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Isolation of aquatic yeasts with the ability to neutralize acidic media, from an extremely acidic river near Japan's Kusatsu-Shirane Volcano.

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1 **Running title:** Acid-tolerant yeasts possessed a neutralizing ability

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3 *Regular Paper*

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5 **Isolation of aquatic yeasts with the ability to neutralize acidic media, from**
6 **an extremely acidic river near Japan's Kusatsu-Shirane Volcano**

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22

23 **Key words:** acid-tolerant yeast; acidic river; acid tolerance; *Candida fluvialtilis*;

24 neutralization

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26

1 **ABSTRACT**

2 The Yukawa River is an extremely acidic river whose waters on the east foot of the
3 Kusatu-Shirane Volcano (in Gunma Prefecture, Japan) contain sulfate ions. Here we
4 isolated many acid-tolerant yeasts from the Yukawa River, and some of them
5 neutralized an acidic R2A medium containing casamino acid. *Candida fluviatilis* strain
6 CeA16 had the strongest acid tolerance and neutralizing activity against the acidic
7 medium. To clarify these phenomena, we performed neutralization tests with strain
8 CeA16 using casamino acid, a mixture of amino acids, and 17 single amino acid
9 solutions adjusted to pH 3.0, respectively. Strain CeA16 neutralized not only acidic
10 casamino acid and the mixture of amino acids but also some of the acidic single amino
11 acid solutions. Seven amino acids were strongly decomposed by strain CeA16 and
12 simultaneously released ammonium ions. These results suggest strain CeA16 is a
13 potential yeast as a new tool to neutralize acidic environments.

14

15

1 INTRODUCTION

2 The various extreme environments on Earth include physical environments (e.g.,
3 temperature, radiation and pressure extremes) and geochemical environments
4 (desiccation, salinity, pH, oxygen species and redox potential environments) in which
5 various organisms have adapted and thrived (1). For example, the biodiversity and
6 ecology of eukaryotic organisms living in extreme acidic environments near volcanos
7 have been studied (2). Yeast diversity has been extensively investigated (3). Several
8 types of yeast have been isolated from these extreme acidic environments to date; e.g.,
9 *Rhodotorula glutinis* (4), *Candida maltosa* (5), and *Cryptococcus tepidarius* (6). In
10 *Saccharomyces cerevisiae*, yeast genes involved in responses to acid stress have been
11 studied (7–9). It has also been reported that acidification of the external medium during
12 yeast growth caused the activation of the plasma membrane ATPase (10). Additionally,
13 several studies on mechanisms underlying the prevention of cell damage from acid
14 stress in various acidic environments and proteomic analyses of such stress have been
15 performed for bacteria related to food and the human body (11, 12). However, to our
16 knowledge, there are no reports on the mechanisms of acid resistance in environmental
17 acid-tolerant yeasts.

18 Mount Kusatu-Shirane is an active volcano whose soil contains an abundance of
19 sulfides (13). The Yukawa River, whose water at the east foot of Mt. Kusatu-Shirane
20 contains sulfate ions, is a strongly acidic river (pH 2–3). The Yukawa River water
21 originates from the Kusatsu Hot Spring area and flows downward to the Shinaki Dam.
22 In order to remove damage due to the river's acidity for the purpose of river
23 conservancy and to provide water for agricultural, industrial and other uses, the Yukawa
24 River water is neutralized by adding milk of lime (i.e., calcium carbonate) from the

1 Kusatsu neutralization plant (14). The Yukawa River thus has an unusual aquatic
2 environment composed of both the natural aquatic environment and artificial elements.

3 In this study, we isolated many acid-tolerant yeasts from both upstream and
4 downstream of the Kusatsu neutralization plant in the Yukawa River. Interestingly, some
5 of these isolated acid-tolerant yeasts possessed a neutralizing ability against an acidic
6 medium. We discuss the mechanisms and the optimum conditions for the neutralization
7 of acidic media by these novel yeasts.

8 9 **MATERIALS AND METHODS**

10 **Collection of environmental samples**

11 In June 2010, we collected water samples from the Yukawa River in Agatsuma-gun,
12 Gunma Prefecture, Japan. The samples were transported to the laboratory in sterile
13 bottles in contact with ice. The water temperature was measured at each of the two
14 sampling stations, and the pH value of each sample was measured in our laboratory.

15 16 **Medium cultures**

17 For the medium cultures, we used an R2A medium consisting of: 0.1% yeast extract
18 (Becton Dickinson [BD], Franklin Lakes, NJ), 0.1% proteose peptone (BD), 0.1%
19 casamino acid (Nihon Pharmaceutical, Tokyo), 0.1% D(+)-glucose, 0.1% soluble starch
20 (Kokusan Chemicals, Tokyo), 0.06% sodium pyruvate, 0.03% K₂HPO₄, and 0.005%
21 MgSO₄·7H₂O. We prepared the solid medium (plate) by adding 1.2% gellan gum
22 (Kanto Chemical, Tokyo) at pH 3.0 or agar (Kokusan Chemicals) at pH 7.0 to the R2A
23 medium. The YDC medium consisted of: 1.0% yeast extract (BD), 2.0 % D(+)-glucose,
24 and 2.0 % casamino acid (Nihon Pharmaceutical).

1

2 **Isolation of bacteria and yeast strains**

3 For the bacterial and yeast isolation, we used both R2A(i) medium (R2A plate medium
4 adjusted to pH 3.0 with sulfuric acid) and R2A(ii) medium (R2A plate medium without
5 any addition). The water samples were concentrated 100-fold using an acid-resistant
6 0.2- μ m PTFE membrane-filter (H020A047A, Advantec, Tokyo). The enriched water
7 samples were appropriately diluted when needed, and a 100- μ l portion of each
8 preparation was spread on an R2A medium. After several days' incubation at 10°C and
9 25°C, colonies growing on the plate were picked up.

10 With the colonies thus obtained, we carried out the yeast isolation procedure.
11 R2A(iii) (R2A plate medium adjusted to pH 2.0, 1.5, and 1.0 with sulfuric acid) was
12 prepared to select acid-tolerant yeasts from the isolates. Using R2A(iv) (R2A plate
13 medium at pH 3.0 with the addition of 0.02% bromocresol purple), we separated the
14 yeasts capable of the neutralization of acid from the acid-tolerant yeasts by observing
15 the change of medium color from yellow to purple.

16

17 **Neutralization of casamino acid or amino acid solution at pH 3.0**

18 After strain CeA16 was precultured at 25°C for 1 day in YDC medium at pH 3.0 with
19 shaking, the yeast pellet obtained by centrifugation at 3,000 rpm was washed three
20 times with saline adjusted to pH 3.0 with sulfuric acid. The washed cell pellet was
21 added to 10 mL of each solution: 0.5% (w/v) casamino acid, a 0.8–2.2 mM 17 amino
22 acids mixture (Asp, Glu, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu,
23 Phe, Lys), or 20 mM single amino acid adjusted to pH 3.0 with sulfuric acid, and it was
24 then incubated at 25°C for 3 days with shaking.

1

2 **Identification of the yeasts by an ITS1 sequence analysis**

3 The isolated yeasts were identified by an ITS1 sequence analysis for efficiently
4 identifying yeast belonging to the genus *Candida* (15). To amplify the internal
5 transcribed spacer 1 (ITS1) region, we performed a polymerase chain reaction (PCR)
6 with the yeast-chromosomal DNA extracted by bead-beating, using the universal
7 primers ITS1F (5'-GTAACAAGGT(T/C)TCCGT-3') and ITS1R (5'-
8 CGTTCTTCATCGATG-3') and Premix Ex Taq (Takara Bio, Shiga, Japan). After the
9 purification of the amplified DNA fragments by a MinElute PCR Purification Kit
10 (Qiagen, Venlo, Netherlands), we determined their nucleotide sequence with a BigDye
11 Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA) using an
12 ABI Prism 3130 Genetic Analyzer (Applied Biosystems). We analyzed the similarities
13 among the ITS1 sequences by using the BLAST program (16).

14

15 **Amino acid analysis and measurement of the ammonium ion and pH values**

16 The amounts of amino acids were measured by the detection of the phenylthiocarbamyl
17 (PTC)-amino acids (17). The following procedure for the PTC derivatization was
18 performed. Aliquots of 20 μ l of ethanol/water/triethylamine (2/2/1, v/v/v) were added to
19 each vacuum-dried sample and then vacuum-dried again. For the PTC derivatization of
20 amino acids, aliquots of 20 μ l of ethanol/water/triethylamine/phenylisothiocyanate
21 (PITC) (7/1/1/1, v/v/v/v) were added to each sample and left standing at room
22 temperature for 20 min. After vacuum drying, 1.0 mL of PTC-derivatized amino acid
23 mobile phase A (Wako Pure Chemical Industries, Osaka, Japan) was added to each
24 sample for the high-performance liquid chromatography (HPLC) sample preparation of

1 the PTC-derivatized amino acid samples. The PTC-derivatized amino acid samples
2 were analyzed using a Prominence HPLC system (Shimadzu, Kyoto, Japan) through a
3 Wakosil-PTC column (200×4.0 mm; Wako) at 40°C at 1.0 mL min⁻¹ using an amino
4 acid mixture standard solution, Type H (Wako) as a standard.

5 The analytical conditions for the HPLC were as follows: mobile phase A, PTC-
6 amino acid mobile phase A; mobile phase B, PTC amino acid mobile phase B (Wako);
7 flow rate, 1.0 mL min⁻¹; gradient of mobile phase B, 0%–70% from 0–15.00 min
8 (linear), 75%–100% from 15.00–15.01 min; injection volume, 15 µl; and UV detection,
9 254 nm.

10 The amounts of ammonium ion were measured by the enzymatic method with F-
11 kit ammonium (JK International, Tokyo). The pH value was measured using a LAQUA
12 pH meter (F-72, Horiba, Kyoto, Japan).

13

14 **Nucleotide sequence accession number**

15 We have submitted the nucleotide sequences to DNA Data Bank of Japan (DDBJ)
16 under the accession numbers LC133464, LC133465, LC133466, LC133467, LC133463,
17 LC133468 and LC133469.

18

19 **RESULTS**

20 **Isolation of yeast strains possessing the ability to neutralize an acidic medium**

21 With the 94 isolates and 230 isolates obtained from upstream and downstream of the
22 neutralization plant in the Yukawa River using R2A(i) or (ii) medium, we performed a
23 screening to identify the microorganisms that could neutralize acid, using the R2A(iv)
24 medium at pH 3.0. As a result, 10 isolates from upstream and 22 isolates from

1 downstream of the neutralization plant in the Yukawa River changed the color of
2 medium containing a pH indicator from yellow to purple after 7 days' cultivation.
3 Therefore, we selected these 32 isolates as the yeasts capable of the neutralization of
4 acid. Then, among these 32 strains, five (CeA14, CeG17, EeB28, EeC21, GeC45) and
5 two strains (AeA6, CeA16) capable of growing on R2A(iii) medium at pH 1.5 and 1.0
6 respectively were identified on the basis of the DNA sequence analysis included
7 complete sequence of their ITS1 in this study (Table 1). Six of these seven isolates
8 showed identical sequence with *Candida fluviatilis* type strain and the other showed
9 identical sequence with *Candida palmioleophila* type strain. In particular, out of two
10 strains grown at pH 1.0, strain CeA16 of *C. fluviatilis* showed faster growth than strain
11 AeA6. We therefore focused on strain CeA16 to clarify the mechanism underlying the
12 acid neutralization in this study. Strain CeA16 grew well at 20°–25°C and showed a
13 strong ability to neutralize acid (data not shown).

14

15 **Neutralization of amino acid solutions by the isolated yeast strain**

16 To clarify the mechanism underlying the acid neutralization, we performed a
17 neutralization test with strain CeA16 using casamino acid, which is a component of
18 R2A medium, adjusted to pH 3.0 with sulfuric acid. Strain CeA16 elevated the pH from
19 3.0 to 7.3 in the casamino acid solution after 1 day (Fig. 1A). Strain CeA16 also
20 reduced the total amount of the 17 amino acids from 18.2 mM to 5.3 mM, whereas it
21 increased the ammonium ion from 1.4 mM to 15.9 mM.

22 As shown in Figure 1B, we detected 17 amino acids (Asp, Glu, Ser, Gly, His,
23 Arg, Thr, Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu, Phe and Lys) in the casamino acid
24 solution before the incubation of strain CeA16 in the HPLC analysis by the PTC

1 derivatization using an amino acid mixture standard solution, Type H, as a standard.
2 After 1 day, >95% of Ser, Arg, Thr and Ala+Pro and 76%–77% of Asp and Glu
3 decreased and were thought to be consumed by strain CeA16 (Fig. 1B). And, 87% of
4 Met and 73% of Lys decreased for two days.

5 To investigate the relationship between the consumption of amino acids and the
6 production of ammonium ions, we performed a neutralization test with strain CeA16
7 using a mixture of 17 amino acids (Asp, Glu, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val,
8 Met, Cys, Ile, Leu, Phe and Lys) and using single amino acid solutions adjusted to pH
9 3.0, respectively. After autoclaving and inoculating cells, the initial pH value changed a
10 little around 3 in the solutions. Strain CeA16 elevated the pH value from pH 3.1 to 7.3
11 in the mixture of 17 amino acids after 2 days, as well as in casamino acid. A decrease of
12 amino acids and an increase of ammonium ions were simultaneously observed in the
13 medium (Fig. 2A).

14 The results of the HPLC analysis showed that 10 amino acids (Asp, Glu, Ser, Gly,
15 Arg, Thr, Ala, Pro, Cys and Lys) were preferably consumed, whereas most of the
16 hydrophobic amino acids and His were hardly consumed (Fig. 2B). In the experiments
17 with single amino acids, seven (Glu, Ser, Lys, Ala, Pro, Asp and Arg) contributed to an
18 elevation of pH values from 2.9–3.1 to 7.7–8.4, and Ile showed an elevation of pH value
19 from 3.1 to 6.0 (Fig. 3A). Therefore, we monitored the consumption of these seven
20 amino acids (Glu, Ser, Lys, Ala, Pro, Asp and Arg) that contributed largely to an
21 elevation of pH values and the production of ammonium ions. The amounts of the
22 ammonium ions increased as these seven amino acids were consumed by strain CeA16
23 (Fig. 3B).

24

1 **DISCUSSION**

2 In this study, we isolated strain CeA16, which was capable of growing in a strongly
3 acidic medium and neutralizing the acidic medium by using several amino acids, and
4 identified as *Candida fluviatilis*. In previous reports, some yeast strains identified as *C.*
5 *fluviatilis* (18, 19), *C. intermedia* (19), and *R. glutinis* (4) were isolated from the various
6 extreme acidic environments and some of them had the ability to neutralize the acidic
7 medium (pH 2.5-3.0) (4, 19). However, there have been no reports of their mechanisms.
8 In the present study, strain CeA16 neutralized oligotrophic medium such as R2A medium,
9 but could not neutralize nutritious YPD medium (2% glucose, 1% polypeptone and 1%
10 yeast extract) adjusted to pH 3.0 (data not shown). These results might indicate that the
11 neutralization ability of strain CeA16 is mainly in oligotrophic environments.

12 As shown in Figure 3B, we observed that seven amino acids (Glu, Ser, Lys, Ala,
13 Pro, Asp and Arg) were preferably utilized by strain CeA16 as the single amino acid
14 source was decomposed to release ammonium ion. However, not only these seven
15 amino acids (Glu, Ser, Lys, Ala, Pro, Asp and Arg) but also three amino acids (Gly, Thr
16 and Cys), which did not contribute to an elevation of pH value in the experiments with
17 single amino acids (Fig. 3A), were preferably consumed under the mixture of amino
18 acids (Fig. 2B). In addition, the decrease rates of Met, Gly and Cys under the mixture of
19 amino acids (Fig. 2B) were different from those under casamino acid (Fig. 1B). These
20 differences might be also involved any metabolism related to amino acid in strain
21 CeA16 according to a ratio of amounts of amino acid.

22 In bacteria, several common mechanisms of acid tolerance are known, such as the
23 GAD system, in which glutamate is converted to GABA by GadA/B with the
24 consumption of one proton and GadC transports out GABA in an exchange of one

1 molecule of glutamate, and the Aid system, in which arginine is converted to CO₂ and
2 ammonia by enzymes encoded by the genes *arcABC* (11). Unlike these systems in
3 bacteria, the yeast strain CeA16 was able to utilize not only Glu and Arg but also Asp,
4 Ala, and Ser to neutralize the acidic medium. These results might indicate that strain
5 CeA16 tolerated acid by digesting one or more amino groups of several amino acids and
6 releasing ammonium ions.

7 In addition, there are other mechanisms of acid tolerance such as a proton pump,
8 which releases protons to extracellular fluid with ATPase in the cells (10, 11). Though
9 the detailed relationship between the proton pump and the neutralizing activity remains
10 unknown, strain CeA16 was observed to resist the acid stress for 1–2 days, as shown in
11 Figures 1A and 2A, until its surrounding pH became neutral by releasing ammonium
12 ions in the test tube. Therefore, strain CeA16 also might cope with acid by other
13 mechanisms such as the proton pump until its surrounding pH becomes neutral.

14 In conclusion, *C. fluviatilis* strain CeA16 neutralized strongly acidic solution
15 using not only an amino acid mixture but also a single amino acid of the seven amino
16 acids (Glu, Ser, Lys, Ala, Pro, Asp and Arg). *C. fluviatilis* strain CeA16 cells might have
17 acquired a wide substrate specificity for amino acids in order to neutralize their
18 surrounding as a survival strategy against strongly acidic environments. Our study
19 indicates that strain CeA16 is a potential yeast for bioremediation instead of chemical
20 neutralization by calcium carbonate.

21

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3

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- 10
- 11

1 **Figure legends**

2 **Fig. 1.** Neutralization of casamino acid solution by *C. fluviatilis* strain CeA16.

3 Cultivation was carried out for 3 days at 25°C under casamino acid solution. The initial
4 pH of the medium was adjusted to pH 3.0 with sulfuric acid before autoclaving. **(A)** The
5 measurements of the pH values and the amounts of ammonium ion and amino acids
6 over time. **(B)** The measurements of the amount of each amino acid over time. Each
7 value is the mean±SD (n=3).

8

9 **Fig. 2.** Neutralization of a mixture of amino acids by *C. fluviatilis* CeA16. Cultivation

10 was carried out for 3 days at 25°C under the mixture of amino acids. The initial pH of
11 the medium was adjusted to pH 3.0 with sulfuric acid before autoclaving. **(A)** The
12 measurements of pH values and the amounts of ammonium ion and amino acids over
13 time. **(B)** The measurements of the amount of each amino acid over time. Each value is
14 the mean±SD (n=3).

15

16 **Fig. 3.** Neutralization of single amino acid solutions by *C. fluviatilis* CeA16. Cultivation

17 was carried out for 3 days at 25°C under single amino acid solution. The initial pH of
18 the medium was adjusted to pH 3.0 with sulfuric acid before autoclaving. **(A)** The
19 measurements of the pH value of each amino acid solution over time. **(B)** The
20 measurements of the amount of each amino acid over time. Each value is the mean±SD
21 (n=3).

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