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Engineering *Corynebacterium ammoniagenes* for urocanic acid production from glucose

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ABSTRACT

Urocanic acid (UA), an intermediate in L-histidine catabolism, is a unique heterocyclic compound with various bioactivities. This study demonstrates UA production from glucose as a carbon source using engineered *Corynebacterium ammoniagenes*. The wild-type strain *C. ammoniagenes* NBRC 12071 was subjected to disruption of *hutU*, which encodes urocanate hydratase. After 7 days of cultivation with a minimum medium containing 2% glucose, the resulting strain MM1 produced 2.0 ± 0.1 mg/L UA with 0.29 ± 0.00 mg/g-cells/day specific production rate, while the wild-type produced a trace amount of UA. This indicated that the disruption arrested UA consumption. MM1 was spontaneously mutated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, forming 3-amino-1,2,4-triazole-resistant strains, some of which produced 0.16–0.29 g/L UA from 2% glucose. This indicates that the disruption of *hutU* was useful for constructing spontaneous mutants that produce UA. To improve UA production, *hutH*, which encodes histidine ammonia lyase, was overexpressed in MM1 using the strong *C. ammoniagenes* promoter P_{rpl21}. After 7 days of cultivation, the resulting strain MM5 produced 7.7 ± 0.3 mg/L UA with 1.73 ± 0.12 mg/g-cells/day specific production rate. This indicates that overexpression strengthened the L-histidine catabolism. The use of a semi-synthetic medium would help improve the growth of engineered strains. To the best of our knowledge, this is the first report on UA production by engineered *C. ammoniagenes*.

1. INTRODUCTION

Metabolites exhibit diverse chemical structures and bioactivities. To utilize these metabolites industrially, a production system should be developed [1,2]. Urocanic acid (UA) is a heterocyclic compound found on animal skin. Its *trans* isomer exhibits UV-protective activity by absorbing ultraviolet (280–310 nm) radiation while isomerizing to the *cis* isomer [3,4]. In addition, UA has potential as a pharmaceutical compound because it strongly inhibits natural killer cell activity [5]. Furthermore, 4-vinylimidazole, which is obtained by decarboxylating UA, can be polymerized into a vinyl monomer in polymer materials [6]. Thus, UA is a promising metabolite for use in the pharmaceutical and industrial fields. UA is biosynthesized as follows: First, glucose is metabolized through the pentose phosphate pathway (PPP) to 5-phospho-α-D-ribose 1-diphosphate (PRPP), which is then metabolized to L-histidine by a series of enzymes encoded by the L-histidine synthesis (*his*) gene cluster. Finally, L-histidine is converted into L-glutamic acid by a series of enzymes encoded by the L-histidine degradation (*hut*) gene cluster [Figure 1]. L-Histidine is degraded to UA by L-histidine ammonia lyase (*hutH*) and further to imidazol-4-one-5-propionic acid (IP) by urocanate hydratase (*hutU*). To date, few studies have investigated microbial UA production. Kisumi *et al.* reported that *Serratia marcescens* SR41 mutants produced 10.5 g/L UA from 70 g/L glucose [7,8]. In contrast, Kobayashi *et al.* reported that HutU activity-deficient *Corynebacterium ammoniagenes* ATCC 6872 produced 7.2 g/L UA from 10 g/L L-histidine [9]. Furthermore, a mutant conferring resistance to histidine analogs such as 3-amino-1,2,4-triazole (3-AT) produced 7.3 g/L UA from 120 g/L glucose. This study suggests that UA can be produced by disrupting *hutU* and overexpressing *hutH* in *C. ammoniagenes*. However, UA production using genetically engineered *C. ammoniagenes* has not yet been reported, as the genetic engineering of *C. ammoniagenes* has only recently been established.

C. ammoniagenes is a coryneform bacterium used in the industrial production of various metabolites such as amino acids and nucleotides [10]. *C. ammoniagenes* has high ammonia production capacity and shows optimal growth at pH 7.0–8.5 [11]. In 2017, the complete genome sequence of *C. ammoniagenes* 9.6 (ATCC 6871) was deposited in GenBank. This revealed that *C. ammoniagenes* has *his* and *hut* gene clusters and produces UA as an intermediate metabolite in the histidine degradation pathway. In contrast, *Corynebacterium glutamicum* does not possess the *hut* gene cluster. Thus, L-histidine degradation is a characteristic metabolic process in

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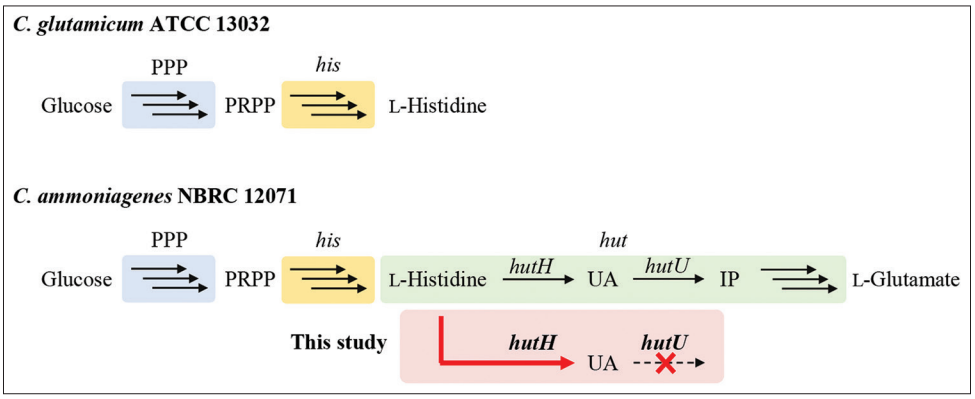

Figure 1: Biosynthesis of L-histidine and urocanic acid from glucose in *Corynebacterium glutamicum* ATCC 13032 and *Corynebacterium ammoniagenes* NBRC 12071. The box indicates the engineering demonstrated in this study. PPP: Pentose phosphate pathway, PRPP: 5-phospho-α-D-ribose 1-diphosphate, IP: 4-imidazolone-5-propanoate, *his*: L-histidine synthesis genes, *hut*: L-histidine degradation genes.

C. ammoniagenes [12,13]. Several studies on the genetic engineering of *C. ammoniagenes* have been reported. Koizumi and Teshiba overexpressed the riboflavin synthesis gene in *C. ammoniagenes* and consequently succeeded in producing 15.3 g/L riboflavin [14]. They used a DNA fragment from *C. ammoniagenes* ATCC 6872 genomic DNA, which showed high homology with the valine tRNA promoter from *Bacillus subtilis*, as the promoter. Stolle *et al.* overexpressed the small subunit of ribonucleotide reductase in *C. ammoniagenes* ATCC 6872 using the tac promoter, which is functional in both *Escherichia coli* and *C. ammoniagenes* [15]. Hou *et al.* cloned various promoters from *C. ammoniagenes* ATCC 6871 and examined the fluorescence intensities after fusion with the red fluorescent protein gene [16]. Consequently, we identified a strong 50S ribosomal protein promoter (P_{rpl21}). As the expression vector for *C. ammoniagenes*, pXMJ19 has been constructed [17]. This shuttle vector contains both pUC and pBL1 replicons that function in *E. coli* and *C. glutamicum*. Electroporation can also be used to transform *C. ammoniagenes* [18]. To determine the role of the cysteine methionine regulator gene in sulfur metabolism in *C. ammoniagenes*, Lee *et al.* demonstrated gene disruption in *C. glutamicum* through homologous recombination [19].

These findings suggest that genetically engineered *C. ammoniagenes* can produce UA. This study aimed to demonstrate UA production from glucose by disrupting *hutU* and overexpressing *hutH* in *C. ammoniagenes* [Figure 1]. The goal of this study is to develop an industrial UA production process. Glucose was used in this study because it is a fundamental substrate. In addition, it would be possible to make UA production a sustainable process by using alternative low-cost sources, such as raw materials and organic waste, instead of glucose. UA is expected to be more soluble under alkaline conditions than under neutral conditions because the p*K*a of UA is 6.1 [20,21]. This suggests that alkaline conditions are suitable for microbial UA production. As noted previously, *C. ammoniagenes* grows under weakly alkaline conditions, suggesting that it is suitable for UA production [11]. In addition, to demonstrate the usefulness of the *hutU* disruptant constructed in this study, we attempted to construct spontaneous mutants from the disruptant with 3-AT.

2. MATERIALS AND METHODS

2.1. Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. The primer sequences and plasmid maps are presented in Table S1 and Figure S1, respectively. A schematic diagram of the construction of engineered *C. ammoniagenes* NBRC 12071 is shown in Figure S2. In this study, the type strain *C. ammoniagenes* NBRC 12071 (ATCC 6871) was used as the parent strain for UA production. The backbone of plasmid pUC18 [22] and *hutU* derived from *C. ammoniagenes* NBRC 12071 were amplified by PCR with pUC18 and *C. ammoniagenes* NBRC 12071 genome, respectively, as templates using the primer sets pUC18_F and pUC18_R and hutU-5′-F and hutU-3′-R, respectively. PCR was performed using Q5 DNA polymerase (New England Biolabs Inc., Ipswich, MA, USA). The amplified fragments were ligated using Gibson Assembly Master Mix (New England Biolabs Inc.) to obtain the plasmid pUC18–hutU. To insert the kanamycin resistance gene into *hutU* on pUC18–hutU, PCR amplicons were obtained with pUC18–hutU and pK18mobsacB [23], respectively, as the templates using the primer sets hutU-3′-F and hutU-5′-R and KanR_F and KanR_R, respectively, and then ligated using the Gibson Assembly, resulting in a plasmid pUC18–ΔhutU. The backbone of plasmid pXMJ19 [17], P_{rpl21} derived from *C. ammoniagenes* NBRC

Table 1: Plasmids and strains used in this study.

Plasmids and strains	Description	Source
Plasmids		
pUC18	*Escherichia coli* vector, Ap^R	[21]
pK18mobsacB	Km^R source	[22]
pUC18–ΔhutU	pUC18 harboring *hutU::*Km^R	This work
pXMJ19	*Escherichia coli–Corynebacterium glutamicum* shuttle vector, Cm^R	[16]
pGreenTIR	*gfp* source	[23]
pXMJ19–P_{rpl21}–gfp	pXMJ19 harboring P_{rpl21} and *gfp*	This work
pXMJ19–P_{rpl21}–hutH	pXMJ19 harboring P_{rpl21} and *hutH*	This work
Strains		
Escherichia coli DH5α	Cloning host	Nippon Gene Co.
Corynebacterium ammoniagenes NBRC 12071	Type strain	NBRC
MM1	NBRC 12071 (*hutU*::Km^R)	This work
MM2	NBRC 12071 (pXMJ19)	This work
MM3	NBRC12071 (pXMJ19–P_{rpl21}–gfp)	This work
MM4	NBRC12071 (pXMJ19–P_{rpl21}–hutH)	This work
MM5	MM1 (pXMJ19–P_{rpl21}–hutH)	This work

12071, and green fluorescent protein gene (*gfp*) were amplified by PCR from pXMJ19, *C. ammoniagenes* NBRC 12071 genome, and pGreenTIR [24], respectively, as templates using the primer sets pXMJ19-F and pXMJ19-R, P_{rpl21}-F and P_{rpl21}-R, and pXMJ19–P_{rpl21}–gfp-F and pXMJ19–P_{rpl21}–gfp-R, respectively. The amplified fragments were ligated using Gibson Assembly, forming the plasmid pXMJ19–P_{rpl21}–gfp. *C. ammoniagenes* NBRC 12071 was transformed with pXMJ19 and pXMJ19–P_{rpl21}–gfp through electroporation [18], resulting in the MM2 and MM3 transformants, respectively. Electroporation was done under the condition of 12.5 kV/cm, 25 μF, and 200 Ω. To replace the *hutH* derived from *C. ammoniagenes* NBRC 12071 with *gfp* on pXMJ19–P_{rpl21}–gfp, PCR amplicons were obtained with pXMJ19–P_{rpl21}–gfp and *C. ammoniagenes* NBRC 12071 genome as templates using the primer sets pXMJ19-F3 and pXMJ19-R3 and hutH-F and hutH-R, respectively, and then ligated using Gibson Assembly, resulting in the plasmid pXMJ19–P_{rpl21}–hutH. The pXMJ19–P_{rpl21}–hutH plasmid was introduced into *C. ammoniagenes* NBRC 12071 and MM1 to generate MM4 and MM5 transformants, respectively. While culturing the transformants and gene disruptants, 20 mg/L chloramphenicol and 30 mg/L kanamycin were added to the medium. The *hutU* and *hutH* sequences encoded in the genome of *C. ammoniagenes* NBRC 12071 were obtained from the GenBank database under accession number CP009244.

After *C. ammoniagenes* NBRC 12071 was treated with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG), the cell viability was determined [Figure S3]. *C. ammoniagenes* MM1 was similarly treated with 2000 μg/mL NTG and cultivated on minimum medium [0.1 g/L KH_2PO_4, 0.3 g/L K_2HPO_4, 2 g/L urea, 3 g/L NH_4Cl, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 10 mg/L $FeSO_4{\cdot}7H_2O$, 1 mg/L $ZnSO_4{\cdot}7H_2O$, 4 mg/L $MnSO_4{\cdot}H_2O$, 0.2 mg/L $CuSO_4{\cdot}5H_2O$, 10 mg/L $CaCl_2{\cdot}2H_2O$, 40 mg/L L-cysteine hydrochloride·H_2O, 10 mg/L thiamin hydrochloride, 0.06 mg/L biotin, 20 mg/L calcium pantothenate, 9 g/L tricine-NaOH (pH 8.5)] containing 2% glucose [25] and 1 mg/mL 3-AT. After cultivating at 37°C for 3 days, 12 3-AT resistant colonies were obtained.

2.2. Effect of Culture pH on *C. ammoniagenes* NBRC 12071 Growth

C. ammoniagenes NBRC 12071 was pre-cultivated in 5 mL IFO 802 medium (10 g/L hipolypepton, 2 g/L yeast extract, 1 g/L $MgSO_4{\cdot}7H_2O$) for 1 d at 30°C with shaking. The culture was inoculated into 30 mL IFO 802 medium supplemented with 0.1 M MOPS (pH 7.0), tricine-NaOH (pH 8.5), or CAPS (pH 10.0) at OD_{600} of 0.1. The OD_{600} and pH of the cultures were monitored during cultivation at 30°C with shaking. This cultivation was performed in triplicate, and the average was determined with errors indicating the standard deviations.

2.3. Gene Disruption

C. ammoniagenes NBRC 12071 was transformed with pUC18–ΔhutU by electroporation method [18]. The resulting transformant MM1 was obtained after cultivation on the brain heart infusion (BHI; BD Difco, NJ, USA) agar plates containing 91.1 g/L sorbitol and 30 mg/L kanamycin for 3 days at 30°C. *hutU* disruption was confirmed by PCR [Figure S4].

2.4. UA Production

A series of strains were pre-cultured in 5 mL BHI medium for 1 day at 30°C with shaking. The culture was inoculated into 30 mL BHI medium at OD_{600} of 0.1, cultivated for 1 day at 30°C with shaking, and then washed with saline solution. For UA production using resting cells, the cells were resuspended in 0.1 M sodium phosphate buffer (pH 7.0) containing 10 mM L-histidine at OD_{600} of 5.0 and then incubated for 3 days at 30°C with shaking. This cultivation was performed in duplicate, and the average was determined with error bars indicating the standard deviations. For UA production using growing cells, the cells were inoculated into 50 mL minimum medium supplemented with 2% glucose at OD_{600} of 0.1 and then cultivated for 7 days at 30°C with shaking. The supernatants of the cell suspensions and cultures were analyzed for UA and L-histidine production. This cultivation was performed in duplicate, and the average was determined with error bars indicating the standard deviations. The UA titer, specific production rate, and yield from glucose obtained in this study are summarized in Table S2.

2.5. Evaluation of GFP Expression with P_{rpl21}

After MM2 and MM3 were cultivated in 5 mL BHI medium containing 20 mg/L chloramphenicol for 1 day at 30°C with shaking, the cells were washed and resuspended in saline solution. The fluorescence of the cell suspensions was monitored using a spectrofluorometer FP-6500 (JASCO, Tokyo, Japan). A 490-nm light was used for excitation, and the emission wavelength was set at 510 nm.

2.6. Analysis Methods

A glucose CII Test Kit (Fujifilm Wako Co., Osaka, Japan) was used to determine the glucose concentration in the cultures. L-Histidine and UA in the culture supernatant were derivatized with dabsyl chloride [26] and analyzed using high-performance liquid chromatography. The analytes were eluted with solvent A (50 mM sodium acetate buffer [pH 6.5]) and solvent B (the same buffer containing 70% acetonitrile) under gradient conditions. The gradient was 100–30% solvent A from 0 to 1 min, 30–0% solvent A from 1 to 25 min, 0% solvent A from 25 to 30 min, 100% solvent A from 30 to 40 min, and 0% solvent B from 30 to 40 min. Calibration curves of L-histidine and UA after derivatization are shown in Figure S5.

The dry cell weight of *C. ammoniagenes* was calculated by correlating it with the OD_{600} value (1 OD_{600} = 0.29 g-cells/L). In detail, *C. ammoniagenes* were dried at 80°C for 18 h and then the dry cell weight was obtained to determine the correlation of OD_{600} to dry cell weight (g). The specific growth rate (μ) was calculated as the slope of the regression line from a plot of $\ln(X_t/X_0)$ and time (t) during the exponential growth period, where X_t (g-cells/L) and X_0 (g-cells/L) are the cell concentrations at t (h) and at the beginning of the exponential phase, respectively. The specific production rate of UA was calculated as follows: Specific production rate = $(UA_t - UA_0)/(X_t - X_0) \times \mu$; UA_t, the UA concentration (mg/L) at t (h); UA_0, the UA concentration (mg/L) at the beginning of the exponential phase.

3. RESULTS AND DISCUSSION

3.1. Effect of Culture pH on *C. ammoniagenes* NBRC 12071 Growth

C. ammoniagenes grows under weak alkaline conditions [11]. To confirm *C. ammoniagenes* growth under alkaline conditions believed to be suitable for microbial UA production, wild-type strain *C. ammoniagenes* NBRC 12071 was cultivated at pH 7.0, 8.5, and 10.0. As expected, the strain grew at pH 7.0 (μ = 0.30 ± 0.12 h^{-1}) and pH 8.5 (μ = 0.37 ± 0.07 h^{-1}) but did not grow at pH 10.0 [Figure 2]. While the pH of the designed medium remained relatively stable across all treatments owing to its buffering capacity, *C. ammoniagenes* growth was well supported at an initial pH of 8.5. Based on this result,

pH 8.5 was used as the culture pH in subsequent experiments for UA production.

3.2. Effect of *hutU* Disruption on UA Production

It was confirmed that *hutU* was disrupted as expected because *the hutU* disruption locus was amplified by PCR without an unspecific amplicon [Figure S4]. To evaluate the effect of *hutU* disruption on UA production, resting cells of *C. ammoniagenes* NBRC 12071 and the *hutU*-disruptant MM1 were incubated for 3 days with 10 mM L-histidine. *C. ammoniagenes* NBRC 12071 and MM1 consumed 5.1 ± 0.8 and 4.0 ± 0.4 mM L-histidine and produced 0.2 ± 0.0 µM and 4.2 ± 0.3 mM UA, respectively [Figure 3a]. Thus, *hutU* is suggested to be expressed in *C. ammoniagenes* NBRC 12071, and UA yield from L-histidine by MM1 was nearly 100%. This finding suggests that *hutU* is not expressed in MM1 and that *hutU* is a unique gene for UA conversion in *C. ammoniagenes* NBRC 12071 and that *hutU* disruption is effective for UA production. Interestingly, L-histidine consumption in MM1 was reduced than in the wild-type strain. This suggests that HutH activity was reduced by product inhibition, and consequently, L-histidine consumption was reduced. Such inhibitory effects of UA on HutH have been observed in some microbes, such as *Pseudomonas putida* [12] and *Aspergillus nidulans* [27].

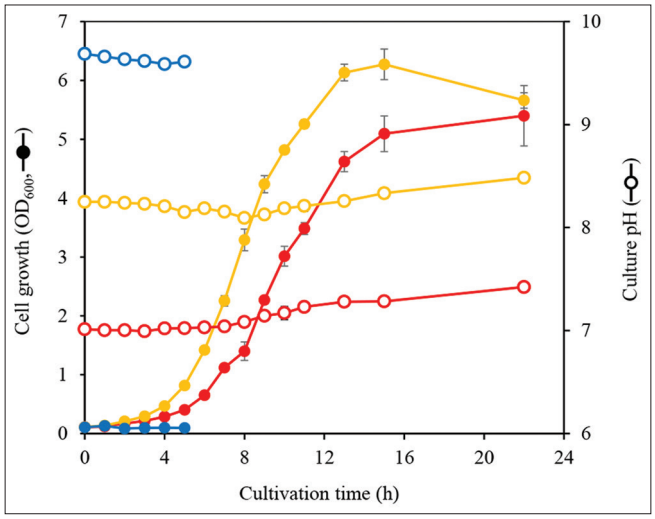

Figure 2: Effect of culture pH on growth of *Corynebacterium ammoniagenes* NBRC 12071. The strain was cultivated in IFO 802 medium supplemented with 0.1 M MOPS (pH 7.0, red), tricine-NaOH (pH 8.5, yellow), or CAPS (pH 10.0, blue). Solid symbols, cell growth (OD_{600}); open symbols, culture pH. The cultivation at pH 10.0 was stopped at 5 h because the strain showed no growth. This cultivation was performed in triplicate, and the average was represented with error bars indicating the standard deviations.

C. ammoniagenes NBRC 12071 growing cells produced a trace amount of UA (0.62 mg/L) from 2% glucose even after 7 days of cultivation, while MM1 produced 2.0±0.1 mg/L UA with 0.29±0.00 mg/g-cells/day specific production rate [Figure 3b]. This indicates that *hutU* disruption in *C. ammoniagenes* enables the direct UA production from glucose. Both strains showed similar profiles of glucose consumption and cell growth; however, their UA production and L-histidine consumption profiles were different. Cell growth showed a lag time in the initial phase, which may be due to the use of minimum medium in this study. Particularly, MM1 showed less cell growth (OD_{600} = 2.23 ± 0.36) than the wild-type strain (OD_{600} = 6.55 ± 1.90) after 120 h cultivation. This may be due to the lack of L-glutamate in MM1 because UA is the precursor of L-glutamate. *C. ammoniagenes* NBRC 12071 genome contains a gene encoding glutamate dehydrogenase (Gdh), which generates L-glutamate from 2-oxoglutarate. Gdh may supply L-glutamate to MM1, and consequently, partially support cell growth. *C. glutamicum* produces L-glutamate from 2-oxoglutarate through Gdh in the same manner [28]. These findings also suggest that L-glutamate supplementation or *gdh* overexpression in the MM1 strain improves cell growth and UA production. The kanamycin resistance marker derived from pK18mobsacB, which has often been used for gene disruption in *C. glutamicum* [29,30], was used for *hutU* disruption in this study, suggesting that the resistance marker did not affect the metabolism of *C. ammoniagenes*.

Kobayashi *et al.* have constructed a spontaneous mutation from HutU activity-deficient *C. ammoniagenes* ATCC 6872 using a series of histidine analogs, including 3-AT, which consequently enhanced

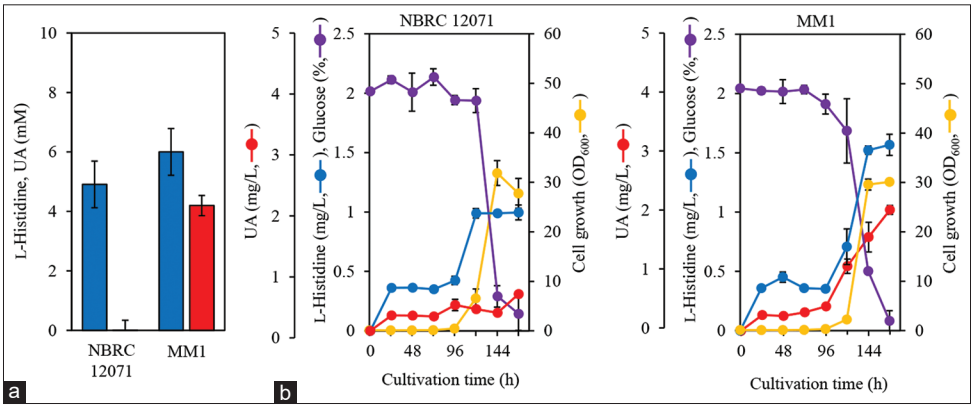

Figure 3: (a) Production of urocanic acid (UA; red bars) from 10 mM L-histidine (blue bars) in the resting cells of *Corynebacterium ammoniagenes* NBRC 12071 and MM1. The cells were resuspended in 0.1 M sodium phosphate buffer (pH 7.0) containing 10 mM L-histidine at OD_{600} of 5.0 and then incubated for 3 days at 30°C with shaking. This assay was performed in triplicate, and the average was presented with error bars representing standard deviations. (b) Cell growth and L-histidine and UA production from 2% glucose in the growing cells of *C. ammoniagenes* NBRC 12071 and MM1. The cells were inoculated to 50 mL minimum medium supplemented with 2% glucose at OD_{600} of 0.1 and then cultivated for 7 days at 30°C with shaking. Red, UA concentration; blue, L-histidine concentration; yellow, cell growth (OD_{600}); purple, glucose concentration. This cultivation was performed in duplicate, and the average was represented with error bars indicating the standard deviations.

UA production [9]. To prove that the *hutU*-disruptant MM1 can be also used for constructing UA-producing spontaneous mutants, we constructed a 3-AT resistant MM1 mutant using NTG treatment. Prior to the construction of the mutant, the conditions for NTG treatment of *C. ammoniagenes* NBRC 12071 were optimized. The results clarified that treating with 2000 µg/mL NTG for 30 min resulted in a 0.97% cell viability [Figure S3]. We concluded that this condition was suitable for the mutation. Using these conditions, 12 3-AT resistant strains were obtained from the *hutU* disruptant. Interestingly, of which, 3 3-AT resistant strains produced 0.16–0.29 g/L UA from 2% glucose (data not shown). The isolation efficiency of mutants producing UA at non-negligible levels was calculated to be 25%. This indicates that the *hutU* disruptant was useful for constructing UA-producing spontaneous mutants.

3.3. Effect of *hutH* Overexpression on UA Production

hutH overexpression was used to improve UA production. Recently, it was reported that the strong promoter P_{rpl21} works in *C. ammoniagenes* ATCC 6871. Therefore, we selected this promoter for *hutH* overexpression. To evaluate the function of P_{rpl21}, its fluorescence level was investigated by measuring *gfp* expression using the promoter in *C. ammoniagenes* NBRC 12071. The fluorescence intensity of the *gfp*-expressing strain MM3 was 5.7 times higher than that of the non-expressing strain MM2 [Figure S6]. This indicated that P_{rpl21} functions in *C. ammoniagenes* NBRC 12071.

To evaluate the effect of *hutH* overexpression on UA production, resting cells of MM4 (*hutH*-overexpressing wild-type strain) and MM5 (*hutH*-overexpressing MM1) were incubated for 3 days with 10 mM L-histidine. MM4 and MM5 consumed 9.2 ± 0.6 and 8.1 ± 0.4 mM L-histidine and produced 3.3 ± 0.2 µM and 8.0 ± 0.6 mM UA, respectively [Figure 4a]. Thus, the UA yields for L-histidine consumption in MM4 and MM5 were 0.04% and 98.8%, respectively. Comparing the wild-type strain and MM4 clarified that *hutH* overexpression resulted in 80.4% enhanced L-histidine consumption. This indicates that *hutH* expression level in the wild-type strain was insufficient for UA production. In addition, the UA produced in MM4 was mostly degraded, indicating that *hutU* disruption is essential for UA production in *C. ammoniagenes*. However, L-histidine consumption in MM5 was double that of MM1. This indicates that the effect of *hutH* overexpression on UA production was limited. Compared to MM4, MM5 increased UA production (8.0 ± 0.6 mM) but decreased L-histidine consumption (8.1 ± 0.4 mM). This decreased L-histidine consumption may be due to product inhibition. Similar levels of UA production and L-histidine consumption indicate that *hutH* overexpression has an additive effect on UA production in *hutU* mutant.

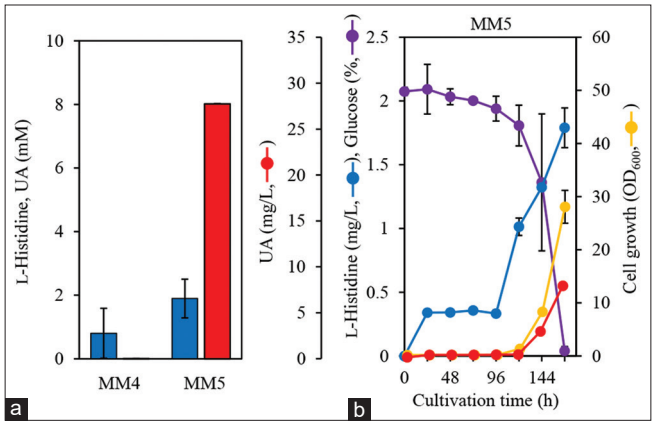

Figure 4: (a) Production of urocanic acid (UA; red bars) from 10 mM L-histidine (blue bars) in the resting cells of MM4 and MM5. The cells were resuspended in 0.1 M sodium phosphate buffer (pH 7.0) containing 10 mM L-histidine at OD_{600} of 5.0 and then incubated for 3 days at 30°C with shaking. This assay was performed in triplicate, and the average was presented with error bars representing standard deviations. (b) Cell growth and L-histidine and UA production from 2% glucose in growing cells of MM5. The cells were inoculated to 50 mL minimum medium supplemented with 2% glucose at OD_{600} of 0.1 and then cultivated for 7 days at 30°C with shaking. Red, UA concentration; blue, L-histidine concentration; yellow, cell growth (OD_{600}); purple, glucose concentration. This cultivation was performed in duplicate, and the average was represented with error bars indicating the standard deviations.

MM5 cells cultured in minimum medium containing 2% glucose for 7 days produced 7.7 ± 0.3 mg/L UA with 1.73 ± 0.12 mg/g-cells/day specific production rate. The specific production rate was 6.0 times higher than that of MM1 [Figure 4b]. This indicates that *hutH* overexpression partially improved UA production when glucose was used as the carbon source. One possible reason for the lack of a significant enhancement in UA production is that L-histidine production may be one of the rate-limiting steps in UA production. In addition, MM5 showed significant less growth (OD_{600} = 8.1 ± 0.4) than MM1 (OD_{600} = 29.6 ± 1.1) after 144 h. During cultivation, the L-histidine level in MM5 was similar to that in MM1. These findings suggest that *hutH* overexpression has negative effects on cell growth and energy consumption. Kobayashi *et al.* demonstrated to produce UA using a *C. ammoniagenes* mutant cultivated in a semi-synthetic medium supplemented with 10 g/L meat extract [9]. The negative effects observed in this study would be recovered by adding such nutrient components to the medium.

4. CONCLUSION

In this study, we demonstrated the production of UA from glucose by disrupting *hutU* and overexpressing *hutH* in *C. ammoniagenes* NBRC 12071. Alkaline conditions were suitable for growing *C. ammoniagenes* NBRC 12071. After 7 days of cultivation with 2% glucose, *C. ammoniagenes* NBRC 12071 and its *hutU* disruptant produced 0.62 and 2.0 mg/L of UA, respectively. This indicates that *hutU* disruption improved UA production. The NTG mutation test revealed that the disruption of *hutU* was useful for constructing UA-producing spontaneous mutants. After 7 days of cultivation with 2% glucose, *hutU*-deleted and *hutH*-overexpressed *C. ammoniagenes* NBRC 12071 produced 7.7 mg/L of UA. This indicates that *hutH* overexpression strengthened the L-histidine catabolism. The use of a semi-synthetic medium would help improve the growth of engineered strains. To the best of our knowledge, this is the first report on UA production by engineered *C. ammoniagenes*.

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6. AUTHOR'S CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in

drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data are available to the authors and shall be provided upon request.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI) tools for writing and editing the manuscript, and no images were manipulated using AI.

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