Oral Microbiology Immunology 2002: 17: 50–54
Printed in Denmark. All rights reserved

Oral Microbiology and Immunology
ISSN 0902-0055

Dipeptide utilization by the periodontal pathogens *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Fusobacterium nucleatum*

N. Takahashi, T. Sato
Division of Oral Biochemistry, Department of Oral Biology, Tohoku University, Graduate School of Dentistry, Sendai, Japan

Takahashi N, Sato T. Dipeptide utilization by the periodontal pathogens *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Fusobacterium nucleatum*.
Oral Microbiol Immunol 2002: 17: 50–54. © Munksgaard, 2002.

Porphyromonas gingivalis, *Prevotella intermedia*, *Prevotella nigrescens* and *Fusobacterium nucleatum*, which can frequently be isolated from periodontal pockets, preferentially utilize proteins and peptides as growth substrates. In this study, we determined the size of peptide that is preferentially utilized as a source of energy and material for cell growth by *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *F. nucleatum* using various sizes of poly amino acids consisting of two to approximately 100 molecules of aspartate or glutamate. Resting cells of *P. gingivalis*, *P. intermedia* and *P. nigrescens* utilized aspartylaspartate, while cells of *P. gingivalis* and *F. nucleatum* utilized glutamylglutamate. The addition of aspartylaspartate to the culture medium increased the growth of *P. gingivalis*, *P. intermedia* and *P. nigrescens*, while the addition of glutamylglutamate promoted the growth of *P. gingivalis* and *F. nucleatum*. These results clearly indicate that dipeptides such as aspartylaspartate and glutamylglutamate can be utilized as growth substrates for *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *F. nucleatum*.

Key words: *Porphyromonas gingivalis*; *Prevotella intermedia*; *Prevotella nigrescens*; *Fusobacterium nucleatum*; dipeptide; protease

Nobuhiro Takahashi, Division of Oral Biochemistry, Department of Oral Biology, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
Accepted for publication July 18, 2001

Periodontal pathogens such as *Porphyromonas gingivalis* are known to induce periodontitis through their biological activities, which produce, for example, cytotoxic metabolic end products, proteases and immunoactive cell components (5, 11, 12). These bacteria are frequently found in periodontal pockets and preferentially utilize proteins and peptides as sources of energy and cell material, as these amino acid compounds are supplied abundantly and continuously from gingival crevicular fluid or inflammatory exudate.

P. gingivalis cells grow efficiently on peptides rather than free amino acids (9, 15, 16, 21, 26). Other periodontal pathogens, including *Prevotella intermedia*, *Prevotella nigrescens* (16, 24) and *Fusobacterium nucleatum* (4, 14), also prefer peptides to free amino acids as growth substrates. In particular, *P. gingivalis* is reported to degrade the dipeptides L-aspartyl-L-aspartate and L-glutamyl-L-glutamate (22, 23), while *F. nucleatum* is reported to degrade the dipeptides L-cysteinylglycine and L-methionyl-L-methionine (25). However, the sizes and types of peptides utilized as growth substrates by these periodontopathic bacteria are still unknown. Although Wyss (27) showed that aspartame (L-phenylalanyl-L-

methylaspartate) can support the growth of *P. gingivalis* in his chemically defined medium lacking phenylalanine, there is little information on simple peptides as growth substrates for periodontal pathogens. Such information may be useful in exploring the metabolic and growth properties of peptide-dependent bacteria and in developing simple chemically defined media for these bacteria.

Therefore, we determined the sizes of peptides preferentially utilized as metabolic substrates by *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *F. nucleatum* using various sizes of poly amino acids consisting of two to approximately 100 molecules of the single amino acids aspartate and glutamate. Using this approach, we have demonstrated that dipeptides can support the growth of these periodontal pathogens.

Materials and methods

Bacterial strains and growth conditions

P. gingivalis ATCC 33277 and W83, *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 25261, and *F. nucleatum* ATCC 25586 and ATCC 10953 were used in this study. The bacteria were grown in modified BM medium (23) containing 1% tryptone (Difco, Detroit, MI), 1% proteose peptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, 5 μg/ml of hemin and 0.5 μg/ml of menadione in 38 mM potassium phosphate buffer (pH 7.0) in an anaerobic chamber (N_2, 80%; H_2, 10%; CO_2, 10%; NHC-type, Hirasawa Works, Tokyo, Japan) at 37°C. Bacterial purity was regularly confirmed by microscopic examination of gram-stained smears and by culturing on blood agar plates containing hemin and menadione.

Utilization of aspartate, glutamate, polyaspartate and polyglutamate by bacterial cells

The bacterial cells were harvested by centrifugation during the logarithmic growth phase (15–18 h after inoculation). Unless otherwise indicated, the experiments described below were carried out in another anaerobic chamber (N_2, 90%; H_2, 10%; NH-type, Hirasawa Works). During centrifugation and transportation between the anaerobic chambers, the cells were protected from oxygen exposure in double-sealed centrifuge tubes. The bacterial cells were washed twice with 25 mM potassium phosphate buffer (pH 7.0) containing 50 mM NaCl plus 5 mM $MgCl_2$ and suspended in the same buffer (2.0–2.2 mg dry weight/ml). The cell suspension (1.5 ml) was incubated at 37°C for 10 min and then mixed with 1.5 ml of prewarmed aspartate, glutamate, polyaspartate or polyglutamate solution. The polyaspartates used in this study were aspartylaspartate, tri-aspartate, tetra-aspartate, hexa-aspartate and $(Asp)_{100}$ consisting of approximately 100 aspartates. Polyglutamates were glutamylglutamate $(Glu)_{5-10}$ consisting of approximately 5–10 glutamates and $(Glu)_{10-20}$ consisting of approximately 10–20 glutamates. The final concentration of each substrate in the reaction mixture was approximately 10 mM when converted to free amino acids. At 0 and 60 min after addition of substrates, the cell suspension was sampled, mixed with perchloric acid at a final concentration of 6% and stored at 4°C for the analysis of carboxylic acids and ammonia.

Assay of metabolic end products

Carboxylic acids, including formic, acetic, propionic, lactic, malic, succinic, butyric, isobutyric, valeric and isovaleric acids, were analyzed using a carboxylic acid analyzer (model S-3000X, Tokyo Rikakikai, Tokyo, Japan), as described previously (20, 21). Ammonia

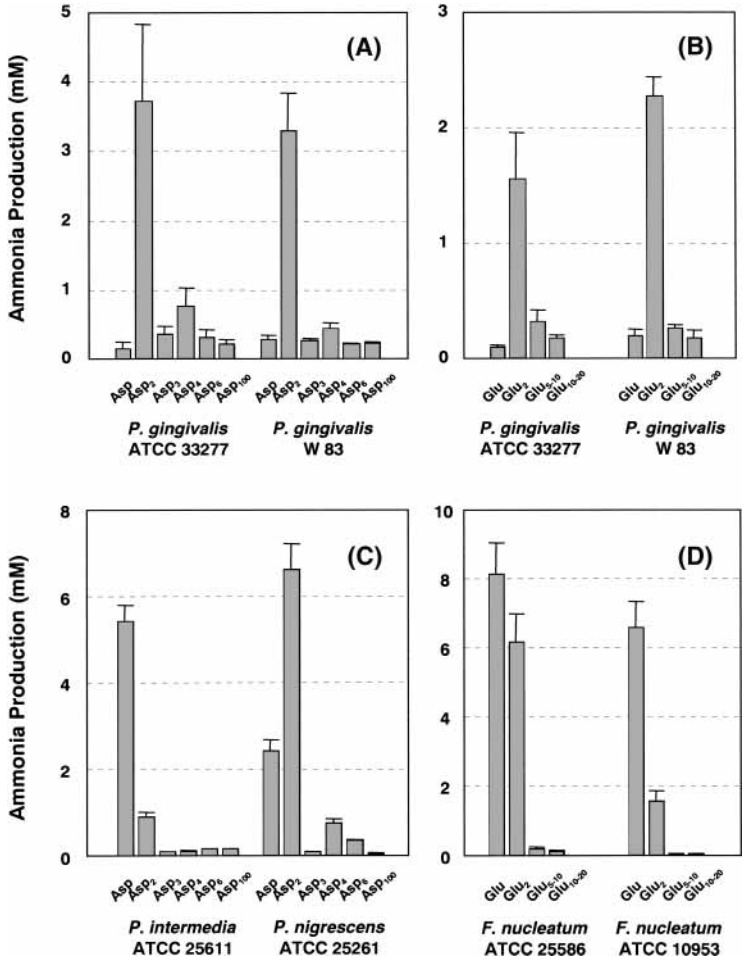

Fig. 1. Ammonia production from free amino acids and peptides by resting cells of *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *F. nucleatum*. (**A**) aspartate (Asp) and polyaspartate (Asp_n) utilization by *P. gingivalis*; (**B**) glutamate (Glu) and polyglutamate (Glu_n) utilization by *P. gingivalis*; (**C**) aspartate (Asp) and polyaspartate (Asp_n) utilization by *P. intermedia and P. nigrescens*; (**D**) glutamate (Glu) and polyglutamate (Glu_n) utilization by *F. nucleatum*; Asp_n, polyaspartate consisting of *n* aspartates; Glu_n, polyglutamate consisting of *n* glutamates. Data are given as means with standard deviations obtained from three independent experiments. Little ammonia production (>0.1 mM) was observed in the absence of free amino acids or peptides.

was assayed enzymatically using glutamate dehydrogenase (1).

Bacterial growth response to aspartate, glutamate, aspartylaspartate and glutamylglutamate

BM media whose concentrations of tryptone and proteose peptone were decreased to 0.25% were defined as 1/4 BM media. They were aseptically supplemented with 0, 2, 5 or 10 mM aspartate or glutamate, or with 0, 1, 2.5 or 5 mM aspartylaspartate or glutamylglutamate. Each well of sterile 96-well plastic plates was filled with 200 µl of the supplemented 1/4BM media, and inoculated with the bacterial cell culture (2 µl) grown until the logarithmic growth phase, as described above. After anaerobic incubation in the NHC-type chamber at 35°C for 48 h, bacterial growth was determined using a microplate reader at 650 nm.

Assay of bacterial dipeptidase activity

The bacterial cells were harvested and washed as described above, and suspended in 25 mM potassium phosphate buffer (pH 7.0) containing 50 mM NaCl and 5 mM $MgCl_2$ at an optical density (at 660 nm) of 1 in the NH-type anaerobic chamber. After preincubation at 35°C for 10 min, 2 ml of cell suspension and 2 ml of 2 mM L-aspartyl-β-naphtylamide or L-glutamyl-*p*-nitroanilide were mixed and additionally incubated for at 35°C. These mixtures were sampled at 0, 30 and 60 min after incubation. Cell-bound dipeptidase activities were estimated as the amount of β-naphtylamine (18) or *p*-nitroaniline (3) released from the substrate.

Part of the cell suspensions was oscillated anaerobically (2A, 190 W, 4°C, 6 min) and centrifuged (10 000 ***g***, 4°C, 10 min) as described previously (24). The resultant cell extracts were assayed for intracellular dipeptidase activities in the NH-type anaerobic chamber. The reaction mixture (2 ml) contained cell extracts and 1 mM aspartylaspartate or glutamylglutamate in 0.1 M Tris-HCl buffer (pH 7.0). After preincubation at 35°C for 10 min, the cell extracts were added to start the reaction. Aliquots of the mixture were sampled (300 µl) periodically and mixed immediately with 30 µl of perchloric acid in order to stop the reaction. The amounts of aspartate or glutamate released from aspartylaspartate or glutamylglutamate were quantified enzymatically (7, 10).

Chemicals and enzyme preparations

Polyaspartates, polyglutamates and aspartyl-β-naphtylamide were purchased from Sigma Chemical Co., St. Louis, MO. Enzyme preparations were purchased from Roche Diagnostics, Basel, Switzerland. Glutamyl-*p*-nitroanilide was obtained from Peptide Institute, Inc., Osaka, Japan.

Results and Discussion

P. gingivalis strains utilized aspartylaspartate and glutamylglutamate efficiently and produced significant amounts of ammonia (Fig. 1). *P. intermedia* and *P. nigrescens* strains utilized aspartylaspartate, while *F. nucleatum* strains utilized glutamylglutamate. These results clearly indicate that the periodontal pathogens *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *F. nucleatum* preferentially utilize dipeptides as metabolic substrates over various other sizes of peptides. After dipeptides, *P. gingivalis* and *P. nigrescens* preferentially utilized tetra-aspartate. *P. gingivalis* also utilized the other peptides slowly. *P. intermedia* and *P. nigrescens* did not utilize glutamylglutamate, while *F. nucleatum* did not utilize aspartylaspartate.

From the dipeptides, *P. gingivalis* strains produced significant amounts of carboxylic acids in addition to ammonia (Table 1). The acidic end products of *P. gingivalis* from aspartylaspartate and glutamylglutamte were consistent with the results of our previous experiments (22, 23), in which acetate, propionate, succinate and butyrate were mainly produced. As reported previously (2, 24), *P. intermedia* and *P. nigrescens* degraded aspartate to formate, acetate, succinate and ammonia, while *F. nucleatum* metabolized glutamate to acetate, butyrate and ammonia (Fig. 1 and Table 1). The end-product profiles of *P. intermedia*, *P. nigrescens* and *F. nucleatum* from dipeptides were similar to those from the corresponding free amino acids, implying that dipeptides can be hydrolyzed to amino acids and subsequently degraded in the same way as the corresponding free amino acids. In contrast to *Prevotella* and *Fusobacterium* species, *P. gingivalis* did not util-

Table 1. End products from dipeptides and/or free amino acids for *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *F. nucleatum*

Strain	Substrate	Formate	Acetate	Propionate	Malate	Succinate	Butyrate
P. gingivalis ATCC 33277	Asp_2	nd	1.87 ± 0.27	0.06 ± 0.02	nd	0.33 ± 0.06	0.81 ± 0.12
	Glu_2	nd	0.05 ± 0.01	0.34 ± 0.07	nd	0.01 ± 0.00	1.02 ± 0.24
P. gingivalis W83	Asp_2	nd	1.23 ± 0.35	0.16 ± 0.02	nd	0.06 ± 0.01	0.65 ± 0.13
	Glu_2	nd	0.03 ± 0.01	0.31 ± 0.06	nd	0.03 ± 0.01	1.61 ± 0.21
P. intermedia ATCC 25611	Asp	1.20 ± 0.11	2.23 ± 0.26	nd	0.13 ± 0.01	3.26 ± 0.46	nd
	Asp_2	0.17 ± 0.02	0.36 ± 0.01	nd	0.01 ± 0.00	0.54 ± 0.03	nd
P. nigrescens ATCC 25261	Asp	0.17 ± 0.01	0.89 ± 0.07	nd	0.02 ± 0.00	1.63 ± 0.02	nd
	Asp_2	1.43 ± 0.35	2.78 ± 0.32	nd	0.39 ± 0.02	4.12 ± 0.21	nd
F. nucleatum ATCC 25586	Glu	0.05 ± 0.02	9.94 ± 0.73	nd	nd	0.01 ± 0.00	3.99 ± 0.51
	Glu_2	0.04 ± 0.01	7.34 ± 0.52	nd	nd	0.01 ± 0.00	2.89 ± 0.81
F. nucleatum ATCC 10953	Glu	0.04 ± 0.01	9.81 ± 1.00	nd	nd	0.02 ± 0.00	2.92 ± 0.35
	Glu_2	nd	1.92 ± 0.12	nd	nd	nd	0.46 ± 0.05

Values are given as the means ± standard deviations obtained from three independent experiments.
Asp, aspartate; Asp_2, aspartylaspartate; Glu, glutamate; Glu_2, glutamylglutamate.
nd, not detected.

Table 2. Intracellular dipeptidase activity of P. gingivalis, P. intermedia, P. nigrescens and F. nucleatum

Strain	Substrate	Activity (U/g of protein)
P. gingivalis ATCC 33277	Asp_2	41.0 ± 23.9
	Glu_2	42.2 ± 28.6
P. gingivalis W83	Asp_2	22.8 ± 3.5
	Glu_2	35.4 ± 12.5
P. intermedia ATCC 25611	Asp_2	33.5 ± 7.3
P. nigresecns ATCC 25261	Asp_2	68.1 ± 12.1
F. nucleatum ATCC 25586	Glu_2	36.0 ± 6.5
F. nucleatum ATCC 10953	Glu_2	24.6 ± 5.2

Values are given as the means ± standard deviations obtained from three independent experiments.
Asp_2, aspartylaspartate; Glu_2, glutamylglutamate.

ize free amino acids, aspartate or glutamate (Fig. 1).

There are two possible pathways for dipeptide metabolism: (1) transport in the dipeptide form and subsequent intracellular hydrolysis to amino acids, or (2) extacellular hydrolysis to amino acids and subsequent transport of amino acids. In this study, all the tested strains had intracellular dipeptidase activities (Table 2), while none of the strains had cell-bound dipeptidase activity, except *F. nucleatum* ATCC 10953 which had weak aspartate dipeptidase activity (data not shown). In addition, *P. gingivalis* strains did not utilize the free amino acids aspartate and glutamate, and one of the tested strains (*P. nigrescens* ATCC 25261) had a higher metabolic rate for aspartylaspartate than for aspartate (Fig. 1 and Table 1). Together, these results suggest that *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *F. nucleatum* strains incorporate dipeptides via a transport system specific for dipeptides, as do other peptide-utilizing bacteria (13), and subsequently degrade the dipeptides into free amino acids using intracellular dipeptidases.

In addition to a dipeptide transport system, most peptide-utilizing bacteria have been reported to have tripeptide and/or oligopeptide transport systems capable of transporting peptides longer than dipeptides (13). However, *P. intermedia* and *F. nucleatum* appeared to have no transport activity for oligopeptides longer than dipeptides. This may depend on the amino acid composition of peptides, but further study is needed to elucidate this. The utilization of tetrapeptides by *P. gingivalis* and *P. nigrescens* may suggest that these bacteria have a transport system for tetrapeptides. However, it is also possible that *P. gingivalis* and *P. nigrescens* degrade tetrapeptides into dipeptide using extracellular proteases, for example dipeptidyl peptidases (5, 18). The slow metabolism of longer peptides by *P. gingivalis* also suggests the involvement of extracelular proteases in the hydrolysis of the peptides into dipeptides.

Dipeptides supported the growth of

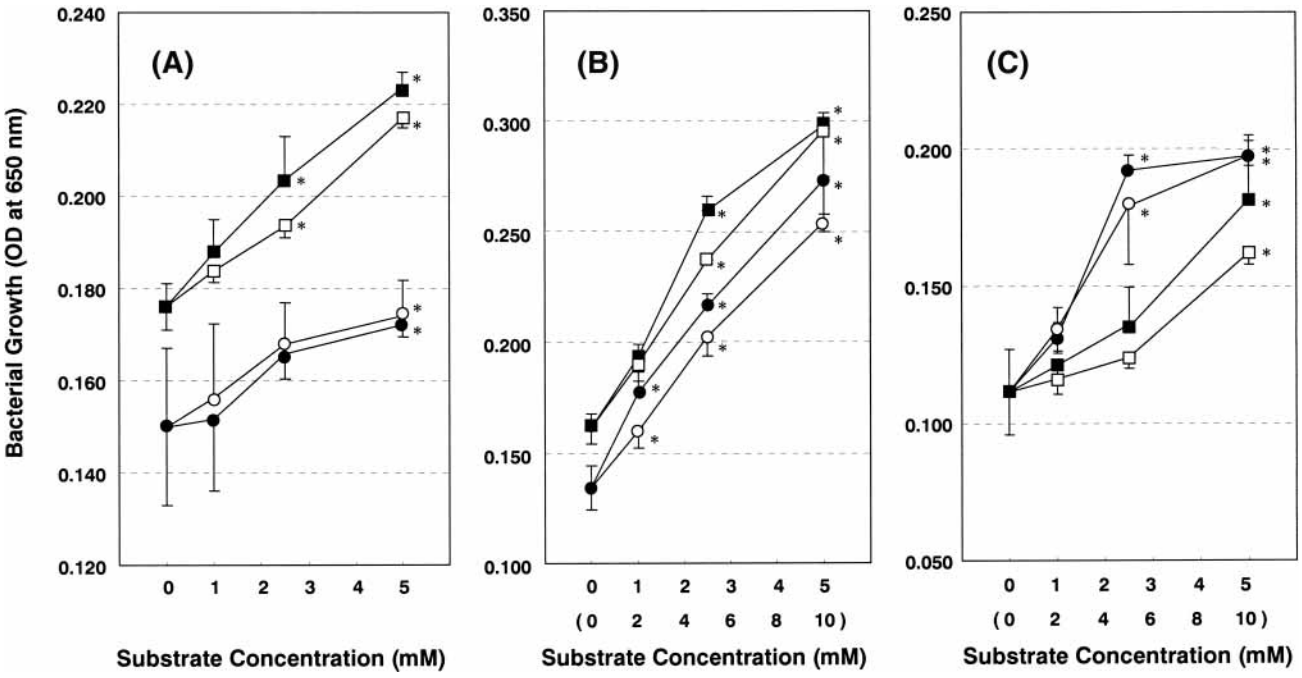

Fig. 2. Growth response to free amino acids and dipeptides of *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *F. nucleatum*. (**A**) Growth of *P. gingivalis* ATCC 33277 (circle) and W83 (square) in the presence of aspartylaspartate (open symbol) and glutamylglutamate (closed symbol); (**B**) growth of *P. intermedia* ATCC 25611 (circle) and *P. nigrescens* ATCC 25261 (square) in the presence of aspartate (open symbol) and aspartylaspartate (closed symbol); (**C**) growth of *F. nucleatum* ATCC 25586 (circle) and ATCC 10953 (square) in the presence of glutamate (open symbol) and glutamylglutamate (closed symbol). *Significantly different from controls ($P < 0.05$ by Dunnett test). The substrate concentrations in parentheses are for aspartate or glutamate. Data are given as means with standard deviations obtained from three independent experiments.

all the tested strains of *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *F. nucleatum* in a concentration-dependent manner up to 5 mM (Fig. 2), indicating that dipeptides can be utilized as a source of energy and cell growth material. *P. intermedia*, *P. nigrescens* and *F. nucleatum* also increased their growth in the presence of the corresponding free amino acids (Fig. 2), while aspartate and glutamate had no effect on the growth of *P. gingivalis*. Wyss (28) reported that some strains of *P. gingivalis* grew in his chemically defined medium consisting of free amino acids as nitrogen sources, although these bacteria required fetal calf serum for maximum growth. This discrepancy may be a result of differences in bacterial strains and bacterial growth conditions.

The growth response of *P. gingivalis* ATCC 33277 was greater than that of strain W83 (Fig. 2A), although the resting cells of these two strains had similar ammonia production (Fig. 1A). This may be a result of bacterial strain-dependent differences in energy efficiency and/or phenotypic characteristics such as nutrition requirements.

The periodontal pathogens tested in this study can grow on aspartate, glutamate and/or dipeptides of these amino acids. Both aspartate and glutamate are among the major amino acids contained in saliva (19), gingival crevicular fluid (19) and dental plaque fluid (6, 8, 17). It is possible that *P. intermedia*, *P. nigrescens* and *F. nucleatum* adapt to the oral environment where such amino acids are continuously and abundantly supplied. These oral fluids also contain various proteins and glycoproteins such as albumin and mucin, which contain aspartate and glutamate. *P. gingivalis*, *P. intermedia* and *P. nigrescens* may generate aspartate- and/or glutamate-containing dipeptides from these proteins and glycoproteins by proteolytic activity (5, 18), and supply these peptides to both themselves and other dipeptide-utilizing but nonproteolytic bacteria such as *F. nucleatum* and *Peptostreptococcus micros* (25).

Acknowledgments

This study was supported in part by the Inamori Foundation, the Inoue Foundation for Science, and Grants-in-Aid for Scientific Research (B) (no. 11470386) and for Exploratory Research (no. 11877321) from the Japan Society for the Promotion of Science. This study was also partly supported by a Health Science Research Grant (no. H12-RHTA-015).

References

1. Bergmeyer HU. Ammonia. Methods Enzym Anal 1983: **8**: 454–461.
2. Buckel W, Barker HA. Two pathways of glutamate fermentation by anaerobic bacteria. J Bacteriol 1974: **117**: 1248–1260.
3. Fujimura S, Nakamura T. Isolation and characterization of a protease from *Bacteroides gingivalis*. Infect Immun 1987: **55**: 716–720.
4. Gharbia SE, Shah HN. Glucose utilization and growth response to protein hydrolysates by *Fusobacterium* species. Curr Microbiol 1988: **17**: 229–234.
5. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. Periodontology 2000 1999: **20**: 168–238.
6. Hyatt AT, Hayes ML. Free amino acids and amines in human dental plaque. Arch Oral Biol 1975: **20**: 203–209.
7. Lund P. L-Glutamine and L-glutamate: UV-method with glutaminase and glutamate dehydrogenase. Methods Enzym Anal 1983: **8**: 357–363.
8. Margolis HC, Moreno EC. Composition and cariogenic potential of dental plaque fluid. Crit Rev Oral Biol Med 1994: **5**: 1–25.
9. Milner P, Batten JE, Curtis MA. Development of a simple chemically defined medium for *Porphyromonas gingivalis*: requirement for α-ketoglutarate. FEMS Microbiol Lett 1996: **140**: 125–130.
10. Möllering H. L-Asparagine and L-aspartate. Methods Enzym Anal 1983: **8**: 350–357.
11. Niederman R, Brunkhorst B, Smith S, Weinreb RN, Ryder MI. Ammonia as a potential mediator of adult human periodontal infection: inhibition of neutrophil function. Arch Oral Biol 1990: **35**: 205–209.
12. Niederman R, Zhang J, Kashket S. Short-chain carboxylic-acid-stimulated, PMN-mediated gingival inflammation. Crit Rev Oral Biol Med 1997: **8**: 269–290.
13. Payne JW, Smith MW. Peptide transport by micro-organisms. Adv Microbial Physiol 1994: **36**: 1–80.
14. Rogers AH, Gully NJ, Pfennig AL, Zilm PS. The breakdown and utilization of peptides by strains of *Fusobacterium nucleatum*. Oral Microbiol Immunol 1992: **7**: 299–303.
15. Seddon SV, Shah HN, Hardie JM, Robinson JP. Chemically defined and minimal media for *Bacteroides gingivalis*. Curr Microbiol 1988: **17**: 147–149.
16. Shah HN, Williams RAD. Utilization of glucose and amino acids by *Bacteroides intermedius* and *Bacteroides gingivalis*. Curr Microbiol 1987: **15**: 241–246.
17. Singer DL, Kleinberg I. Free amino acids in human dental plaque. Arch Oral Biol 1983: **28**: 873–878.
18. Suido H, Nakamura M, Mashimo PA, Zambon JJ, Genco RJ. Arylaminopeptidase activities of oral bacteria. J Dent Res 1986: **65**: 1335–1340.
19. Syrjänen SM, Alakuijala L, Alakuijala P, Markkanen SO, Markkanen H. Free amino acid levels in oral fluids of normal subjects and patients with periodontal disease. Arch Oral Biol 1990: **35**: 189–193.
20. Takahashi N, Abbe K, Takahashi-Abbe S, Yamada T. Oxygen sensitivity of sugar metabolism and interconversion of pyruvate formate-lyase in intact cells of *Streptococcus mutans* and *Streptococcus sanguis*. Infect Immun 1987: **55**: 652–656.
21. Takahashi N, Saito K, Schachtele CF, Yamada T. Acid tolerance of growth and neutralizing activity of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum*. Oral Microbiol Immunol 1997: **12**: 323–328.
22. Takahashi N, Sato T. Preferential utilization of dipeptides by *Porphyromonas gingivalis*. J Dent Res 2001: **80**: 1425–1429.
23. Takahashi N, Sato T, Yamada T. Metabolic pathways for cytotoxic end-product formation from glutamate- and aspartate-containing peptides by *Porphyromonas gingivalis*. J Bacteriol 2000: **182**: 4704–4710.
24. Takahashi N, Yamada T. Pathways for amino acid metabolism by *Prevotella intermedia* and *Prevotella nigrescens*. Oral Microbiol Immunol 2000: **15**: 96–102.
25. Tang-Larsen J, Claesson R, Edlund, M-B, Carlsson, J Competition for peptides and amino acids among periodontal bacteria. J Periodont Res 1995: **30**: 390–395.
26. Wahren A, Gibbons RJ. Amino acid fermentation by *Bacteroides melaninogenicus*. Antonie van Leeuwenhoek 1970: **36**: 149–159.
27. Wyss C. Aspartame as a source of essential phenylalanine for the growth of oral anaerobes. FEMS Microbiol Lett 1993: **108**: 255–258.
28. Wyss C. Growth of *Porphyromonas gingivalis*, *Treponema denticola*, *T. pectinovorum*, *T. socranskii*, and *T. vincentii* in a chemically defined medium. J Clin Microbiol 1992: **30**: 2225–2229.