Isolation and Characteristics of an Acetone–Butanol-negative, Ethanol–Isovaleric Acid-producing Mutant of *Clostridium saccharoperbutylacetonicum* N1-4 ATCC 13564

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A mutant, AS2-1, was isolated by UV irradiation from *Clostridium saccharoperbutylacetonicum* N1-4 ATCC 13564 producing hyper butanol at the molar ratio of acetone–butanol–ethanol (3.3:9.5:1). The mutant proved to be deficient in the production of acetone, butanol, and acetic acid, but enhanced the ethanol (17.7 times increase) and newly produced isovaleric acid at a molar ratio of 1:1. The maximum production of ethanol and isovaleric acid reached 10.6 g/l (0.23 M) and 19.8 g/l (0.20 M) from glucose at 77.7 g/l (0.43 M) in TYA medium. As the equimolar ratio of ethanol and isovaleric acid was also produced in an amino acid-free minimal medium containing ammonium acetate as a nitrogen source, isovaleric acid was not produced from amino acids, but *de novo* synthesized from the ammonium and glucose.

Recently, the acetone–butanol–ethanol fermentation by *Clostridium* species^{1,2)} has been intensively studied for industrial fuel production. It is a problem that the clostridial fermentation is a heterofermentation, producing mainly acetone, butanol, and ethanol as “solvent”, together with acetic acid and butyric acid as by-products. Therefore, we would like to know the possibility of conversion of the solvent ratio of acetone, butanol, and ethanol by mutation of *Clostridium* species.

Genetic information on strict anaerobic Clostridia has not been well known. A few mutants of *Clostridium* has been isolated for genetic studies: mutant strains of *Clostridium thermocellum* and *Clostridium pasteurianum* were isolated by various mutagens such as UV light,^{3,4)} *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG),^{3,5)} and ethyl methane sulfonate (EMS)^{3,6)} as well as by spontaneous mutation^{3,7)}; mutants of *Clostridium acetobutylicum*, however, were only isolated by EMS^{8–11)} and spontaneous mutation.¹²⁾ Interestingly, the mutagenesis of *C. acetobutylicum* in comparison with thermophilic Clostridia was considered difficult by UV

light or MNNG. The relationship between Clostridia and mutagens remains doubtful.

This paper describes a novel type of ethanol–isovaleric acid fermentation by a UV-mutant derived from the hyper-butanol-producing *C. saccharoperbutylacetonicum* N1-4 ATCC 13564 to convert the fermentation products by mutagenesis.

Materials and Methods

Microorganisms. The microorganisms used were *Clostridium saccharoperbutylacetonicum* N1-4 ATCC 13564 as the parental strain and the mutant AS2-1. N1-4 ATCC 13564 had been isolated as the butanol-rich strain by Hongo *et al.*¹³⁾ The spores of N1-4 and AS2-1 mutant from N1-4, which were formed in 10 ml of Potato Glucose (PG) medium for 10 days, were mixed in 5 ml of sterile sea-sand to dry under a vacuum by a desiccator for storage at room temperature. Compared to the storage in humid conditions, a spore dies very slowly in sand storage. The spores were germinated to give rise to vegetative cells by heat shock (100°C, 1 min) and used as inocula.

Media. 1) PG medium contained: mashed potato, 150 g; glucose, 10 g; ammonium sulfate, 1 g; and CaCO₃, 3 g per liter of tap water. The preparation was boiled in a water bath for 45 min, filtered through gauze, and autoclaved at

1 kg/cm² for 1 hr. 2) Tryptone-yeast extract-ammonium acetate (TYA) medium contained: glucose, 40 g; KH₂PO₄, 0.5 g; MgSO₄·7H₂O, 0.3 g; FeSO₄·7H₂O, 10 mg; ammonium acetate, 3 g; yeast extract, 2 g; and bactotryptone (DIFCO), 6 g per liter of deionized water. The pH was adjusted to 6.5 with NaOH before autoclaving at 0.75 kg/cm² for 15 min. 3) Minimal medium for subculture contained: glucose, 40 g; KH₂PO₄, 0.5 g; MgSO₄·7H₂O, 0.3 g; FeSO₄·7H₂O, 10 mg; and ammonium acetate, 3 g per liter of deionized water. Compared to the TYA medium, the minimal medium did not contain yeast extract, and bactotryptone to eliminate amino acids. The pH adjustment and sterilization were done in the same manner as for the TYA medium.

Induction and isolation of mutants. All the processes of inoculation were done under anaerobic conditions by flushing with a 99.999% sterile nitrogen gas. Induction and isolation were done by the following steps. 1) One ml of PG culture of N1-4 was inoculated into 10 ml of fresh TYA medium and incubated at 30°C for 6 hr. 2) This culture (OD₆₆₀ 0.5) was transferred to a centrifuge glass tube (dia 15 mm, length 105 mm) and a butyl rubber stopper was quickly inserted. The cells were harvested by centrifuging (Model KC-20; Kubota Co., Japan) at 1200 × *g* for 6 min at room temperature. The pellet sediment was washed twice with fresh sterile TYA medium by centrifuging as above. 3) The washed cells were serially diluted with fresh sterile TYA medium containing no glucose. One-tenth ml of the dilution was transferred onto TYA medium containing 1.5% agar in a petri dish and spread by a spreader. The petri plates were put on a turntable under aerobic conditions and immediately irradiated under a continuous slow turning turntable beneath a UV lamp (wavelength 253.6 nm, distance 42 cm) at a distance of 20–30 cm for 5–40 sec in the dark. The UV-exposed cells were directly incubated in a BBL Gas Pak Jar at 30°C for 3 days in the dark. 4) After 3 days, the colonies were streaked onto TYA agar medium and incubated at 30°C for 3 days. The irradiated strains were inoculated into 10 ml of PG medium in a test tube and incubated in the BBL Gas Pak Jar at 30°C for 24 hr. One ml of the culture was taken out, anaerobically transferred into 10 ml of fresh TYA medium and incubated in a BBL Gas Pak Jar at 30°C for 24 hr. The cells were again inoculated and incubated until the end of fermentation under the same conditions as mentioned above. 5) The fermented TYA culture was centrifuged, and the products were detected by gas chromatography for selection of mutants.

Chemicals. Isovaleric acid (3-methyl butanoic acid) was purchased at Tokyo Kasei Co., Ltd., Tokyo, Japan. *Meso*-diaminopimelic acid (DAP) and LL-DAP were obtained from Sigma Chemical Co.

Fermentation procedure. TYA and minimal media were

used. Pre- and main cultures were done as the following steps. 1) The spores of parental N1-4 were used as a starting culture. 2) For heat shock, the tube containing the spores was placed in boiling water for 1 min, cooled immediately in running tap water, and incubated in an anaerobic BBL Gas Pak Jar (Becton Dickinson Co.) for 24 hr at 30°C. 3) For seed culture, 1 ml of the culture was inoculated in 10 ml of fresh PG medium and incubated for 24 hr in the BBL Gas Pak Jar. 4) For the main culture, a 10-ml PG culture at the mid exponential growth phase was transferred into 100 ml of fresh sterile TYA medium and incubated as a static culture for 18 hr at 30°C. Ten percent of the unwashed cells from the preculture were used for growth in the TYA medium and subculture in the minimal medium. But the tests of carbohydrate use as the sole carbon source were inoculated with 0.1% cells that had been washed with 0.15 M NaCl by centrifuging at 3000 × *g* for 5 min, and the process was repeated with 0.015 M NaCl.

Methods of analysis. Fermented cultures were centrifuged (Model KR-20000; Kubota Co., Japan) at 9800 × *g* for 15 min at 4°C. 1) Unknown products were analyzed by gas chromatography (Model 163; Hitachi Ltd., Japan) equipped with a flame ionization detector (FID) and connected with an automatic chromatogram-integrator (Shimadzu Chromatopac C-R3A). The U-shaped glass column (3 mm i.d. × 2 m) was packed with Chromosorb 101 (80–100 mesh) and run isothermally at 195°C. Injection and detector temperatures were 200°C. Nitrogen gas used as a carrier gas and flowed at the rate of 30 ml/min. Hydrogen and air flowed at the rate of 1 kg and 0.75 kg per cm², respectively. Also unknown compounds were analyzed by gas chromatography using a U-shaped glass column (3 mm i.d. × 2 m) packed with Chromosorb WAW-DMCS, DEGS+H₃PO₄ (2%+5%) and by the paper chromatography method using hydrazide.^{14,15} For identification of the structure of an unidentified product, the unknown compound was prepared by distillation at 100°C for the infrared spectrometer and separated by preparative gas chromatography using a U-shaped glass column (3 mm i.d. × 2 m) packed with PEGS+H₃PO₄ (2+0.5%) for the mass spectrometer. The column temperature was 80°C. The structure of the unknown product was confirmed by an elemental analyser (240 C Perkin Elmer) by GC-Mass spectrometer (VG 12-250, England), by an infrared spectrometer (Analect FX 6160, U.S.A.), and by nuclear magnetic resonance (proton) spectrometer (Bruker 200 MH2 NMR aspect 3000, Germany). 2) The cell mass concentration was measured at OD₆₆₀. 3) The centrifuged supernatants were used for analyses of fermented glucose and products. The reduced sugar was measured by the Somogyi-Nelson method¹⁶ using a UV-visible recording spectrophotometer (Model UV-240; Shimadzu Co., Japan). Products (acetone, butanol, ethanol, acetic acid, butyric acid, and isovaleric acid) were quantitatively analyzed by gas chromatography. A standard sample was made up from the aqueous mixture of

each 1% (w/v) solution of the authentic reagents of acetone, butanol, ethanol, acetic acid, butyric acid, isovaleric acid, and isobutanol. Preparation of samples were as follows: one ml of centrifuged supernatant was added to 6 ml double deionized water containing 0.84 mg of *meta*-phosphoric acid and 1% (w/v) isobutanol. Isobutanol was used as an internal standard. One ml of the sample was injected into the gas chromatograph. 4) Diaminopimelic acid (DAP) isomers and carbohydrates from hydrolysates of cells were separated by thin-layer chromatography.¹⁷⁾ 5) DNA was isolated by the method of Saito *et al.*¹⁸⁾ Guanine+cytosine (mol% G+C) of the base compositions of the DNA were measured by the melting point (T_m)¹⁹⁾ using an automatic recording spectrophotometer (Model UV-240, Shimadzu Co.). The mol% G+C was calculated from the equation of Marmur and Doty.¹⁹⁾

Results

Selection of mutants

A mutant designated as AS2-1 was selected from 3000 isolated colonies (20 cm, 10 sec, survival rate; $3.39 \times 10^{-2}\%$). The parent N1-4 produced acetone 2.5 g, butanol 9.1 g, and ethanol 0.6 g at the molar ratio of 3.3:9.5:1 and acetic acid 1.0 g and butyric acid 0.8 g per liter in TYA medium, but the AS2-1 mutant simply produced ethanol and an unidentified product. Acetone and butanol were not detected. Ethanol increased to 17.7 times (10.6 g/l) than that of the parent. Lactate was not detected in the culture.

Identification of products

The gas chromatographic identification of products of AS2-1 mutant in TYA medium is shown in Fig. 1. Retention times of isovaleric acid (3-methyl butanoic acid) from the authentic standard solution (Fig. 1A) and the unidentified product in the supernatant of the fermented culture (Fig. 1B) were the same at 5.64–5.65 min by Chromosorb 101 (80–100 mesh).

On the paper chromatography, the R_f values of authentic isovaleric acid (3-methyl butanoic acid), the evaporated supernatant of AS2-1 mutant culture, and the supernatant of the mutant culture were exactly the same at 0.81.

Elementary analysis of an unknown com-

pound found C 59.10, H 9.88%; *Anal.* Calcd. for $C_5H_{10}O_2$, C 58.71, H 9.80%.

The GC-mass spectrum of an unidentified product is shown in Fig. 2. The unidentified compound had a molecular ion at m/z 102. Mass spectrometry yielded a molecular weight that corresponds to the molecular formula $C_5H_{10}O_2$, and the mass spectrum had the following diagnostic peaks of fragment ions; 74, 60, 45, 43, 41, 39, and 29. These specific spectra corresponded to 3-methyl-butanoic

Fig. 1. Gas Chromatogram of Volatile Components from the Culture of AS2-1 Mutant.

Separation was done by Chromosorb 101. A: gas chromatogram of standard solution containing 1% (wt/vol) each solution of authentic ethanol, acetone, acetic acid, isobutanol (IS), *n*-butanol, *n*-butyric acid, and isovaleric acid; B: gas chromatogram of products of AS2-1 mutant in the fermented TYA medium at 30°C. 1, ethanol; 2, acetone; 3, acetic acid; IS, internal standard; 4, *n*-butanol; 5, *n*-butyric acid; 6, isovaleric acid.

Fig. 2. Mass Spectrum of Unidentified Product by AS2-1 Mutant.

Fig. 3. Infrared Spectrum of Unidentified Product by AS2-1 Mutant.

Fig. 4. Nuclear Magnetic Resonance (Proton) Spectrum of Unidentified Product by AS2-1 Mutant.

acid, $(\text{CH}_3)_2\text{-CH-CH}_2\text{-CO}_2\text{H}$.

The IR spectrum of a typical unidentified product is shown in Fig. 3. The broad band, multiple maxima, in the region of $3300\text{--}2500\text{ cm}^{-1}$ was diagnostic of the carboxylic acid as dimers due to strong hydrogen bonding; showed O-H stretching vibration at 3100 cm^{-1} and C-H stretching vibration at 2962 , 2938 , and 2878 cm^{-1} . The strong absorption band at 1710 cm^{-1} was typical for the stretching vibration of a -C=O structure in dimerized saturated aliphatic acids (R-COOH). The absorption at 933 cm^{-1} showed an O-H bond of a carboxylic group absent in monomers.

The NMR spectrum of an unidentified com-

pound is shown in Fig. 4. The carboxyl proton was found as the broad peak (weak) in the region $13\text{--}14\text{ ppm}$ and the other proton is shown in Fig. 4.

On the basis of these results, the unidentified product was identified as 3-methyl butanoic acid, also called isovaleric acid.

Morphological, biochemical, and physiological characteristics

Mutant AS2-1 was a straight rod ($0.5\text{--}0.7 \times 1.3\text{--}2.1\text{ }\mu\text{m}$); in a 24-hr culture in PG medium, motile with peritrichous flagella; no plasmid; and formed spores as did the parent.

As shown in Table I, the mutant had the same chemical components as the parent in the

Table I. CHARACTERISTICS OF AS2-1 MUTANT AND THE PARENT STRAIN

Characteristics	AS2-1 mutant	Parent strain
mol% G+C of DNA	25-28 (T_m^a)	25-29 (T_m)
DAP ^b isomer	<i>meso</i> -DAP	<i>meso</i> -DAP
Wall sugars	Glucose, Galactose, Rhamnose, Mannose	Glucose, Galactose, Rhamnose, Mannose
Motility ^c	+	+
Hydrogen	+	+
H ₂ S	-	-
Lecithinase	-	-
Indol	-	-
Lactic acid	-	-
Caproic acid	-	-
Propionic acid	-	-
Gelatin ^d	+	+

^a Method of thermal melting point.^b Diaminopimelic acid.^c +, motility; -, nonmotility; +, production; -, no production.^d Liquefaction.

DAP isomers and the carbohydrates of cell walls. The mutant was also the same in the GC content of the DNA bases as the parent. These results were similar to the *Clostridium*²⁰⁾ species which is 27-28 (T_m) of the mol% G+C of the DNA, and usually contains *meso*-DAP in the cell walls.

AS2-1 showed good use of mono- and disaccharides such as D(+)-glucose, mannose, and sucrose in the same manner as the parent (Table II). The mutant, however, could not ferment starch, glycogen, dextran, dextrin, lactose, methyl- α -D-glucoside, amygdalin, and melezitose, which were fermented by the parent (Table II). Characteristics of N1-4 and AS2-1 mutant were identical except for the glucosidase activities.

Growth and ethanol-isovaleric acid fermentation

As shown in Fig. 5, the log phase continued for 23 hr. Generation time of the mutant was slow at 4 hr while that of the parent was 1.6 hr. The fermentation products were ethanol, isovaleric acid, carbon dioxide, hydrogen, and a trace of *n*-butyric acid. Ethanol and isovaleric

Fig. 5. Course of Fermentation and Cell Growth of AS2-1 Mutant.

Symbols: ●—●, isovaleric acid; ■—■, ethanol; ○—○, Cell growth (OD): △—△, glucose; □—□, pH. Fermentation was done in TYA medium at 30°C.

Fig. 6. Effects of Temperature on Fermentation of AS2-1 Mutant.

Symbols: ●—●, isovaleric acid; ■—■, ethanol.

Fig. 7. Effects of Initial pH on Fermentation of AS2-1 Mutant.

Symbols: ●—●, isovaleric acid; ■—■, ethanol.

acid from 0.2 M consumed glucose reached 0.11 M (5.46 g/l) and 0.11 M (11.8 g/l), respectively, at 34 hr. And 0.28 M carbon dioxide was produced (Table III). The yields of ethanol and isovaleric acid from consumed sugar were 13.9% and 30.0%, respectively. Carbon recovery was 92.4% (Table III).

Table II. USE OF CARBOHYDRATES BY AS2-1 MUTANT

Carbohydrates	Products (g/l)						
	AS2-1 mutant		Parent strain				
	EtOH ^a	C ₅ acid ^b	EtOH	Acetone	BuOH ^c	C ₂ acid ^d	C ₄ acid ^e
Arabinose	5.65	9.17	0.83	1.44	10.46	0.86	—
Fructose	5.68	9.73	1.01	1.70	12.60	0.79	—
Galactose	5.00	6.74	0.72	1.05	8.74	0.66	0.20
Glucose	6.01	11.99	0.60	2.50	9.10	1.00	0.80
Mannose	5.79	11.73	1.03	1.55	12.01	0.36	—
Rhamnose ^g	0.25	—	0.12	0.61	1.24	2.34	2.31
Ribose ^f	1.58	1.23	0.01	0.41	1.00	2.20	0.49
Xylose	4.35	5.23	1.57	1.41	11.90	0.50	—
Sorbose ^g	0.23	—	—	0.31	0.63	1.94	0.44
Cellobiose	5.95	9.18	0.46	1.88	11.22	0.54	—
Lactose	—	—	0.52	1.13	8.04	0.71	0.41
Maltose	2.80	2.45	1.45	1.63	12.37	0.36	—
Sucrose	5.87	12.06	1.00	1.63	12.34	0.40	—
Melibiose	2.56	3.01	0.56	1.43	13.46	0.54	0.34
Trehalose	5.68	10.30	0.77	1.47	9.46	0.58	—
Raffinose	5.10	7.20	0.57	1.23	8.22	0.53	0.50
Melezitose ^g	—	—	—	0.31	0.96	1.75	0.68
Dextran	—	—	—	—	—	—	—
Dextrin	—	—	0.96	1.68	10.84	0.60	—
Glycogen	—	—	0.97	1.28	10.12	0.71	0.29
Inulin ^f	—	—	—	0.31	0.92	1.99	0.60
Starch ^f	—	—	0.53	1.59	9.50	0.53	0.58
Pectin ^f	0.53	0.62	0.12	1.10	3.49	1.21	0.58
Amygdalin	—	—	—	0.24	2.35	1.48	2.04
Esculin ^f	0.98	0.40	0.57	1.00	5.44	1.42	0.45
Salicin	1.70	1.95	0.14	1.02	5.03	1.25	—
α-Methyl-D-glucose	—	—	0.83	1.41	9.54	0.62	0.32
meso-erythritol	—	—	—	0.27	0.61	1.88	0.41
Inositol ^g	0.16	—	—	0.30	0.92	1.92	0.54
Mannitol ^g	5.86	3.49	1.07	0.42	7.24	0.46	—
Glycerol	—	—	—	0.33	1.11	1.89	0.56
Dulcitol	—	—	—	0.32	0.91	1.81	0.59
Sorbitol ^g	5.23	2.96	—	0.28	1.03	1.81	0.71
Adonitol ^f	—	—	—	0.25	0.67	1.85	0.46
Milk	—	—	0.21	0.26	1.67	1.56	1.55
Cellulose	—	—	—	0.31	0.75	1.73	0.60

^a Ethanol. ^b Isovaleric acid. ^c Butanol. ^d Acetic acid. ^e Butyric acid. ^f One gram of the substrate was added to 100 ml TYA medium as the carbon source. ^g Two grams of the substrate was added to 100 ml TYA medium. Four grams of the others were added as the substrate to the TYA medium.

On the other hand, ammonium acetate in TYA medium was completely used in 12 hr in the mid-exponential phase.

Culture conditions for ethanol-isovaleric acid fermentation

AS2-1 grew in a wide range of temperature,

20 to 40°C (Fig. 6) and the initial pH was 5.0 to 9.5 (Fig. 7). The maximum productions of ethanol and isovaleric acid were 10.6 g/l (0.23 M) and 19.8 g/l (0.20 M), and the yields of ethanol and isovaleric acid from consumed glucose (77.6 g/l, 0.43 M) were 13.6% and 25.5%, respectively. Compared to the parent,

Table III. CARBON RECOVERY AND HYDROGEN RECOVERY ON FERMENTATION BY AS2-1 MUTANT

Glucose consumed	200 mM	Carbon consumed 1200 mM	H ₂ consumed 1200 mM
	Product formed (mM)	Carbon recovered (mM)	H ₂ recovered (mM)
Ethanol	118.51	237.02	355.53
Isovaleric acid	115.53	577.65	577.65
<i>n</i> -Butyric acid	3.06	12.24	12.24
CO ₂	282	282	
H ₂	101.78		101.78
Total		1108.91	1047.20
Carbon recovery		92.4%	
H ₂ recovery			87.3%

Table IV. SUBCULTURE OF AS2-1 MUTANT IN MINIMAL MEDIUM FREE FROM AMINO ACIDS

Succession times of subcultures ^a	Products (g/l)	
	Ethanol	Isovaleric acid
1st	3.57	7.61
2nd	3.28	7.88
3rd	3.10	7.56
4th	3.00	7.41
5th	3.06	7.39
6th	3.21	7.55
7th	3.10	7.53

^a Transferred every two days.

ethanol was increased 17.7 times.

In the fermented TYA medium with 8% (w/v) sucrose as the sole carbon source, the maximum production of ethanol and isovaleric acid reached 10.3 g/l (0.22 M) and 20.0 g/l (0.20 M), respectively. Upon the addition of 14% sucrose into the TYA medium, the mutant grew well, but the parent autolyzed.

Subculture in the minimal medium free from amino acids

In the minimal medium free from amino acids, AS2-1 mutant produced the same products of ethanol and isovaleric acid as in TYA medium, to the extent of 0.07 M (3.2 g/l) and

0.07 M (7.6 g/l), respectively, during subculturing in minimal medium (Table IV). The yields of ethanol and isovaleric acid from consumed glucose (15.3 g/l) were 12.9% and 30.7%, respectively. The molar ratio of ethanol and isovaleric acid was the same at 1:1 as in the fermented TYA medium.

Discussion

It has been reported that the resting cells of *Clostridium bifermentans* or *C. difficile* produced isovaleric acid from valine, leucine, and isoleucine by a reaction of transamination or oxidative deamination in the cell suspension containing the amino acids.^{21,22} *E. coli* assimilated isovaleric acid as an intermetabolite in the biosynthesis of L-valine and L-leucine from pyruvate.²³ Hongo *et al.*²⁴ and Ueda *et al.*²⁵ reported that the parent of the AS2-1 mutant, *Clostridium saccharoperbutylacetonicum* N1-4 ATCC 13564, produced alanine through pyruvate and *o*-butylhomoserine, passing acetyl-CoA from glucose and ammonium acetate as the nitrogen source in the biotin-containing TYA medium.

On the other hand, AS2-1 constantly produced ethanol and isovaleric acid at the molar ratio of 1:1 in the amino acid-free minimal medium containing glucose as the sole carbon source and ammonium acetate as the sole nitrogen source. These results indicated that AS2-1 produced isovaleric acid through pyruvate as an intermediary metabolite of valine biosynthesis.

Dürre²⁶ reported that spontaneous mutants (strains No. 1–10) of *C. acetobutylicum* DSM 1732 were defective in butanol formation, but produced acetone and ethanol. AS2-1, however, did not produce any butanol, acetone, and acetic acid, but enhanced the ethanol and newly produced isovaleric acid. Therefore, we suggest that AS2-1 had a new clostridial isovaleric acid pathway causing blockage of the pathways of butanol, acetone, and acetic acid.

We will describe the metabolic mechanism of the novel type clostridial ethanol–isovaleric acid fermentation of AS2-1 mutant in our next

paper.

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