulphate ions during growth in the sports drink. Indeed, the concentration of sulphate ions was far lower in the sports drink than in a medium that allows the optimal growth of yeast. To prove the starvation of sulphate ions of yeast, several ions were added to the beverage and its effects were investigated. The addition of sulphate ions, but not chloride ions or sodium ions, to the beverage stimulated yeast growth in the beverage in a dose-dependent manner. Moreover, the addition of sulphate ions to the sports drink increased the biosynthesis of sulphur-containing amino acids in yeast cells and hydrogen sulphide in the beverage. These results indicate that sulphate ion concentration should be regulated to prevent microbial spoilage of sports drinks.

Key Words—beverage; gene expression; microarray; spoilage; sulphate ion; yeast

Introduction

The demand for and popularity of sports drinks (car-

bohydrate-electrolyte replacement drinks) is rapidly increasing in the world. Generally sports drinks provide the carbohydrates and electrolytes to rehydrate the body fluids. They contain 5.5–8.2% carbohydrates such as glucose, fructose and dextrin and 30 and 5 mmol L⁻¹ of sodium and potassium salts, respectively (Mettler et al., 2006). Based on the sugar and the salt content, they are categorized into isotonic, hypotonic and hypertonic sports drinks. However, spoilage of these beverages accounts for about \$1 billion in losses annually among the beverage industries worldwide

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Abbreviations: OD₆₆₀, optical density at 660 nm; YPD, yeast peptone dextrose.

(Ibribe Network, 2011). Moreover, a diverse range of microbes causes spoilage of beverages (Juvonen et al., 2011). Among these, yeasts are the most prominent spoilage species in sports drinks because they are able to grow at low pH and under anaerobic conditions (Stratford, 2006). *Zygosaccharomyces* and some of the *Saccharomyces* strains have been characterized as the spoilage microorganisms of beverages due to their high resistance to preservatives, their high fermentative production of CO₂ in up to 6 atmospheres of pressure, their osmotolerance, and their fructophilic nature. Yeast spoilage of beverages is characterised by the production of haze, off-flavors, off-odors, sediment, and excessive gas (Davenport, 1996; Statford et al., 2002; Steels et al., 1999; Stratford et al., 2006). The amount of generated CO₂ is sufficient to cause the bottles to explode and cans and packaging to rupture (Stratford et al., 2006). On the other hand, yeast rarely causes toxic injuries to humans but only causes minor gastrointestinal disorder (Stratford et al., 2006). In addition to *Zygosaccharomyces* and *Saccharomyces* species, *Brettanomyces* such as *Dekkera anomala*, *Dekkera bruxellensis*, and *Dekkera naardenensis* (Juvonen et al., 2011), *Kloeckera*, *Rhodotorula*, *Torulopsis*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Lodderomyces*, *Pichia*, *Saccharomycopsis* species (Henritte et al., 1996), and *Kloeckera* species have been reported as spoilage yeasts (Stratford et al., 2006).

It has been widely accepted that nitrogen compounds such as amino acids and vitamins regulate microbial contamination. However, sulphur components have not been paid attention in terms of microbial contamination. Sulphur contamination risk depends on the place and environment where the beverage is produced. Fossil fuel combustion and volcanic activities emit massive quantities of sulphur species into the environment. It has been reported that sulphate content in rainwater ranges from 10.41 mmol L⁻¹ to 142.62 mmol L⁻¹ due to variations in the atmospheric sulphur content (Xiao et al., 2011). Sulphate directly contaminates both ground and surface water bodies. The concentration of sulphate ions in drinking water in different regions of the world has been continuously reported to exceed 1.76 mmol L⁻¹, which is the recommended level by World Health Organization (WHO, 2004).

In order to prevent microbial contamination of sports drinks, it is important to know the physiology of mi-

crobes as they grow in the actual liquids. The recently developed Affymetrix DNA chip, which elucidates the genome-wide gene expression, is a powerful tool to obtain insight into the physiology of yeast cells, and many researchers have used this tool to investigate the physiology of yeast cells in various experimental nutrition conditions (Boer et al., 2003; Kitagaki et al., 2009; Wu et al., 2006). Moreover, genes encoding nutrient uptake and gene expressions involved in nutrient starvation are shared among several yeasts. For example, the amino acid identity of Gap1, which is responsible for uptake of general amino acids, shows 75% identity between *Saccharomyces cerevisiae* and *Candida glabrata*, and 63% identity between *S. cerevisiae* and *Pichia stipitis*. A gene expression response to amino acid starvation of *Candida albicans* has been reported to be similar to that of *S. cerevisiae* (Renard, 2008). Therefore, the gene expression profile of *S. cerevisiae* was considered to represent that of various yeasts and fungi that contaminate beverages. *S. cerevisiae* that was isolated from the natural environment in Japan was adopted as the model yeast to explore gene expression during growth in the representative sports drink, since laboratory strains are considered to have lost their genetic response to low nutrition due to many generations of growth in rich artificial media (Homann et al., 2005). As a result of this analysis, it was suggested that yeast cells suffer from a deficiency of extracellular sulphate ions during growth in sports drinks. Consistent with this hypothesis, the concentration of sulphate ions in the beverage was far lower than the optimal concentration for growth, and addition of sulphate ions stimulated yeast growth in the beverage. Our study has for the first time shown the significant role of the sulphate ion in the growth of yeast in sports drinks and the importance of decreasing the concentration of sulphate ions in sports drinks.

Materials and Methods

Yeast strain. A strain of the yeast *Saccharomyces cerevisiae* that was isolated from a natural environment in Japan (AHU4426) was used in this study (Sekiguchi et al., 2008).

Beverage. One of the most popular sports drink in Japan was selected as the representative sports drink for the study. All of the nutritional information including the amino acid profile is given in Tables 1 to 3. Tables 1 and 2 were obtained from the manufacturer of the

Table 1. Nutritional information about the representative sports drink.

Component	Amount in 100 ml of beverage
Energy	27 kcal
Carbohydrate	6.7 g
Protein	0
Fat	0
Sodium	49 mg
Potassium	20 mg
Calcium	2 mg
Magnesium	0.6 mg

The product ingredients are sugar, high-fructose corn syrup, fruit juice, salt, sour agent, vitamin C, potassium chloride, calcium ion, lactic acid, flavoring (amino acid), magnesium chloride, and fragrance. The pH value of the beverage is 3.6 and preservatives are not included in the product.

Table 2. Electrolyte concentrations of the representative sports drink.

Ions	Concentration (mEq L ⁻¹)
Positive ions	
Na ⁺	21
K ⁺	5
Ca ²⁺	1
Mg ²⁺	0.5
Negative ions	
Cl ⁻	16.5
Citrate ³⁻	10
Lactate ⁻	1

beverage.

Growth and media. Culture of AHU4426 for all the experiments was prepared by overnight incubation of the yeast at 30°C in yeast peptone dextrose (YPD) medium (Becton Dickinson, Franklin Lakes, NJ). The sports drink in a sterile plastic container was inoculated with yeast cells. The initial OD₆₆₀ value was set at 0.05. As a control, YPD medium was inoculated with the yeast cells in the same type of container. The culture was incubated for 2 doubling times at 4°C without shaking, and the cells were sampled, centrifuged, and immediately frozen at -80°C for microarray analysis. Growth was monitored by homogenizing and measuring OD₆₆₀ of the culture. The cultures used for measurements were not used for subsequent cultures. Four different sports drinks that are commercially available in Japan were used for inoculation.

RNA isolation. Total RNA was extracted by the hot

Table 3. Amino acid concentrations of the representative sports drink.

Amino acid	mg/100 ml of beverage	SD
Asp	0.62	0.01
Glu	6.50	0.15
H-Pro	0.04	0.01
Ser	0.07	0.00
Gly	0.01	0.00
His	0.05	0.02
Tau	nd	
Thr	0.23	0.01
Arg	0.16	0.00
Ala	0.41	0.06
Pro	1.02	0.08
Tyr	3.10	0.30
Val	0.61	0.07
Met	nd	
Cys	nd	
Ile	nd	
Leu	nd	
Phe	1.95	0.34
Lys	0.04	0.00

Amino acid concentrations were analyzed by HPLC as described in MATERIALS AND METHODS. The results are averages and standard errors of 2 measurements. "nd" represents not detected.

phenol method (Kohrer and Domdey, 1991). After the extraction, RNA was further purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). The purified RNA was stored at -80°C. The quality of the total RNA samples was checked using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA extraction and its purification experiments were performed 2 times independently from respective independent starter cultures for gene expression analysis.

Expression analysis using microarray. Total RNA was labeled using the GeneChip one-cycle target labeling and control reagents (Affymetrix, Santa Clara, CA). Labeling of total RNA, hybridization to the DNA microarray, and scanning with the GeneChip scanner 2500 (Affymetrix) were performed in accordance with the manufacturer's instructions. Microarray analysis of the samples from each time point was repeated two times using total RNA samples obtained from two independent experiments. Microarray data were analyzed using GeneSpring 7 software (Agilent Technologies) as previously described (Wu et al., 2006). Before expression profile analysis, the correlation coefficients

for each set of gene expression data (two replicates from respective culture conditions) were calculated to evaluate the reproducibility of the experiments. A high correlation coefficient (greater than 0.90) was obtained for comparisons between all pairs of samples for each set of culture conditions, indicating good reproducibility. The microarray data have been deposited online in the MAGE-TAB ArrayExpress database (<http://www.ebi.ac.uk/cgi-bin/microarray/magetab.cgi>) as "low nutrition."

Analysis of the expression of genes by quantitative real-time reverse transcriptase (RT)-PCR. Total RNA was extracted by the hot phenol method (Kohrer and Domdey, 1991), after 1 day and 8 days incubation of yeast strain (which corresponds to 2 doubling times) at 4°C in 10 ml of YPD media and the representative sports drink, respectively. The extracted RNA was further purified by using an RNeasy Mini Kit (Qiagen, Valencia, CA) and frozen at -80°C until further analysis. Expression of *MET1*, *SUL2* and *APG3* genes was analyzed by quantitative real-time RT-PCR using the Roche LightCycler™ (Roche Applied Biosystem) with the one-step SYBR® Prime script™ PLUS RT-PCR KIT (Perfect Real Time) (TaKaRa Biotechnology (Dalian) Co., Ltd.), according to the manufacturer's protocol. The purified 30 ng of template total RNA and the *MET1*_{fw} 5'-CAA AGC AGG AAA CTG TCA CA-3' and *MET1*_{rw} 3'-TCC GAC TAA AGA AAT CTT CCC-5' for *MET1*, *SUL2*_{fw} 5'-TCC TCC AAT GCT TAC CCA GC-3' and *SUL2*_{rw} 3'-TGC GCC GGT CAA ACA ATA TA-5' for *SUL2*, *APG3*_{fw} 5'-TTC TGG AAC GGA ATC AGT TGC-3' and *APG3*_{rw} 3'-CGG AAC AGT TGC TCC ATA GAA-5' for *APG3* primer pairs were used for quantitative real-time RT-PCR analysis. *RDN1* was used as an internal control gene for normalization (Teste et al., 2009) with the primer set of *RDN1*_{fw} 5'-CCA TGG TTT CAA CGG GTA ACG G-3' and *RDN1*_{rw} 5'-GCC TTC CTT GGA TGT GGT AGC C-3'. As templates, 30 ng of total RNA was used. RT reaction was carried out at 42°C for 5 s followed by heating at 95°C for 10 s. The genes were amplified in 40 cycles of 95°C for 5 s followed by 60°C for 20 s. The relative gene expressions of *MET1*, *SUL2* and *APG3* were calculated by following the method described by Livak and Schmittgen (2001).

Addition of mineral ions to sports drinks. The effect of the addition of ions on the growth of the yeast strain was assessed in a sterile plastic tube containing 10 ml of the representative sports drink that was supplemented with 8.5 mmol L⁻¹ sodium sulphate or 17 mmol

L⁻¹ sodium chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The samples were incubated without shaking at 4°C. The OD₆₆₀ of the cultures was monitored at 24 h intervals with a UV spectrophotometer (UV-1800; Shimadzu Scientific Instruments, Kyoto, Japan). To assess the effect of sulphate ions on cell growth at a cell concentration that mimics that of the actual contamination situation, 100 cells ml⁻¹ were inoculated into the representative sports drink with or without 8.5 mmol L⁻¹ of sulphate ions and incubated at 4°C. The cell growth was monitored by colony forming units.

Analysis of amino acids. Amino acids were determined by HPLC with pre-column derivatisation according to the method of Bidlingmeyer et al. (1984). For the pre-column derivatization, 4 µl of sample or standard solution (2.5 mmol L⁻¹ tryptophan or common amino acids mixture (Type H, Wako Pure Chemical Industries Ltd.)) was added with 4 µl of internal standard (2.5 mmol L⁻¹ γ-amino butyric acid). The mixture was dried using a centrifugal evaporator (myVac Quattro, Genevac, Ltd., Ipswich, UK), dissolved in 20 µl counteragent (triethylamine : water : methanol = 1 : 1 : 2 (v/v)), and dried again. The concentrate was added with 20 µl phenylisothiocyanate (10% (v/v) in triethylamine : water : methanol = 1 : 1 : 7 (v/v/v)), sonicated for 10 s and stored for 20 min at room temperature. After removal of solvent using a centrifugal evaporator, the resulting phenylthiocarbamyl derivative of amino acids was dissolved in HPLC mobile phase A. For HPLC analysis, mobile phases A and B were 50 mmol L⁻¹ sodium acetate (pH 6.0)/acetonitrile=97/3 (v/v) and water/acetonitrile=40/60 (v/v), respectively. Separations were performed using an ODS column (Shimpack XR-ODS, 2.0 × 100 mm, Shimadzu Corp., Kyoto, Japan) with elution using a gradient of 100% mobile phase A from zero to 1.5 min, 60% mobile phase B from 1.5 to 16 min, and 100% mobile phase B from 16 to 20 min. Other conditions were as follows: apparatus, Ultimate U-3000 (Dionex Corp., Sunnyvale, CA); column temperature, 45°C; flow rate, 0.2 ml min⁻¹; detection, UV at 254 nm; injection volume, 1 µl. The internal standard method was used to quantify the analytes.

Measurement of amino acid concentration. Yeast cells were grown statistically at 4°C in sterile plastic tubes containing 40 ml of the representative sports drink supplemented with 8.5 mmol L⁻¹ of sodium sulphate. The initial OD₆₆₀ value was set at 0.05. After 8

days of incubation, the samples were centrifuged and 2×10^7 cells were separated. The cells were washed with sterile water twice and the supernatant was discarded. Seven hundred microliters of 10% TCA was added to the cells and the suspension was vortexed for 1 min. The samples were kept on ice for 15 min. The sample was centrifuged and the supernatant was discarded. To eliminate TCA in the supernatant, 700 μ l of diethyl ether was added and vortexed for 10 s, centrifuged at 14,000 rpm for 5 min, and the supernatant was discarded. This procedure was repeated 3 times. The samples were heated at 100°C for 1 min to eliminate any remaining diethyl ether. The amino acid contents of 50 μ l of the individual samples were analyzed using the method described in the previous section.

Analysis of the concentration of sulphate ions. Sulphate ions in the representative sports drink were determined with an ion chromatography (IC) system (DXC-500, Dionex Corp., Sunnyvale, CA). IC conditions were as follows: column, AS-12A (4.6 mm $\times$ 250 mm, Dionex Corp., Sunnyvale, CA); column temperature, 35°C; flow rate, 1.5 ml min⁻¹; detection, conductivity detection with a suppressor (ASRS-300, Dionex Corp., Sunnyvale, CA); eluent, 2.7 mmol L⁻¹ Na₂CO₃/0.3 mmol L⁻¹ NaHCO₃; sample injection volume, 25 μ l.

Analysis of the concentration of hydrogen sulphide.

The concentrations of hydrogen sulphide in beverages were analyzed according to the method described by Shen et al. (2011) with slight modifications.

Briefly, the reaction mixture was prepared by mixing 400 μ l of 20 mmol L⁻¹ *N,N*-dimethyl-*p*-phenylenediamine sulphate (Nacalai Tesque, Inc., Kyoto, Japan) in 7.2 mmol L⁻¹ HCl and 400 μ l of 30 mmol L⁻¹ FeCl₃ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 1.2 mmol L⁻¹ HCl. The reaction mixture was mixed with 225 μ l of sample and incubated for 10 min at room temperature. The absorbance at 670 nm was measured by UV spectrophotometer (UV-1800; Shimadzu Scientific Instruments, Kyoto, Japan) and the concentration of hydrogen sulphide was determined with a calibration curve constructed with standard methylene blue solution.

Statistical analysis. Unpaired, one-sided Student's *t*-test was performed by using the Microsoft Office Excel 2007 to evaluate the differences in the microarray results between the two culture conditions of two independent cultures from respective independent starter cultures and those in the real-time RT-PCR results between two culture conditions of three independent cultures from respective independent starter cultures.

Results

Analysis of the nutrition in the representative sports drink

First, the nutritional information of a representative commercially available beverage categorized as an isotonic sports drink was investigated (Tables 1 to 3). Although the carbohydrate concentration was relative-

Fig. 1. Growth curve of yeast cells in the sports drink and YPD medium.

Yeast growth in the representative sports drink (A) and YPD medium (B). The representative sports drink (40 ml) and YPD medium (10 ml) were inoculated with 0.5×10^6 cells ml⁻¹ of the yeast strain AHU4426. The samples were incubated at 4°C without shaking. The growth was monitored by measuring OD₆₆₀ of the culture broth after thorough homogenization.

ly high, the concentrations of other constituents such as electrolytes and amino acids were significantly low.

Growth of yeast cells in the representative sports drink

Next, yeast growth in the sports drink was investigated. It was clearly showed that yeast cells could grow in the beverage (Fig. 1A), although they grew very slowly relative to that in the YPD medium (Fig. 1B). In order to investigate the gene expression profile, yeast cells were incubated for 2 doubling times, sampled and used for further analyses.

Overrepresented gene categories during growth in the sports drink

Using microarray analysis, of 5,925 genes in total, genes that were differentially expressed between yeast cells that were incubated in a rich medium (YPD medium) and a poor medium (the representative sports drink) were identified (Fig. 2). In order to analyze the genes in detail, 453 genes that had significantly ($p < 0.05$) different expression levels (increased or decreased) in the 2 nutrition conditions were selected (Table S1). The selected genes were analyzed by the FunSpec web-based clustering tool (Robinson et al., 2002), and the significantly enriched categories were extracted (Table 4). The analysis revealed that cate-

ries such as C4-dicarboxylate transport; Homeostasis of sulphate; Amino acid/amino acid derivatives transport; C-2 compound and organic acid catabolism were significantly overrepresented in a Munich Information Center for Protein Sequences (MIPS) functional classification. These results suggest perturbation of the metabolism of carbohydrate compounds due to a

Fig. 2. Frequency distribution of the gene expression levels in the sports drink relative to those in YPD medium.

The gene expression ratio is defined as the ratio of the gene expression in the sports drink relative to that in YPD medium. Numbers of genes whose expression ratios are within the ranges are indicated.

Table 4. Munich Information Centre for Protein Sequences (MIPS) functional classification of genes whose expressions were significantly increased or decreased during growth in the sports drink relative to that in YPD medium.

Category	<i>p</i> -value	In Category from Cluster	<i>k</i>	<i>f</i>
C-2 compound and organic acid catabolism	0.0003328	<i>ACS1 ACS2 ALD6</i>	3	9
C4-dicarboxylate transport (e.g., malate, succinate, fumarate)	0.001557	<i>JEN1 DIC1</i>	2	4
Biosynthesis of vitamins, cofactors, and prosthetic groups	0.002058	<i>ACS1 ADE3 ACS2 NRK1 ABZ1 COQ2 THI22</i>	7	110
Energy conversion and regeneration	0.01572	<i>ACS1 ACS2</i>	2	12
C-compound and carbohydrate transport	0.01763	<i>ADY2 MCH1 YAT2</i>	3	34
Regulator of transcription factor	0.0221	<i>CYC8 ADA2 WTM2</i>	3	37
Cell wall	0.02293	<i>ACS1 CAX4 BGL2 ECM29 CWP1 CCW12 GAS2 ECM30</i>	8	213
Mitochondrial transport	0.02735	<i>ATP16 AFG3 YAT2 SSA2 DIC1</i>	5	104
Glycolysis and gluconeogenesis	0.02893	<i>GPM2 FBA1 ADH6</i>	3	41
Amino acid/amino acid derivatives transport	0.03676	<i>BAP2 ERS1 TAT2</i>	3	45
Protein/peptide degradation	0.04104	<i>RRT12 AFG3 ECM29</i>	3	47
Homeostasis of sulphate	0.04828	<i>YPR003C</i>	1	3

The 110 genes whose expressions were significantly ($p < 0.05$) altered between a sports drink beverage and YPD medium were analyzed by the FunSpec web-based clustering tool. Categories that contain less than 3 genes were not included in this classification.

k: number of genes from the input cluster in given category. *f*: number of genes total in given category.

Fig. 3. Expression of genes in sulphate assimilation pathway remarkably increases in the sports drinks beverage.

Relative gene expression level of *MET1* (A), *SUL2* (B) and *APG3* (C) in the representative sports drink was analyzed by quantitative real-time reverse transcriptase (RT)-PCR. The results are represented as mean values $\pm$ standard deviations of 3 independent experiments from respective starter cultures. Statistical significance levels of the difference of relative gene expression are indicated (***) ($p < 0.001$).

lack of sulphate ions, nitrogen compounds such as amino acids and vitamins, during growth in the sports drink. Since it is well known that amino acids and vitamins regulate microbial growth, the genes in the sulphate ion starvation response were further analyzed in detail.

Sulphate ion starvation response in the sports drink

We realized that many genes involved in sulphate ion starvation response were upregulated significantly ($p < 0.05$) in the representative sports drink (Table S1). *MET1*, which encodes S-adenosyl-L-methionine uro-

porphyrinogen III transmethylase (Hansen et al., 1997), was increased 2.13-fold, *APG3*, a gene that encodes a low-affinity amino acid permease (Schreve and Garrett, 2004) was increased 2.9-fold and *SUL2*, which encodes a permease of the sulphur ion (Thomas and Surdin-Kerjan, 1998), was 2.35-fold upregulated in the sports drink relative to YPD medium. To confirm the microarray results, we analyzed the expression of genes by quantitative real-time RT-PCR. It turned out that *MET1*, *APG3* and *SUL1* expression significantly increased (Fig 3A–C, $p < 0.001$, $n=3$) by 3.31-, 7.78- and 6.39-fold in the representative sports drink relative to

those of YPD medium, respectively. These results coincided with the microarray results.

Since these genes coincided with those upregulated in sulphur-limited condition (Boer et al., 2003), these results inspired us to hypothesize that yeast cells growing in the sports drink suffer from sulphate ion deficiency.

Addition of sulphate ions to a sports drink stimulates yeast growth

Based on the above information, we hypothesized that a sulphate ion deficiency was the factor that prevented microbial spoilage of these beverages. In order to verify this hypothesis, the concentration of sulphate ions in the representative sports drink was analyzed. Consistent with our hypothesis, the concentration of sulphate ions in the representative sports drink was $9.3 \pm 0.31 \mu\text{mol L}^{-1}$ ($n=3$), which was far less than the optimal concentration for growth (approximately 39.6 mmol L^{-1}) (Boer et al., 2003). This result supports our hypothesis that yeast cells growing in the sports drink are deficient in sulphate ions.

To further confirm sulphate ion deficiency during yeast growth in the sports drink, the representative sports drink was supplemented with sulphate ions and yeast growth was monitored. Because the sports drink already contained approximately 20 mmol L^{-1} sodium ions, sodium sulphate was adopted as the salt for supplementation. It turned out that the OD_{660} of the beverage supplemented with 8.5 mmol L^{-1} sodium sulphate was significantly higher than that of the unsupplemented beverage (Fig. 4; 16–19 days; $p < 0.01$). The effect of the addition of sulphate ions was dose-dependent (Table 5). In order to elucidate if the addition of sulphate ions is effective in other sports drinks, the growth of yeast was examined in several commercially available sports drinks (Fig. S1A). The results indicated that supplementation of sulphate ions increased the growth of yeast in all the tested sports drinks. In order to rule out the possibility that Na^+ stimulated growth, the same molar concentration of Na^+ was added ($17 \text{ mmol L}^{-1} \text{ NaCl}$) to the representative sports drink, and growth was monitored at OD_{660} . Yeast growth in the sports drink that was supplemented with sodium chloride was slower than that in the untreated drink (Fig. 5). This result indicates that Na^+ is not involved in stimulating yeast growth in the sports drink; therefore, the sulphate ion is responsible for stimulating yeast growth in the sports drink. In order to verify that the

Fig. 4. Effect of the addition of sodium sulphate on the growth of yeast cells in the sports drink.

The representative sports drink (10 ml) supplemented with 8.5 mmol L^{-1} sodium sulphate was inoculated with 0.5×10^6 cells of the yeast strain AHU4426. The beverage was incubated at 4°C without shaking. The growth was monitored by measuring the OD_{660} at 24 h intervals. The results are represented as mean values $\pm$ standard deviations of 3 independent experiments from respective starter cultures. The significance of differences between the untreated and sodium sulphate-supplemented samples was evaluated by a one-sided Student's *t*-test ($n=3$, ** $p < 0.01$, * $p < 0.05$).

Table 5. Effect of different concentrations of sulphate ion on the specific growth rate of yeast in the sports drink.

Sulphate ion concentration (mmol L^{-1})	Specific cell growth rate ($\text{g g}^{-1} \text{ day}^{-1}$)
0	0.050 ± 0.001
8.5	0.054 ± 0.001
25.3	0.057 ± 0.001

Bold value indicates statistically significant value ($p < 0.05$, $n=3$) as compared to other values.

yeast cell number does not influence the effect of added sulphate ions, we inoculated $100 \text{ cells mL}^{-1}$ at the start of the culture and analyzed the effect of the addition of sulphate ions. The addition of sodium sulphate increased the growth at this inoculum level ($p < 0.01$) (Fig. 6), indicating that the effect of adding sulphate ions holds true irrespective of the inoculum level. To investigate the effect of temperature, the addition of sulphate ions on the yeast growth in the sports drink was tested at room temperature. The yeast growth in

Fig. 5. Effect of the addition of sodium chloride on the growth of yeast cells in the sports drink.

The representative sports drink (10 ml) that was supplemented with 17 mmol L^{-1} sodium chloride was inoculated with 0.5×10^6 cells of the yeast strain AHU4426. The beverage was incubated at 4°C without shaking. The growth was monitored by measuring the OD_{660} at 24 h intervals. The results are represented as mean values $\pm$ standard deviations of 3 independent experiments from respective starter cultures. The significance of differences between the untreated and sodium chloride-supplemented samples was evaluated by a one-sided Student's *t*-test ($n=3$, $^{**}p < 0.01$, $^*p < 0.05$).

the sports beverage at room temperature was significantly larger and faster relative to refrigerator temperature (4°C) ($p < 0.05$, $n=3$). This result indicates that the stimulation of yeast growth in sports drinks by the addition of sulphate ions can be generalized not only at refrigerator temperature but also at room temperature. Supplementation of sulphate ions improved the growth of yeast in all the tested sports drinks at room temperature also (Fig. S1B). These results indicate that sulphate ion contamination of water is a risk factor for yeast spoilage of sports drinks both at room temperature and at refrigerator temperature.

Addition of sulphate ions affects sulphur-containing amino acid production

Based on the result that sulphate ion stimulated the yeast growth in the sports drink, we hypothesized that the sulphate ion stimulates growth by producing sulphur-containing amino acids, because sulphate ions have been reported to be incorporated into methionine and cysteine through 5'-adenylylsulphate,

Fig. 6. Effect of the addition of sodium sulphate on the growth of yeast cells with a small cell number in a sports drink.

The representative sports drink (10 ml) that was supplemented with 8.5 mmol L^{-1} sodium sulphate was inoculated with $100 \text{ cells ml}^{-1}$ of the yeast strain AHU4426. The beverage was incubated at 4°C without shaking. The growth was monitored by assessing the colony forming units. The results are represented as mean values $\pm$ standard deviations of 3 independent experiments from respective starter cultures. The significance of differences between the untreated and sodium sulphate-supplemented samples was evaluated by a one-sided Student's *t*-test ($n=3$, $^*p < 0.05$).

Table 6. Effect of sulphate ion addition to the sports drink on production of sulphur-containing amino acids in yeast.

	Methionine (mmol ml^{-1})	Cysteine (mmol ml^{-1})
Untreated	2.57 ± 0.16	0.31 ± 0.04
Sulphate ion added	2.86 ± 0.03	0.35 ± 0.02

Bold value indicates statistically significant value ($p < 0.05$, $n=3$) as compared to those of untreated sample.

3'-phospho-5'-adenylylsulphate, sulphite, sulphide, and homocysteine (methionine), or through homocysteine and cystathionine (cysteine) by means of the sulphate assimilation pathway within yeast cells (Thomas and Surdin-Kerjan, 1998). To support this hypothesis, we analyzed the amino acid profiles of yeast that was incubated with sodium sulphate and compared the profiles to those of untreated yeast. Consistent with our hypothesis, the concentrations of methionine and cysteine were increased in the sample with added sul-

phate ions compared to the untreated sample, although the difference in cysteine was not statistically significant (Table 6). It is estimated that cysteine concentrations were not significantly altered between these samples since the intracellular concentrations of amino acids are strongly regulated by homeostasis.

Addition of sulphate ion increases hydrogen sulphide production in sports drink

Hydrogen sulphide is one of the sulphur-containing metabolites produced by yeast under anaerobic conditions from sulphate ions. Therefore, we analyzed the concentrations of hydrogen sulphide in beverages with or without sulphate ion supplementation. The concentration of hydrogen sulphide in sulphate ion-added beverage was $0.083 \pm 0.005 \text{ mg L}^{-1}$, while that in nontreated beverage was $0.062 \pm 0.004 \text{ mg L}^{-1}$, indicating that hydrogen sulphide increased in the sulphate ion-added beverage ($p < 0.05$, $n = 3$). Moreover, hydrogen sulfide was not detected in a sulphate ion-added sample without yeast inoculation. Hence, it was indicated that yeast cells produce hydrogen sulphide in the sports drinks supplemented with sulfate ions.

Discussion

The market of sports drinks is enlarging worldwide. These beverages are primarily consumed by athletes as well as wide range of people including elders and immuno-compromised individuals. These beverages tend to be easily contaminated by yeast (Davenport, 1996; Statford et al., 2002; Steels et al., 1999; Stratford et al., 2006); thus, it is important to prevent yeast spoilage of these beverages. Moreover, a similar isotonic saline solution used for intravenous infusion has been reported to be contaminated with a wide range of microbes (Mershon et al., 1991). However, there has been no study to date that elucidated the genome-wide gene expression profile of the yeast growing in these beverages or the factors that could prevent yeast spoilage in the sports drink. In this study, we, for the first time, elucidated the genome-wide gene expression profile of yeast during growth in the sports drink and identified the sulphate ion as a candidate substance to prevent yeast spoilage of sports drinks. This result will benefit manufacturers of both beverages and isotonic saline solution.

The concentration of sulphate ions in water has not been paid much attention in the field of food hygiene

and drinking water quality (WHO, 2004). Rather, it has been only considered as an off-flavor component. However, the results obtained in this study clearly indicate that sulphate ions in water are present at a high enough concentration to stimulate yeast growth. Hence, sulphur is identified as the key substance to control yeast spoilage of sports drinks. Indeed, a great deviation in the concentration of sulphate ions in drinking water has been observed in various parts of the world (WHO, 2004). It is considered that many beverage-producing companies use reverse osmosis technology to remove dissolved solids and ions in the constituent water; however, the prevalence of this technology is not known, especially in the developing countries. Furthermore, many beverages are supplemented with ions and amino acids for nutritional enhancement. Our results indicate that it is important to decrease the concentration of sulphate ions in the constituent water for sports drinks and isotonic saline solution.

Analysis of the amino acid profile of yeast cells revealed that sulphate ion addition leads to biosynthesis of elevated levels of methionine and cysteine in the sports drink, although the increase of the level of cysteine was not statistically significant maybe because of the amino acid homeostasis within yeast cells. Indeed, methionine and cysteine were not detected in the analyzed amino acid profile of the representative sports drink (Table 3). Hence, it is suggested that the sulphate ion is utilized by the yeast cells through a sulphate assimilation pathway during growth in the sports drink, and contributes to the total growth. Furthermore, methionine is considered to have a greater influence on yeast growth than other sulphur-containing amino acids (Maw, 1963). The sulphate ion has also been reported to be involved in yeast growth under salt-stressed conditions (Glaser et al., 1993). This knowledge suggests that under the stress condition of low temperature and nutrient starvation, methionine may play an important role in yeast growth in the sports drinks. This hypothesis will be the target of future study.

Synthesis of vitamins and small bioactive molecules and incorporation of mineral ions play central roles in the growth of microbes. Therefore, yeast is considered to upregulate genes required for synthesis of these molecules in the sport drink. Indeed, several genes such as *BIO3*, *NIT1* and *BET2*, which are involved in synthesis of vitamins and small bioactive molecules,

were upregulated in the sport drink (Table S1). Mg^{2+} is required for vital steps of the sulphate assimilation pathway, such as ATP sulphurylation and phosphorylation of 5'-adenylylsulphate (Thomas and Surdin-Kerjan, 1998). Indeed, Mg^{2+} is contained at a concentration of 6.12 mg L^{-1} in the representative sports drink, which is sufficient to support these reactions. These results support our hypothesis that synthesis of vitamins and small bioactive molecules and incorporation of mineral ions play critical roles of microbial contamination of sports drinks.

The sulphate ion is considered to have important effects on the quality of beverages. For example, it has been reported that in nitrogen starvation conditions, yeast cells degrade sulphur-containing amino acids and utilize the nitrogen for cell growth (Linderholm et al., 2008). However, at the same time, the degradation of methionine and cysteine in a reductive environment at low pH generates mainly hydrogen sulphide and other volatile sulphur-containing compounds through the sulphate reduction pathway (Linderholm et al., 2008; Schutz and Kunkee, 1977). Moreover, it has been reported that in *S. cerevisiae*, high amounts of hydrogen sulphide are generated due to the leakage of sulphide from the sulphur accumulation pathway (Linderholm et al., 2008; Spiropoulos et al., 2000). Hydrogen sulfide has been recognized as one of the major sensory-defective compounds produced by yeast in wine fermentation (Schutz and Kunkee, 1977; Spiropoulos et al., 2000). Therefore, the results obtained in this study indicated that yeast cells in the sports drinks with added sulphate ion produce an increased amount of hydrogen sulphide and can cause sensory defects. This information will be important for beverage companies to improve the quality of beverages.

In summary, the gene expression profile of yeast during growth in a sports drink suggested that yeast cells are deficient in sulphate ions. Indeed, the addition of sulphate ions but not sodium ions or chloride ions significantly stimulated yeast growth and production of methionine and hydrogen sulphide in the sports drink. Therefore, it is important to decrease the content of sulphate ions to prevent yeast spoilage of sports drinks.

Supplementary Materials

Fig. S1. Effect of the addition of sodium sulphate on the growth of yeast cells in the sports drinks.

Yeast growth at 4°C (A) and yeast growth at room temperature (B). The sports drinks (10 ml) supplemented with 8.47 mmol L^{-1} sodium sulphate were inoculated with 0.5×10^6 cells of the yeast strain AHU4426. The samples were incubated at 4°C (A) or at room temperature (B) without shaking. The growth was monitored by measuring OD_{660} at different time points. The results are represented as mean values $\pm$ standard deviations of 3 independent experiments from respective starter cultures. Samples 1 to 4 represents different sports drinks commercially available in Japan.

Table S1. Genes of yeast cells whose expressions were statistically different between growth in YPD medium and a sport drink.

Supplementary figure and table are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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