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# Utilization of Ammonia Nitrogen by Intestinal Bacteria Isolated from Pigs

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In a medium containing ammonia, proteose peptone, and cysteine as nitrogen sources, 17 of 24 *Bacteroidaceae* strains, 3 of 3 *Selenomonas* strains, 1 of 7 curved rods, 3 of 7 *Spirochaetaceae* strains, 8 of 20 *Eubacterium* strains, 8 of 13 *Peptococcaceae* strains, 3 of 4 *Clostridium* strains, 19 of 20 *Enterobacteriaceae* strains, and 1 of 8 *Streptococcus* strains utilized ammonia nitrogen preferentially to proteose peptone nitrogen. To determine the ability of intestinal microbes to synthesize amino acids from ammonia, ammonia utilization by *Bacteroides ruminicola* strain 9 was studied in defined media containing ammonia and other nitrogen sources. In another medium containing ammonia, proteose peptone, and cysteine as nitrogen sources, ammonia was preferentially utilized even when the proteose peptone nitrogen content was eight times greater than that of ammonia nitrogen. In a medium containing ammonia, an amino acid, and cysteine, the lowest uptake of ammonia nitrogen was observed when the medium contained aspartic acid, glutamic acid, threonine, or alanine; but ammonia was utilized more effectively than any of the amino acids. Incorporation of $^{15}N$ from [$^{15}N$]ammonia into bacterial amino acids was studied. $^{15}N$ was incorporated into every amino acid of *B. ruminicola* strain 9, and the highest uptake was observed in aspartic acid and alanine.

Not only ruminants but also monogastric animals have been known to utilize nonprotein nitrogen (NPN) in their body protein synthesis (20, 22). Some of this NPN-utilizing ability might be related to their intestinal microbes (21). Some intestinal microbes have the ability to synthesize amino acids and proteins from ammonia for their growth (7), and it has been suggested that microbial protein is digested or autolyzed and absorbed by the host and utilized as a material for protein biosynthesis by the host (14, 21). Therefore, gnotobiotes associated with intestinal bacteria which preferentially utilize ammonia might have the nitrogen-sparing ability. This study was designed to screen bacteria with the ability of preferential ammonia utilization and to determine the incorporation of $^{15}N$ from [$^{15}N$]ammonia into bacterial amino acids.

## MATERIALS AND METHODS

**Microorganisms used.** Seventy-nine strains of strict anaerobes and 29 strains of facultative anaerobes were freshly isolated from colon contents of 90-day-old pigs given a diet by using medium 10 (4), modified Eggerth-Gagnon (EG) agar (12), and glucose blood liver (BL) agar (12). Each medium was inoculated with 0.05 ml of the dilutions representing $10^{-5}$, $10^{-6}$, and $10^{-7}$ g (wet weight) of colon contents. EG and BL agars were incubated at 37°C in an anaerobic steel wool jar (15) filled with an atmosphere of 100% $CO_2$. Medium 10 was prepared by the "plate-in-bottle" method (11) and incubated at 37°C. After incubation for 2 to 3 days, representatives of the different colonial types were isolated onto medium 10, EG, or BL agar. All strains were maintained on prereduced EG liver slants with $H_2CO_3$-$CO_2$ buffer (12) and stored at 4°C. The slant cultures were replated on medium 10, EG agar, or BL agar, and one colony was tested for its ability in utilization of ammonia nitrogen by growth in liquid media mentioned below. After 2 days of incubation, one colony grown on agar plate was picked with a platinum-iridium needle and inoculated into duplicate tubes of each of the liquid media, which served as inoculum. These included 24 *Bacteroidaceae* strains, 3 *Selenomonas* strains, 3 curved-rod strains, 7 *Spirochaetaceae* strains, 20 *Eubacterium* strains, 13 *Peptococcaceae* strains, 4 *Clostridium* strains, 1 *Bifidobacterium* strain, 20 *Enterobacteriaceae* strains, 8 *Streptococcus* strains, and 1 *Lactobacillus* strain.

**Media used in ammonia utilization studies.** The basal medium was similar to the defined medium used by Varel and Bryant (19) with some modifications. Composition of the basal medium was as follows: 0.5% glucose; 5.0% mineral solution (1.8% each $KH_2PO_4$ and NaCl, 0.053% $CaCl_2 \cdot 2H_2O$, 0.04% $MgCl_2 \cdot 6H_2O$, 0.02% $MnCl_2 \cdot 4H_2O$, and 0.002% $CoCl_2 \cdot 6H_2O$); 0.0001% hemin; 0.45% volatile fatty acid mixture (36 ml of acetic acid, 1.8 ml of isobutyric acid, and 2.0 ml each of *n*-valeric, DL-2-methylbutyric, and isovaleric acids); 0.0001% resazurin; 0.5% vitamin solution (20 mg each of thiamine hydrochloride, calcium-D-pantothenate, nicotinamide, riboflavin, and pyridoxine hydrochloride; 1 mg of *p*-aminobenzoic acid; 0.25 mg each of

biotin and folic acid; and 0.1 mg of vitamin $B_{12}$; 100 ml of water); 0.0004% $FeSO_4 \cdot 7H_2O$; 0.012% of $(NH_4)_2SO_4$; 0.4% $Na_2CO_3$; 0.05% cysteine·HCl·$H_2O$; and $CO_2$ gas phase. $(NH_4)_2SO_4$-labeled 19.6 atoms percent excess $^{15}N$ was used in all experiments. Four different media were made by addition of various components to the basal medium as follows (expressed as final percent): (medium 1) 0.07% of proteose peptone no. 3 (Difco Laboratories); (medium 2) 0, 0.009, 0.018, 0.035, 0.07, and 0.14% proteose peptone no. 3; (medium 3) 0.01% amino acid-N of various amino acids—the amino acids used were L-aspartic acid, L-glutamic acid, L-alanine, glycine, L-valine, L-leucine, L-isoleucine, L-serine, L-threonine, L-methionine, L-phenylalanine, L-tyrosine, L-tryptophan, L-proline, L-lysine, L-histidine, and L-arginine; (medium 4) 0.05% cystine·HCl.

**Utilization of ammonia nitrogen by the predominant bacteria isolated from pigs.** Medium 1 tubed in 6-ml amounts was inoculated with one colony. Each tube was incubated until good growth was apparent. The bacterial cultures were then centrifuged at 9,000 × *g* for 10 min. The cells collected were washed with 3 ml of redistilled water and centrifuged twice, and 2 ml of methanol was added to form a cell suspension. A sample of this suspension was used for nitrogen analysis by the micro-Kjeldahl method. The remaining part was concentrated, and a cell suspension containing 3 μg of nitrogen was taken up in a capillary about 1 mm in diameter and 1 cm long and dried in a vacuum oven. The capillary was sealed in a glass tube under $10^{-3}$ mmHg with 0.2 mmHg of xenon and 10 mmHg of helium, and $^{15}N$ was analyzed by the Dumas method of optical spectroscopic analysis (10). The error in $^{15}N$ analysis was under 5% (18).

**Effect of peptone or single amino acid on the uptake of $^{15}N$ from [$^{15}N$]ammonia by *B. ruminicola* strain 9.** Uptake of $^{15}N$ from $(^{15}NH_4)_2SO_4$ by *B. ruminicola* strain 9 in the presence of peptone or amino acid was tested with media 2 and 3. One colony of *B. ruminicola* strain 9 was inoculated into a tube with 6 ml of medium 2 without proteose peptone no. 3 and incubated at 37°C for 3 days. From the resulting culture, 0.2 ml was inoculated into tubes of each of media 2 and 3 and incubated at 37°C for one day. Bacterial nitrogen and $^{15}N$ incorporation were analyzed in the same way as the above experiment.

**Incorporation of $^{15}N$ from [$^{15}N$]ammonia into bacterial amino acid.** One colony of *B. ruminicola* strain 9 was inoculated into a bottle with 50 ml of medium 4 and incubated at 37°C for 5 days. The culture was then poured into 2.5 liters of the same medium and incubated for 3 days. After incubation, bacterial cells were gathered by centrifugation at 6,000 × *g* for 10 min, washed with saline, and centrifuged twice. The bacterial precipitate was hydrolyzed with 17 ml of 6 N HCl at 110°C for 18 h. The hydrolysate was filtered with a glass filter. One aliquot was used for nitrogen analysis by the micro-Kjeldahl method and for amino acid analysis by an amino acid analyzer. The other aliquot was placed on a column of Dowex 50-4X and eluted with 2 N $NH_4OH$. The eluate was dried, and distilled water was added. Amino acids were separated from this sample by thin-layer chromatography. The first dimension was developed with butanol-acetic acid-water (4:2:1, vol/vol/vol) and the second dimension was developed with phenol-water (4:1, vol/vol). When plates had been developed, individual ninhidrin-reactive areas were scraped into a glass test tube for $^{15}N$ analysis. $^{15}N$ determinations were performed by the Dumas optical spectroscopic method as described by Arima and Kumazawa (1).

The relation between the bacterial count and total nitrogen was studied with *B. ruminicola* strain 9. The method of incubation and the medium used were the same as described above. Bacterial counts were determined by the Petroff-Hausser counting chamber, and nitrogen was determined by the micro-Kjeldahl method.

## RESULTS

**Utilization of ammonia nitrogen by the predominant bacteria isolated from pigs.** Tables 1 and 2 show the incorporation of $^{15}N$ from ammonia into bacteria isolated from pigs. It was assumed that bacteria with uptakes of $^{15}N$ of more than 2.98 atoms percent excess can use ammonia nitrogen preferentially, because initial $^{15}N$ abundance in the medium was 2.98 atoms percent excess, and ammonia nitrogen was 15.2% of total nitrogen in the medium. Bacteria which utilize ammonia nitrogen preferentially as the nitrogen source were as follows: 17 strains of *Bacteroidaceae*, 3 strains of *Selenomonas*, 1 strain of curved rods, 8 strains of *Peptococcaceae*, 3 strains of *Clostridium*, 19 strains of *Enterobacteriaceae*, and 1 strain of *Streptococcus*. Strains of *Bacteroidaceae* and *Selenomonas* utilized ammonia nitrogen more effectively than those of the other bacterial groups.

**Effect of peptone on the uptake of $^{15}N$ from [$^{15}N$]ammonia by *B. ruminicola* strain 9.** Figure 1 shows the effect of proteose peptone on the uptake of $^{15}N$ from $^{15}N$-labeled ammonia by *B. ruminicola* strain 9. Although uptake of $^{15}N$ decreased as the peptone increased, $^{15}N$ uptake was 7.8 atoms percent excess, equal to 44% bacterial nitrogen, when eight times more peptone nitrogen than ammonia nitrogen was present. Incorporation of $^{15}N$ was 17.8 atoms percent excess when proteose peptone did not exist in the medium whose initial $^{15}N$ abundance was 19.6 atoms percent excess. Calculated from the result, the uptake of $^{15}N$ was decreased by only 9% by the existence of cysteine, although there was 1.6 times more cysteine nitrogen than ammonia nitrogen.

**Effect of a single amino acid on the uptake of $^{15}N$ from ammonia by *B. ruminicola* strain 9.** Table 3 shows the effect of the addition of single amino acids on uptake of $^{15}N$ from $^{15}N$-labeled ammonia. Even when the medium contained aspartic acid, 34% of bacterial nitrogen was derived from [$^{15}N$]ammonia. The effect of glycine, methionine, tyrosine, phenylalanine,

TABLE 1. *Incorporation of $NH_4$-$^{15}N$ into intestinal strict anaerobes from pigs*

| Bacterial group | Strain no. | Microbial N/tube (μg) | $^{15}N$ incorporation (atoms % excess) | Bacterial group | Strain no. | Microbial N/tube (μg) | $^{15}N$ incorporation (atoms % excess) |
|---|---|---|---|---|---|---|---|
| *Bacteroidaceae* | 100 | 52 | 12.9 | *Eubacterium* | 17 | 88 | 7.16 |
| | 9 | 144 | 12.3 | | 60 | 56 | 6.95 |
| | 51 | 40 | 9.62 | | 102 | 60 | 5.39 |
| | 30 | 48 | 9.31 | | 42 | 140 | 5.33 |
| | 22 | 44 | 7.74 | | 52 | 40 | 3.64 |
| | 77 | 56 | 7.61 | | 66 | 32 | 3.48 |
| | 31 | 44 | 7.48 | | 47 | 68 | 3.40 |
| | 19 | 80 | 7.15 | | 108 | 60 | 3.78 |
| | 21 | 72 | 6.25 | | 35 | 72 | 2.74 |
| | 105 | 40 | 6.06 | | 34 | 60 | 2.78 |
| | 147 | 80 | 5.87 | | 36 | 64 | 2.67 |
| | 25 | 28 | 5.14 | | 26 | 60 | 2.42 |
| | 29 | 28 | 5.14 | | 145 | 92 | 2.03 |
| | 54 | 20 | 4.83 | | 39 | 36 | 1.77 |
| | 101 | 44 | 4.77 | | 107 | 20 | 1.12 |
| | 53 | 40 | 3.47 | | 112 | 24 | 0.87 |
| | 118 | 48 | 3.29 | | 23 | 36 | 0.78 |
| | 75 | 40 | 2.95 | | 16 | 48 | 0.55 |
| | 64 | 44 | 1.02 | | 114 | 52 | 0.44 |
| | 65 | 40 | 0.92 | | 109 | 20 | 0.12 |
| | 55 | 32 | 0.85 | | | | |
| | 103 | 40 | 0.69 | *Peptococcaceae* | 86 | 52 | 7.45 |
| | 11 | 80 | 0.43 | | 84 | 40 | 6.57 |
| | 33 | 36 | 0.21 | | 93 | 60 | 6.50 |
| | | | | | 79 | 40 | 6.40 |
| *Selenomonas* | 37 | 32 | 8.48 | | 92 | 56 | 5.60 |
| | 1 | 100 | 7.62 | | 68 | 80 | 4.82 |
| | 40 | 40 | 6.69 | | 62 | 52 | 4.08 |
| | | | | | 63 | 48 | 3.82 |
| Curved rods | 48 | 48 | 4.85 | | 140 | 60 | 2.65 |
| | 146 | 132 | 2.40 | | 126 | 36 | 2.59 |
| | 69 | 68 | 1.20 | | 142 | 24 | 2.13 |
| | | | | | 120 | 40 | 0.78 |
| *Spirochaetaceae* | 61 | 60 | 7.70 | | 111 | 80 | 0.56 |
| | 87 | 40 | 3.82 | | | | |
| | 78 | 44 | 3.05 | *Clostridium* | 115 | 60 | 6.30 |
| | 104 | 72 | 2.91 | | 18 | 72 | 3.40 |
| | 119 | 48 | 0.55 | | 15 | 108 | 3.72 |
| | 32 | 36 | 0.36 | | 7 | 120 | 2.95 |
| | 41 | 20 | 0.31 | | | | |
| | | | | *Bifidobacterium* | 90 | 40 | 0.88 |

tryptophan, and histidine on the uptake of $^{15}N$ was little, and bacterial nitrogen derived from ammonia nitrogen was 76 to 85% in the media containing them.

**Incorporation of $^{15}N$ from [$^{15}N$]ammonia into bacterial amino acids.** Incorporation of $^{15}N$ from $^{15}N$-labeled ammonia by *B. ruminicola* strain 9 and the concentration of amino acids are shown in Table 4. Not only nonessential amino acids of pigs, such as alanine, glutamic acid, aspartic acid, and glycine, but also essential amino acids, such as lysine, leucine, and threonine, were generally observed among bacterial amino acids. We observed an uptake of $^{15}N$ into every amino acid, quantitatively, in the sequence aspartic acid > alanine > isoleucine and leucine > glutamic acid. Incorporation of $^{15}N$ into tyrosine was the least, but 11% of bacterial tyrosine was derived from ammonia nitrogen. The $^{15}N$ content (in milligrams) of aspartic acid and alanine was more than the other amino acid-$^{15}N$. Under the conditions in this experiment, 31% of the ammonia nitrogen in the initial medium was transferred to the bacterial nitrogen. In $10^9$ cells of *B. ruminicola* strain 9 there was 102 μg of nitrogen, equal to 731 μg of amino acid, assuming the mean molecular weight of amino acids to be about 100.

TABLE 2. *Incorporation of $NH_4$-$^{15}N$ into intestinal facultative anaerobes from pigs*

| Bacterial group | Strain no. | Microbial N/tube (μg) | $^{15}N$ incorporation (atoms % excess) |
|---|---|---|---|
| *Enterobacteriaceae* | 42 | 90 | 6.38 |
| | 17 | 118 | 5.96 |
| | 43 | 100 | 5.26 |
| | 41 | 50 | 4.97 |
| | 25 | 120 | 4.68 |
| | 23 | 162 | 4.59 |
| | 5 | 104 | 4.44 |
| | 30 | 120 | 4.40 |
| | 27 | 54 | 4.37 |
| | 29 | 94 | 4.29 |
| | 3 | 126 | 4.16 |
| | 4 | 128 | 4.06 |
| | 39 | 72 | 3.98 |
| | 8 | 172 | 3.97 |
| | 6 | 152 | 3.94 |
| | 28 | 82 | 3.83 |
| | 7 | 140 | 3.82 |
| | 24 | 128 | 3.40 |
| | 26 | 80 | 3.29 |
| | 40 | 52 | 2.56 |
| *Streptococcus* | 21 | 124 | 3.86 |
| | 9 | 112 | 2.49 |
| | 16 | 118 | 2.28 |
| | 35 | 44 | 2.06 |
| | 37 | 36 | 1.44 |
| | 32 | 30 | 1.21 |
| | 33 | 32 | 0.72 |
| | 31 | 40 | 0.32 |
| *Lactobacillus* | 44 | 52 | 0.00 |

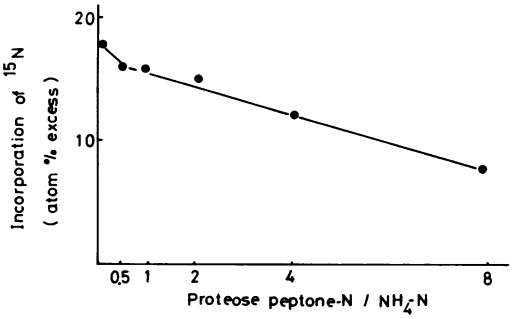


FIG. 1. *Uptake of $^{15}N$ from $^{15}NH_4$ medium with proteose peptone 3 by B. ruminicola strain 9.*

## DISCUSSION

The effects of protein level and many kinds of NPN on utilization of NPN by monogastric animals and uptake of NPN into animals have been studied for the purpose of sparing protein sources in feeds (5, 6). It is also known that monogastric animals utilize NPN under low-protein diet conditions (22). The microorganisms in the host intestine were thought to be unsuitable for the utilization of NPN (8, 9).

Urea, which is known to diffuse into the gut contents from tissue (20), may be hydrolyzed by bacterial urease to produce further ammonia. Ammonia, which comes from ammonium salts and urea, may be utilized by some microorga-

TABLE 3. *Effect of adding a single amino acid to a medium containing ammonia and cysteine as nitrogen sources on the incorporation of $NH_4$-$^{15}N$ by B. ruminicola strain 9*

| Added amino acid | Microbial N/tube (μg) | $^{15}N$ incorporation (atoms % excess) |
|---|---|---|
| Aspartic acid | 192 | 6.7 |
| Glutamic acid | 144 | 8.4 |
| Alanine | 168 | 10.3 |
| Glycine | 108 | 15.7 |
| Valine | 128 | 11.6 |
| Leucine | 192 | 11.9 |
| Isoleucine | 132 | 11.5 |
| Serine | 208 | 11.5 |
| Threonine | 140 | 9.4 |
| Methionine | 168 | 15.8 |
| Phenylalanine | 160 | 16.7 |
| Tyrosine | 160 | 14.9 |
| Tryptophan | 132 | 14.8 |
| Proline | 152 | 11.6 |
| Lysine | 108 | 12.2 |
| Histidine | 140 | 16.2 |
| Arginine | 164 | 11.9 |
| None | 124 | 17.8 |

TABLE 4. *Incorporation of $NH_4$-$^{15}N$ into different amino acids and concentration of amino acids by B. ruminicola strain 9*

| Bacterial amino acid | N (mg) | $^{15}N$ (atom % excess) | $^{15}N$ (mg) |
|---|---|---|---|
| Alanine | 1.90 | 11.91 | 0.244 |
| Glutamic acid | 1.69 | 8.76 | 0.159 |
| Aspartic acid | 1.59 | 14.63 | 0.249 |
| Proline | 0.41 | 2.91 | 0.013 |
| Glycine | 1.32 | 6.81 | 0.096 |
| Histidine | 0.36 | 4.15 | 0.016 |
| Arginine | 0.58 | 4.73 | 0.030 |
| Tyrosine | 0.46 | 2.13 | 0.010 |
| Serine + Threonine | 0.66 + 0.86 | 7.74 | 0.126 |
| Valine | 0.70 | 8.39 | 0.063 |
| Methionine | 0.64 | 2.52 | 0.017 |
| Isoleucine + Leucine | 0.68 + 1.11 | 10.45 | 0.202 |
| Phenylalanine | 0.56 | 3.34 | 0.020 |
| Lysine | 1.11 | 4.33 | 0.051 |
| Total nitrogen | 30.38 | 12.22 | 4.03 |

nisms in the intestine of the host, and microbial amino acids are in part absorbed (14, 17) and utilized in the synthesis of protein by the host (14). Some bacteria might be useful but others might not be useful for the utilization of NPN; gnotobiotes associated with NPN-assimilating bacteria, therefore, might utilize NPN better than conventional animals. For this purpose, screening of the ammonia nitrogen-assimilating bacteria was performed. Contaminating germ-free pigs with ammonia-assimilating bacteria and urease-producing bacteria, production of an "NPN-assimilating gnotobiotic pig" might be possible.

Pittman and Bryant (16) found that *B. ruminicola* can utilize peptide nitrogen and ammonia nitrogen but not utilize a significant amount of free amino acid nitrogen for growth in medium which contains them as the main nitrogen source. Our results also indicate that a significant proportion of the bacterial population in the colon of pigs utilize ammonia nitrogen preferentially to proteose peptone nitrogen. On the other hand, *B. ruminicola* was known to be of primary importance in ammonia production from hydrolyzed protein (2). Bryant and Robinson suggested that a considerable amount of dietary protein in the rumen was broken down to $NH_3$, $CO_2$, and volatile fatty acid before being utilized in microbial protein synthesis (3). In our experiment, ammonia might be utilized more than the incorporation of $^{15}N$ (Fig. 1) because $^{15}NH_3$ should be considerably dilute due to ammonia production when peptone nitrogen was increased. The efficiency of utilization of ammonia nitrogen was relatively low in the presence of aspartic acid and glutamic acid, probably because they release ammonia by glutamate dehydrogenase and glutamate oxaloacetate transaminase.

Our data showed that *B. ruminicola* strain 9 has a high content of lysine, which is important as an essential amino acid for the growth of the pig (13). Ammonia-$^{15}N$ in the medium was incorporated into every amino acid of *B. ruminicola* strain 9. If these bacterial amino acids are used as a source of amino acids for protein synthesis of the host, the existence of ammonia-assimilating bacteria such as *B. ruminicola* strain 9 in the intestine might be useful for sparing of a limiting amino acid.

It was found that *B. ruminicola* strain 9 contains about 0.7 mg of amino acids per $10^9$ bacterial cells. If $10^{11}$ bacteria per gram of intestinal contents are present, intestinal contents contain 7% microbial protein. Since the quantity of intestinal contents passing through the lower intestine of the pig was unknown, total microbial protein could not be calculated, but because the protein requirement of the pig was 14% according to nutrient requirements of swine (13), 7% of microbial protein per gram of intestinal contents might have an important role as a protein source for the host.

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