

Identification of Two Major Ammonia-Releasing Reactions Involved in Secondary Natto Fermentation

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Natto is a traditional Japanese food made from soybeans fermented by strains of *Bacillus subtilis* natto. It gives off a strong ammonia smell during secondary fermentation, and the biochemical basis for this ammonia production was investigated in this study. When natto was fermented by strain r22, ammonia production was shown to involve degradation of soybean proteins releasing amino acids, and only the glutamate contained in the natto obviously decreased, while the other amino acids increased during secondary fermentation. Strain r22 has two active glutamate dehydrogenase genes, *rocG* and *gudB*, and inactivating both genes reduced ammonia production by half, indicating that deamination of glutamate was one of the major ammonia-releasing reactions. In addition, urease encoded by *ureABC* was found to degrade urea during secondary fermentation. A triple mutant lacking *rocG*, *gudB*, and *ureC* exhibited minimal ammonia production, suggesting that the degradation of urea might be a further ammonia-releasing reaction.

Key words: natto; *Bacillus subtilis*; ammonia; glutamate dehydrogenase; urease

Strains of *Bacillus subtilis* natto are gram-positive bacteria used in the fermentative production of natto.^{1–3)} The strains are also known as rich producers of poly- γ -glutamate (γ -PGA),^{4–6)} which is synthesized by polymerizing D- and L-glutamate.^{7,8)} Natto strains usually exhibit higher protease activities than *B. subtilis* 168, a well-characterized laboratory strain.⁹⁾ In addition, natto strains are able to secrete large amounts of proteases, breaking down soybean proteins into amino acids, which serve not only as nutrients for bacterial growth, but also as substrates producing two important characteristics of natto, the viscous texture and a specific smell. The viscous texture of natto depends on the γ -PGA in the biofilm that forms on the surface of soybeans after

fermentation; this is why spiderweb-like strings are produced when natto products are stirred. Around 100 volatile compounds emitted from natto have been identified,¹⁾ indicating the complexity of its specific smell. Tanaka and colleagues¹⁰⁾ have reported that the smell of natto foods was mainly characterized by 10 volatile compounds, including ethanol, 2,3-butanedione (diacetyl), pyrazine, 2-methylpyrazine, 3-hydroxy-2-butanone (acetoin), 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2-methylpropanoic acid (iso-butyric acid), 2-methylbutanoic acid, and 3-methylbutanoic acid (iso-valeric acid). Among these compounds, diacetyl and pyrazines are thought to bring out the preferred natto aroma, while the others cause unpleasant and very strong smells that mask the aroma. Two branched-chain fatty acids, iso-valeric acid and iso-butyric acid, are thought to be the main compounds responsible for the unpleasant smell. Recently, these fatty acids were found to be synthesized by leucine degradation involving a reaction catalyzed by leucine dehydrogenase (LeuDh) in a natto strain,¹¹⁾ and a LeuDh-defective mutant exhibited a dramatic decrease in these fatty acids. Already, inactivation of LeuDh has been used successfully to breed a new generation of natto starters to produce odorless and low-odor natto products commercially.

On the other hand, natto often gives off a very strong smell of ammonia, similarly to strong cheese. This odor sometimes causes problems in the quality control of natto products. Ammonia is not usually detected just after the main natto fermentation process,¹⁰⁾ but depending on the conditions of storage, a large amount of ammonia is readily released through excess (so-called secondary) fermentation. Once a natto product is exposed to relatively high temperatures (25–35 °C), secondary fermentation commences to emit ammonia into the air. This is one of the reasons a natto food must be usually chilled to lower than 10 °C after the main fermentation; this chilling minimizes ammonia produc-

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Abbreviations: γ -PGA, poly- γ -glutamate; LeuDh, leucine dehydrogenase; 2-OG, α -ketoglutarate; GlutDH, glutamate dehydrogenase

Table 1. Bacterial Strains and Plasmids Used in This Study

	Relevant genotype or description	References
<i>E. coli</i> strain		
JM109	<i>recA1, endA1, gyrA96, thi, hsdR17, relA1, Δ (lac-proAB)/F' [traD36, proAB⁺, lacI^q, lacZΔM15]</i>	35)
Natto starter strain		
r22	<i>bio</i>	11)
SKAPR1	<i>bio aprE::ermC</i>	This study
SKGUD1	<i>bio rocG::ermC</i>	This study
SKGUD2	<i>bio gudB::aad</i>	This study
SKGUD3	<i>bio rocG::ermC gudB::aad</i>	This study
SKURE1	<i>bio ureC::tetL</i>	This study
SKRGU1	<i>bio rocG::ermC gudB::aad ureC::tetL</i>	This study
Plasmid		
pUC19	Cloning vector	35)
pT7Blue	Cloning vector	36)
pAE194	Carrying erythromycin resistance (<i>ermC</i>) gene	21)
pIC333	Carrying spectinomycin resistance (<i>aad</i>) gene	22)
pHY300PLK	Carrying tetracyclin resistance (<i>tetL</i>) gene	23)

tion. Koguchi and colleagues¹²⁾ measured ammonia production from liquid cultures of a natto strain grown on various nitrogen sources and predicted that ammonia is released from the degradation of at least six amino acids: glutamate, alanine, glutamine, arginine, aspartate, and asparagine. Although the true sources of ammonia remain to be identified, the possible ammonia-producing reactions involving these amino acids are as follows: Glutamate can be converted to α -ketoglutarate (2-OG) with the generation of ammonium by catabolic glutamate dehydrogenase (GlutDH).¹³⁾ At least two genes coding for catabolic GlutDH, *rocG* and *gudB*, have been identified in *B. subtilis* 168, but only RocG is functional, because *gudB* was found to encode an inactivated form with the insertion of three additional amino acid residues.¹⁴⁾ Glutamine is degraded by glutaminases, which might be encoded by *ybgJ* and/or *ylaM*, to yield glutamate and ammonium.¹⁵⁾ In other organisms, it is known that the amino group of alanine can be transferred to 2-OG to yield glutamate, although this has not been demonstrated in strains of *B. subtilis*. Arginine is also converted to glutamate through its catabolic pathway, with the generation of urea as a byproduct in the conversion of arginine to ornithine, and then two molecules of ammonium are released from one urea molecule by urease.¹⁶⁾ Asparaginase releases ammonium from asparagine to produce aspartate, and subsequently aspartase converts aspartate to fumarate and ammonium.¹⁷⁾ Thus, there are at least five possible ammonia-releasing reactions involving the six amino acids listed above.

Because ammonia production is easily noticed by the specific smell and because it clearly indicates the deterioration of a natto product, a promising strategy to prevent ammonia production is required to guarantee quality in commercial production. Therefore, in the food industry, product storage has been stringently managed.

Nevertheless, the ultimate solution to this problem will not be achieved until the biochemical basis for ammonia production is identified and eliminated. Here, we report that combined biochemical and genetic analysis demonstrate two major ammonia-releasing reactions involving three key enzymes. A mutant lacking all these enzymes was shown to lose the major part of its ammonia-releasing ability during the secondary natto fermentation process.

Materials and Methods

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* natto strain r22 was used as the parental strain, a mutant derived from *B. subtilis* natto strain O-2, a natto starter with no genetic competence¹⁸⁾ used commercially.¹¹⁾ r22 has acquired competence by mutagenesis and is able to make natto with the same quality as that made by the parental strain, O-2. Natto strains were grown in a nutrient broth medium (1% polypeptone (Nihon Seiyaku, Tokyo, Japan), 0.5% NaCl (Sigma-Aldrich Japan, Tokyo, Japan), and 0.5% meat extract (OXOID, Hampshire, England) for proliferation, in modified Spizizen's minimal medium (0.25% glucose (Kanto Chemical, Tokyo, Japan), 5 mM MgSO₄·7H₂O (Sigma-Aldrich Japan), 0.6% KH₂PO₄ (Kanto Chemical), 1.4% K₂HPO₄ (Kanto Chemical), 0.1% sodium citrate (Sigma-Aldrich Japan), 0.1 µg/ml biotin (Wako Pure Chemical Industries, Osaka, Japan))¹⁹⁾ with various amino acids as nitrogen sources (0.2%) to test their availability, and in a sporulation medium (0.0068% KH₂PO₄ (Kanto Chemical), 0.0535% NH₄Cl, (Sigma-Aldrich Japan), 0.0106% Na₂SO₄ (Wako), 0.00965% NH₄NO₃ (Wako), 0.000006% FeCl₂·4H₂O (Wako), 0.00126% MnCl₂·4H₂O (Katayama Chemical, Osaka, Japan), 0.934%

Table 2. DNA Primers Used in This Study

Primer	Sequence ^a
rocG-N	CCAAAAAACAATGAGGTGAAAAAG
rocG-C	TATTTTGGCGAGGCTTCTCAAATTA
gudB-N	AGAAGATTCTAGGAGGTTAACTCAA
gudB-C	TATTTTATGCAAATCATCAACTTA
ureABC-N	TATGACAAAAGAAGGAGGACTACAG
ureABC-C	GATGCACTCCGCCGCGTCTTATCA
aprE-F1	ACATGCATGCTTAAAGCTTACAATCAC
aprE-F2	GCTCTAGACAATTTTGTCTTCTCAC
aprE-B1	CGGGATCCCGCAGCTGCACAATAATAG
aprE-B2	GGggtaccCGGCCGCGCCATTACG
rocG-F1	CGGCGAGCTCAATATGTTTCATATCAGCACG
rocG-F2	CGGGATCCCTTTTTCACCTATTGTTTT
rocG-B1	GCTCTAGATTGAGAAGCCTCCGCAAAA
rocG-B2	ACATGCATGCTAAGGATAGCTCGTGATCA
gudB-F1	ACATGCATGCTAAGGATAGCTCGTGATCA
gudB-F2	GCTCTAGACCGGTGTTTTCGATCGGCTGCC
gudB-B1	CGGGATCCCTTTCGCGTTTTAGAGGCTGG
gudB-B2	GGggtaccTAATCGATCCTTTGGACTTC
ureC-F	TGAAACTGACACCAGTTGAACAAG
ureC-B	TTATTCCGCTTCGCTTACCCTGTG
ermC-F	CGGGATCCCGTGTCTATAAATTACTAATT
ermC-B	GCTCTAGATCCCTTAACCTACTTATTAATA
aad-F	GCTCTAGAGTATAATAAAGAATATTAATC
aad-B	GCGGATCCCTAAATTAAGTAATAAAGCGTTCTC
tetL-F	TCCCCGCGGCTGTATAAAAAAAGGATCAA
tetL-B	TCCCCGCGGTATTATTGCAATGTGGAATTG

^aUnderline, doublet underline, dotted underline, small letters, italic letters, and bold letters indicate *SphI*, *XbaI*, *BamHI*, *KpnI*, *SacI*, and *SacII* sites respectively.

MgSO₄·7H₂O (Sigma-Aldrich Japan), 0.238% sodium glutamate (Sigma-Aldrich Japan), 0.025% CaCl₂·2H₂O (Sigma-Aldrich Japan), 0.152% yeast extract (Becton, Dickinson and Company, Sparks, MD)) to prepare spores. *E. coli* JM109 was used as the cloning host to construct recombinant plasmids and was grown in LB²⁰⁾ containing the required antibiotics.

Nucleotide sequencing. Nucleotide sequences of the *rocG*, *gudB*, and *ureABC* regions of strain r22 were determined as follows: To determine the *rocG* region, a 1.3-kb long DNA fragment was amplified from the r22 chromosome DNA by PCR with primer pair rocG-N/rocG-C (Table 2), which was designed based on the sequence of strain 168 *rocG*. The amplified fragment was cloned into *E. coli* JM109 on pT7Blue with ampicillin resistance (50 µg/ml), and sequenced using a DNA sequencer (Applied Biosystems 3130xl Genetic Analyzers, Foster City, CA). Similarly, one DNA fragment corresponding to the *gudB* region and another to the *ureABC* region¹⁶⁾ were amplified with specific-primer pairs of gudB-N/gudB-C and ureABC-N/ureABC-C (Table 2) respectively, cloned, and then sequenced. The nucleotide sequences determined in this study are available in the GSDB, DDBJ, EMBL, and NCBI databases under accession nos. AB370869, AB370870, and AB370871, respectively.

Construction of *B. subtilis natto* mutant strains. The

mutant strains listed in Table 1 were constructed from the parent strain r22 as follows: For construction of SKAPR1 with inactivation of *aprE*, two DNA fragments were amplified by PCR with specific primer pairs and chromosomal DNA of r22 as a template; one fragment corresponded to an upstream flanking stretch of the *aprE* coding region, and the other corresponded to a downstream one. The specific primer pairs used were aprE-F1/aprE-F2 (Table 2) for the *aprE*-upstream fragment, and aprE-B1/aprE-B2 for the *aprE*-downstream one. The *aprE*-upstream fragment was trimmed with *SphI* and *XbaI*, and the *aprE*-downstream one with *BamHI* and *KpnI*. Another 0.8-kb fragment containing an erythromycin resistance gene (*ermC*) was also amplified by PCR using plasmid pAE194²¹⁾ as a template and the primer pair ermC-F/ermC-B (Table 2), and it was trimmed with *XbaI* and *BamHI*. The above three fragments were linked tripartitely in the order *aprE*-upstream, *ermC*, and *aprE*-downstream, and then ligated to the *SphI* and *KpnI* arms of pUC19 with ampicillin resistance (50 µg/ml). In this way, a recombinant plasmid in *E. coli* JM109 was obtained. Using the plasmid as a template, a 4.8-kb DNA fragment was amplified by PCR employing the primer pair aprE-F1/aprE-B2. The amplified fragment was used to transform r22 into erythromycin resistance (0.5 µg/ml). In such transformants, the region between the *aprE*-upstream and *aprE*-downstream stretches was deleted and replaced with *ermC* through a double-crossover event. The correct replacement of *aprE* was confirmed by southern hybridization analysis. After this confirmation, one of the transformants was designated SKAPR1.

The construction of SKGUD1 and SKGUD2, with inactivation of *rocG* and *gudB* respectively, was performed similarly to the construction of SKAPR1, except for alterations, as follows: For *rocG* inactivation, *rocG*-upstream and *rocG*-downstream fragments were amplified using two pairs of primers, rocG-F1/F2 and rocG-B1/B2 respectively (Table 2). For *gudB* inactivation, the *gudB*-upstream and *gudB*-downstream fragments were used two pairs of primers, gudB-F1/F2 and gudB-B1/B2 respectively (Table 2). Instead of the *ermC* cassette, a spectinomycin resistance gene (*aad*) cassette was used in the gene replacement of *gudB*. The *add* cassette fragment was prepared by PCR using plasmid pIC333²²⁾ as a template and the primer pair aad-F/aad-B (Table 2). The right transformants were selected on plates containing spectinomycin (100 µg/ml).

In the construction of SKURE1 with inactivation of *ureC*, one DNA fragment containing the *ureABC* locus and another containing a tetracycline-resistance gene (*tetL*) were prepared. The 4-kb *ureABC* fragment was amplified by PCR using the primer pair ureC-F/ureC-B (Table 2), and it was cloned into pT7Blue. The *ureABC* plasmid was digested with *SacII*, thereby cleaving the coding region of *ureC* in the middle. A 1.5-kb *tetL* fragment was amplified from the plasmid pHY300PLK²³⁾ with the primer pair tetL-F/tetL-B

(Table 2). The *tetL* fragment was trimmed with *Sac*II and ligated to the *Sac*II arm of the *ureABC* plasmid to provide another recombinant plasmid. Using the plasmid as a template, a 5.5-kb DNA fragment was amplified by PCR with the primer pair *ureC-F/ureC-B* and subjected to transformation of r22 into tetracycline resistance (10 µg/ml). It was verified by southern analysis that one of the transformants possessing the correct *ureC* disruption by the insertion of *tetL* provided SKURE1.

SKGUD3 was constructed by transforming SKGUD1 with the DNA of SKGUD2 into spectinomycin resistance in addition to the original erythromycin resistance. Finally, SKRGU1 was obtained by transforming SKGUD3 with the DNA of SKURE1 into additional tetracycline resistance.

Preparation of spore and natto fermentation. Spores of natto strains were prepared as starters of natto fermentation in the following manner: Cells of natto strains from fresh colonies on the nutrient broth plate were inoculated into 5 ml of nutrient broth liquid medium containing 0.5% glucose, and were allowed to grow at 37 °C overnight with shaking at 150 rpm. The culture (0.1 ml) was diluted in 10 ml of sporulation medium, further incubated at 37 °C for 24 h with shaking at 150 rpm, and then kept as a stock culture at 4 °C. The number of heat-resistant spores contained in the stock culture was determined by its CFU. Steamed soybean (100 g) was inoculated with 10⁶ spores, incubated at 39 °C for 18 h, and then stored at 4 °C for 24 h.

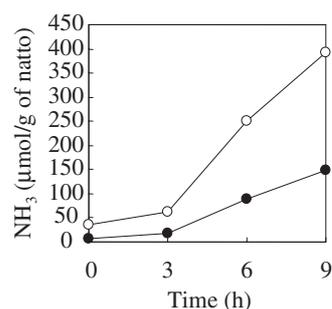
Measurement of ammonia, amino acids, and urea. The natto stored at 4 °C was transferred into an incubator kept at 37 °C to initiate secondary fermentation. At various time points, aliquots of natto (30 g) were extracted from incubator, immediately put into an airtight container with an interior volume of 2 liters, and kept at 20 °C for 30 min. Then the content of ammonia emitted from the natto sample in the container was measured using an ammonia gas detector tube (Gastec, Kanagawa, Japan). To determine the composition and concentration of free amino acids and urea contained in the natto sample, the sample was homogenized, suspended in 50 ml of 0.2 N HCl, and incubated at 4 °C overnight. After paper filtration, 25 ml of filtrate was mixed with 20 ml of 3% sulfosalicylic acid and filtered once again. The filtrate was adjusted to pH 2.2 with LiOH powder, and then its aliquot was subjected to concentration determination using a JCL-500/V AminoTacTM amino acids analyzer (JEOL, Tokyo, Japan). The concentration of metabolite derived from 1 g natto was indicated as µmol per 1 g natto.

Results

Preferential consumption of glutamate during secondary natto fermentation

It has been proposed that the ammonia produced

A



B

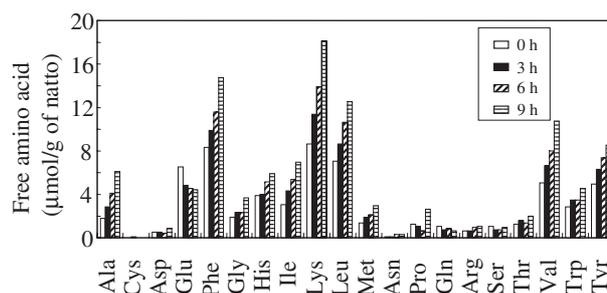


Fig. 1. Ammonia Production and Concentration of Free Proteinic Amino Acids in Natto during Secondary Fermentation.

A, Ammonia emitted from natto fermented by strain r22 (open circles, parental strain) and SKAPR1 (closed circle, *aprE::ermC*) during secondary fermentation. The concentration of ammonia emitted in a container by natto was determined as described at the indicated time points after initiation of secondary fermentation. B, Concentration of the 20 proteinic amino acids in natto fermented by r22. Amino acids were extracted from homogenized natto samples with HCl, and their concentration in the extract solution was determined as described at the indicated time points during secondary fermentation.

during secondary natto fermentation might be derived from soybean proteins,¹²⁾ but this has not been confirmed experimentally. Among the proteases secreted by *B. subtilis* natto, the major alkali protease encoded by *aprE*²⁴⁾ is known to be induced during stationary growth, and it is likely to function during secondary fermentation. A mutant strain, SKAPR1, with inactivation of *aprE* was constructed, and its ammonia production during secondary fermentation was compared with that of the parental strain, r22 (Fig. 1A). The data clearly demonstrated that AprE protease was required for ammonia production. These results suggested that a large part of the ammonia might be derived from the amino acids in soybean proteins degraded by AprE protease secreted from the bacterial cells.

The amino acid-utilizing ability of strain r22 was evaluated by growing it in a minimal medium supplemented with various amino acids instead of ammonium sulfate as a nitrogen source, and it turned out that amino acids belonging to the glutamate family (glutamate, glutamine, arginine, and proline) and aspartate (asparagine) supported faster growth than the other amino acids (data not shown). Since glutamine, arginine, and

GudB	168	76	GPTKGGIRFHPNVTEKEVKAVKALS	SIWMSLKCGIID	111
GudB1	168	76	GPTKGGIRFHPNVTEKEVKA---	LSIWMSLKCGIID	108
GudB	r22	76	GPTKGGIRFHPNVTEKEVKA---	LSIWMSLKCGIID	108
RocG	r22	77	GPTKGGVRFHPNVTEKEVKA---	LSIWMTLKCGIAN	109
RocG	168	77	GPTKGGVRFHPNVTEKEVKA---	LSIWMTLKCGIAN	109
			*****	*****	*****

Fig. 2. Partial Sequence Alignment of GlutDHs of *B. subtilis* Strain 168 and Natto Strain r22.

One of the most conserved regions in the amino acid sequences of the GlutDHs of *B. subtilis*, corresponding to residues from the 76th to the 111th of GudB of strain 168, is shown in an alignment. Asterisks indicate the conserved residues of the five GlutDHs: GudB, GudB1 (a mutant allele), RocG of 168, and GudB and RocG of r22.

proline can readily be converted to glutamate, and asparagine to aspartate, the results indicated that the natto strain might utilize glutamate and aspartate as the most preferable nitrogen source. The concentration of each of the 20 proteinic amino acids released during secondary fermentation was measured, and it was found that only glutamate exhibited an obvious decrease during secondary fermentation, while all the others increased or remained almost constant (Fig. 1B), implying that the bacteria might consume more glutamate than that produced by the degradation of soybean proteins. These results strongly suggest that glutamate is the amino acid consumed most preferentially during secondary fermentation.

Involvement of GlutDH in ammonia production

The glutamate metabolism pathway involves a reaction catalyzed by GlutDH converting glutamate to 2-OG and ammonium.¹³⁾ In *B. subtilis* strain 168, at least two paralogous genes for GlutDH, *rocG* and *gudB*¹⁴⁾ have been characterized within the genome, and the same has been reported for several other bacterial species.^{25,26)} Compared with the *gudB* genes from the other species,^{25,26)} the original 168 *gudB* is known to code for an inactive enzyme with an extra insertion of three amino acid residues (Fig. 2), and its active form is known as a mutant allele of *gudB1* lacking the insertion.¹⁴⁾ DNA fragments corresponding to the *rocG* and *gudB* loci of the r22 chromosome were cloned and sequenced. The predicted amino acid sequence of r22 *rocG* was entirely identical to that of 168 *rocG* (data not shown). On the other hand, the sequence of r22 *gudB* appeared to be identical to that of the *gudB1* allele, without the extra insertion that is found in the 168 *gudB* (Fig. 2). Therefore, it was highly probable that strain r22 possesses at least two active GlutDH, RocG and GudB.

To assess the contributions of RocG and GudB to ammonia production, two mutants, SKGUD1 and SKGUD2, with inactivation of *rocG* and *gudB* respectively, and a double mutant, SKGUD3, inactivating both *rocG* and *gudB*, were constructed from strain r22 (Table 1). Ammonia production during secondary fermentation by these mutants was measured (Fig. 3). SKGUD1 with the *rocG* single inactivation showed only a slight effect, while SKGUD2 with the *gudB* single

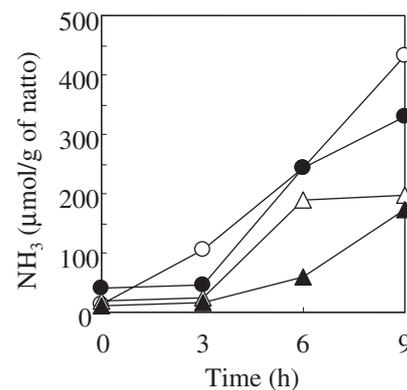


Fig. 3. Ammonia Production by GlutDH-Defective Strains.

Ammonia emitted by natto fermented by r22 (open circles, parental strain), SKGUD1 (closed circles, *rocG::ermC*), SKGUD2 (open triangles, *gudB::aad*), and SKGUD3 (closed triangles, *rocG::ermC gudB::aad*) was determined as described at the indicated time points after initiation of secondary fermentation.

inactivation proved to be more efficient at reducing ammonia production (Fig. 3), suggesting that r22 GudB functions as the major GlutDH. Furthermore, among the three mutants, SKGUD3, lacking both *rocG* and *gudB*, exhibited the most effective repression of ammonia production. All through the 9 h of secondary fermentation, the ammonia production level of SKGUD3 remained less than 50% of that of r22 (Fig. 3). Thus the results suggest that almost half of the ammonia is derived from glutamate degradation by RocG and GudB.

Evidence of consumption of urea during secondary fermentation

In addition to glutamate, arginine and proline are also relatively abundant in soybeans.²⁷⁾ RocG plays an important role in arginine catabolism and is induced in the presence of arginine or proline.¹⁴⁾ Hence we further investigated the possibility that arginine and proline are involved in ammonia production. It is known that proline is readily converted to glutamate through two reaction steps without the generation of ammonia.¹⁴⁾ On the other hand, arginine is converted to glutamate in three steps, and the first step is the conversion of arginine to ornithine, generating urea as a byproduct.

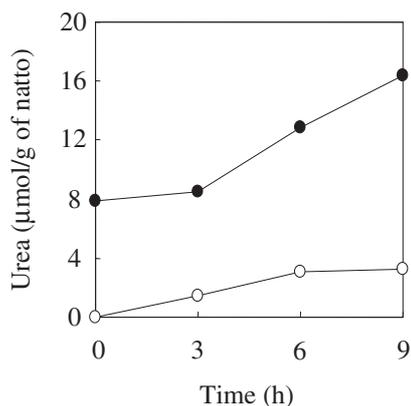


Fig. 4. Accumulation of Urea in Natto.

The concentration of urea in natto fermented by r22 (open circles, parental strain) and SKURE1 (closed circles, *ureC::tetL*) was determined as described at the indicated time points after initiation of secondary fermentation.

Urea is a reasonable ammonia source, since strains of *B. subtilis* are known to possess an active urease, encoded by an operon, *ureABC*, usually expressed during stationary growth, that generates two molecules of ammonia from one molecule of urea.¹⁶⁾ A DNA fragment corresponding to the *ureABC* loci of the r22 chromosome was cloned and sequenced, revealing that the sequence was almost identical to that of strain 168 (data not shown). Since a single knockout of *ureC* was found to be sufficient to inactivate urease completely,¹⁶⁾ the coding region of *ureC* in the r22 chromosome was disrupted by *tetL*-marker insertion to construct a mutant, SKURE1. Within 9 h of secondary fermentation, 4 μmol/g of natto of urea was found to accumulate in the natto of strain r22, while SKURE1 drastically elevated the concentration of urea, up to 16 μmol/g of natto of urea (Fig. 4). These results indicate that urea was consumed during secondary fermentation, due to the active urease.

Ammonia reduction by inactivation of urease

The urease reaction must have produced ammonia during secondary fermentation. Two additional mutants, SKRGU1, lacking *rocG*, *gudB*, and *ureC*, and SKURE1, lacking *ureC* alone, were constructed and subjected to ammonia release assay in parallel with SKGUD3 and r22 (Fig. 5). The single mutation of *ureC* (SKURE1) reduced the ammonia emitted from the natto of SKURE1 down to about 75% of that from r22 after 9 h of secondary fermentation (Fig. 5). Furthermore, the triple mutation inactivating *rocG*, *gudB*, and *ureC* (SKRGU1) was so effective that the ammonia release was only about half of that from SKGUD3, lacking both two GlutDH enzymes, and only 20% of the original ammonia release from r22 (Fig. 5). These data clearly indicate that the degradation of urea catalyzed by urease was another ammonia producing reaction during secondary natto fermentation.

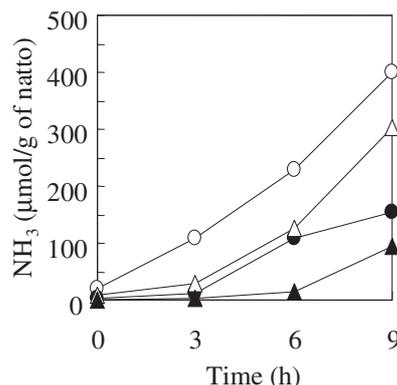


Fig. 5. Ammonia Production by GlutDH- and Urease-Defective Strains.

Ammonia emitted by natto fermented by r22 (open circles, parental strain), SKGUD3 (closed circles, *rocG::ermC gudB::aad*), SKURE1 (open triangles, *ureC::tetL*), and SKRGU1 (closed triangles, *rocG::ermC gudB::aad ureC::tetL*), determined as described at the indicated time points after initiation of secondary fermentation.

Discussion

Strains of *B. subtilis* natto might have evolved a metabolic system using glutamate as one of the amino acids preferentially, since glutamate is one of the most abundant amino acids in soybeans,²⁷⁾ and is not only a nitrogen source but also a carbon source.¹⁴⁾ As we indicated in this report, the two functional GlutDHs, RocG and GudB, of natto strain r22 degraded glutamate so efficiently that the GlutDH reaction produced only about half the total ammonia released during secondary fermentation. Probably in the case of bacteria, the GlutDH reaction degrades glutamate not only for ammonia production but also to obtain 2-OG, because 2-OG can serve as a carbon and energy source for stationary growth. It is known that expression of RocG is under carbon catabolite repression involving CcpA,²⁸⁾ and is induced in the presence of arginine, proline, or ornithine, since GlutDH is required for the catabolism of these amino acids.¹⁴⁾ Thus, in response to glucose depletion, RocG contributes to the utilization of these amino acids as carbon sources for better survival of the bacteria, and GudB might be assumed to function similarly to RocG. However, the laboratory *B. subtilis* strain 168 has *rocG* as the only gene for active GlutDH, since its *gudB* does not encode an active enzyme, while the corresponding mutant allele *gudB1* does (Fig. 2).¹⁴⁾ Additionally, at present, the exact physiological role of *gudB* in strain 168 remains to be understood, but the inactive enzyme might interact with GltC, involved in the regulation of *gltAB* in glutamate synthesis.²⁹⁾ Interestingly, strain r22 appeared to possess a second gene identical to *gudB1*, and this *gudB* of r22 appeared to be more efficient at glutamate degradation than its *rocG*, because SKGUD1 equipped with *gudB* alone produced more ammonia than SKGUD2 with *rocG*

alone (Fig. 3). Strain r22 derived from strain O-2, a commercial natto starter that can be regarded as one of the undomesticated strains in nature. These facts and results imply that most undomesticated strains of *B. subtilis* originally possessed the gene for active GudB enzyme, which is sufficiently efficient at glutamate degradation, releasing ammonia and 2-OG.

Mutant strain SKGUD3, which possesses neither of the two GlutDH enzymes, was kept in more glutamate-rich conditions during secondary fermentation than its parent strain. Since glutamate can be also used as a building block in synthesizing γ -PGA, it can be speculated that the γ -PGA contents increased in the natto of SKGUD3. Indeed, natto fermented by SKGUD3 proved to contain more γ -PGA (7.5 ± 0.5 mg/g of natto) than that by r22 (5.5 ± 0.5 mg/g of natto), suggesting that γ -PGA synthesis is stimulated by glutamate retained upon inactivation of both the two GlutDH enzymes.

On the other hand, previous transcriptome analysis revealed that CodY, a global regulator that senses the intracellular GTP concentration as an indicator of nutritional conditions, repressed transcription of the *ureABC* operon,^{30,31} suggesting that urease is induced in response to the depletion of intracellular GTP. In addition, it has also been found that TnrA, another global regulator under nitrogen-limited conditions, regulated *ureABC* in a positive way.³¹ Therefore, urease is assumed to process urea as an alternative nitrogen source used only when other available nitrogen sources are depleted.¹⁶ However, our data indicate that urease played an important role in the degradation of urea during secondary fermentation. This proved to be contradictory to the previous assumption, because during secondary fermentation, the nitrogen source is not limited. On the contrary, very rich amino acids are provided continuously (Fig. 1B). Moreover, a large amount of ammonia produced by the urease reaction is emitted into the air, and it is useless as a nitrogen source. It is likely that the recruitment of urease during secondary fermentation has some other biological significance. We found that in urease-defective mutants, urea accumulated up to nearly 100 mM after excessive fermentation (data not shown). It is conceivable that higher concentrations of urea can provoke osmotic stress in bacterial cells, and urease might function to cancel this stress by converting urea into volatile compounds such as ammonia and CO₂. This might be a bacterial strategy to survive under higher osmotic conditions.^{32–34}

It is generally believed that the production of ammonia in natto products is unavoidable, because ammonia production is assumed to be a natural consequence of natto fermentation. The amount of ammonia released by natto is great only when the fermentation time is excessive and/or the storage temperature is not properly maintained. Accordingly, for commercial natto production, time and temperature have been the most important factors, and are controlled stringently. In this study, we specified the ammonia releasing reactions

and constructed strain SKRGU1 to minimize ammonia release. Natto products made by SKRGU1 were observed to be stable. Further, they maintained normal levels of γ -PGA content, and had almost the same quality as commercial products (data not shown). However, SKRGU1 itself is a gene-manipulated organism that is not ready for commercial food production. In addition, *ureC* inactivation led to an obvious accumulation of urea in the natto foods (Fig. 4), which is not good enough for commercial products either. During secondary fermentation, urea can be produced mainly as a byproduct of the arginase reaction converting arginine to ornithine, which is the first reaction in the arginine catabolic pathway, although some urea might be made in the other pathways, such as purine and pyrimidine metabolism. Consequently, inactivation of *rocF* encoding arginase might be a clever way to reduce urea production. In any case, ammonia in secondary natto fermentation was found to be produced mainly by two ammonia-releasing reactions involving *rocG*, *gudB*, and *ureABC*. This finding should be useful in breeding a new generation of natto starters to minimize the risk of secondary fermentation releasing ammonia.

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