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Utilization of D-amino acids by *Fusobacterium nucleatum* and *Fusobacterium varium*

M. Ramezani, S. E. MacIntosh, and **R. L. White**

Department of Chemistry, Dalhousie University, Halifax, Nova Scotia, Canada

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Summary. The utilization of D- and L-amino acids with acidic, basic or polar side chains was demonstrated by HPLC. Two species of the anaerobe *Fusobacterium* utilized D-lysine and the L isomers of glutamate, glutamine, histidine, lysine and serine. Only *F. varium* used L-arginine, D-glutamate and D-serine as substrates, whereas *F. nucleatum* specifically utilized D-histidine and D-glutamine. D-Glutamate accumulated in *F. nucleatum* cultures supplemented with D-glutamine, and ornithine was detected when either DL- or L-arginine was included in *F. varium* cultures. Based on literature precedents, D-glutamate and D-histidine are isomerized to their L isomers prior to degradation, but separate catabolic pathways are possible for each enantiomer of lysine and serine.

Keywords: Amino acids – Anaerobic bacteria – Catabolism – HPLC – Stereochemistry

Introduction

Amino acids are important carbon and energy sources for anaerobic bacteria of the genus *Fusobacterium* (Jackins and Barker, 1951; Loesche and Gibbons, 1968; Rogers et al., 1991), and the transport and intracellular accumulation of glucose and galactose by *F. nucleatum* depends on energy provided by the fermentation of glutamic acid, histidine or lysine (Robrish et al., 1987). Amino acids with acidic, basic or polar side chains are preferred over amino acids with nonpolar side chains, but the specific amino acids utilized vary among *Fusobacterium* species and also among strains within a species (Gharbia and Shah, 1991). Moreover, fresh clinical isolates of *F. nucleatum* utilize a broader range of amino acids after repeated subculturing (Gharbia and Shah, 1991), and utilization depends to some extent on the composition of the culture medium (Gharbia and Shah, 1991; Rogers et al., 1991).

While the nutritional role of amino acids in fusobacteria has been established, the utilization of D-amino acids has not been assessed. Amino acids of

unspecified stereochemistry (Gharbia and Shah, 1989; 1991; Gharbia et al., 1989; Jackins and Barker, 1951; Rogers et al., 1991; Shah and Gharbia, 1989; Ushijima, 1992), mixtures of DL-and L-amino acids (Dzink and Socransky, 1990; Loesche and Gibbons, 1968), or only L-amino acids (Bakken et al., 1989) have been employed in the nutritional studies along with analytical methods incapable of separating enantiomeric amino acids. In bacteria, however, D-amino acids are not metabolically inert (Kuhn and Somerville, 1971; Meister, 1965; Rydon, 1948; Tanaka et al., 1996). D-Alanine, D-glutamate and *meso*-diaminopimelic acid are biosynthesized and incorporated into the peptidoglycan crosslinks of bacterial cell walls (Bugg and Walsh, 1992); L-isoleucine is formed from D-threonine in *Serratia marcescens* (Kisumi et al., 1964) and D-aspartate is converted to D- and L-alanine by a *Pseudomonas* species (Markovetz et al., 1966). Isomerization of the L-amino acid to the corresponding D isomer is the first step in the catabolism of L-alanine by enterobacteria (McFall and Newman, 1996) and of L-ornithine by *Clostridium sticklandii* (Tsuda and Friedmann, 1970), whereas separate pathways have been proposed for the catabolism of D- and L-lysine by *Clostridium* species (Stadtman, 1973).

Given the nutritional importance of amino acids to fusobacteria and the precedents for D-amino acid metabolism in bacteria, the present investigation examines whether D-amino acids are substrates for *F. nucleatum* and *F. varium*.

Materials and methods

Microorganisms, growth conditions, and media

Fusobacterium nucleatum (ATCC 25586) and *Fusobacterium varium* (NCTC 10560) were subcultured at weekly intervals on 5% (v/v) sheep-blood agar (Victoria General Hospital, Halifax, NS). All bacterial transfers were performed in air, but both agar and liquid cultures were incubated at 37°C in anaerobic jars under an atmosphere of $H_2/CO_2/N_2$ (10:10:80; v/v).

Peptone medium contained (g/L): trypticase peptone (BBL Microbiology System, Cockeysville, MD, U.S.A.), 5.0; proteose peptone (Difco Laboratories, Detroit, MI, U.S.A.), 5.0; yeast extract (Difco), 5.0; glucose, 5.0; NaCl, 5.0; and L-cysteine hydrochloride, 0.80. The components were dissolved in water, adjusted to pH 7.3–7.4 with 3M NaOH, and autoclaved. Asparagine, citrulline, glutamine, and 5-hydroxy-4-OXO-L-norvaline (White et al., 1994) were dissolved in potassium phosphate buffer (100mM, pH 7.4, 3ml), filter sterilized (0.22μm), and added to sterile peptone medium; all other amino acids were added to the peptone medium prior to autoclaving.

Survey of amino acid utilization

Bacterial cells spread on a 9-cm-diameter plate of sheep-blood agar and grown for 24h were suspended in peptone medium (3ml), and a portion (100–200μl) of the suspension was used to inoculate peptone medium (50ml) supplemented with an amino acid (either 10, 17 or 20mM). The culture was incubated under anaerobic conditions, and 0.5-ml samples were removed aseptically prior to inoculation and at various incubation times up to 50h. Each sample was centrifuged (15,400g, 10min); a portion of the supernatant (20μl) was mixed with water (380μl) and stored at −15°C for HPLC analysis.

Amino acid analysis by HPLC

Fluorescent isoindole derivatives of amino acids were prepared by mixing the sample solution (20 μl) with water (20 μl) and commercial *o*-phthalaldehyde reagent (40 μl, Fluoraldehyde, Pierce Chemical Co., Rockford, IL, U.S.A.). After 1 min at ambient temperature, sodium acetate solution (120 μl, 0.1 M, pH 6.5) was added, and a 20-μl portion was injected onto a Beckman Ultrasphere ODS column (5 μm, 45 × 4.6 mm). Separations were achieved at a total flow rate of 2.5 ml/min using gradients formed between sodium acetate (0.1 M, pH 6.5 with 3 M HCl)-methanol-THF (900:95:5) and methanol (White et al., 1989). Alanine, α-aminoadipic acid, arginine, aspartic acid, glutamic acid, glutamine, isoleucine, leucine, methionine, serine, tyrosine, and valine were analyzed using a gradient of composition (min, % methanol): 0.0, 0; 0.5, 15; 3.0, 15; 3.25, 30; 5.75, 30; 6.0, 100; 6.5, 100; 7.0, 0. The following gradient composition was used for all other amino acid determinations (min, % methanol): 0.0, 0; 0.5, 15; 3.0, 15; 3.25, 30; 4.0, 30; 4.25, 45; 6.25, 45; 6.5, 100; 7.0, 100; 7.5, 0. Amino acid concentrations were calculated as a percentage of the initial concentration by comparing the chromatographic peak areas of samples collected after a period of incubation to those determined prior to inoculation.

Enantiomeric analysis of amino acids by hplc

Derivatization reagent (80 μl), prepared by dissolving *N*-acetyl-L-cysteine (1.0 mg) in commercial, incomplete *o*-phthalaldehyde reagent (1.0 ml, Sigma Chemical Co., St. Louis, MO, U.S.A.), was mixed with sample solution (20 μl), and the reaction was allowed to proceed for 3 min at ambient temperature with occasional agitation. Mobile phase used for the separation (100 μl) was added, and a 20-μl portion was injected. The fluorescent isoindole derivatives were separated by gradient elution at a total flow rate of 2.0 ml/min on a Nucleosil 5 C18 column (4.6 × 250 mm, Phenomenex, Torrance, CA, U.S.A.) (Lam, 1986; White et al., 1994). D-and L-Arginine had retention times of 17.0 and 14.2 min, respectively, when a gradient of composition (min, % acetonitrile) 0.0, 0; 3.0, 5; 13.0, 5; 14.0, 7; 21.0, 7; 23.0, 0 was created by mixing acetonitrile with a solution of acetonitrile (5%) – copper (II) acetate (2.5 mM) – L-proline (5 mM) – ammonium acetate (26 mM), adjusted to pH 7.0 with 5-M NaOH. For the enantiomeric analysis of glutamate and serine, gradients of composition (min, % acetonitrile) 0.0, 0; 3.0, 4; 10.0, 4; 11.0, 7; 16.0, 7; 18.0, 0 and 0.0, 0; 4.0, 6; 9.0, 6; 10.0, 10; 14.0, 10; 16.0, 0, respectively, were formed between acetonitrile and acetonitrile (2%) – copper (II) acetate (2.5 mM) – L-proline (5 mM), adjusted to pH 6.0 with ammonium acetate. Retention times of 14.2 and 11.6 min were obtained for D- and L-glutamate, while D- and L-serine had retention times of 9.1 and 7.6 min, respectively.

Results

Amino acid concentrations in *Fusobacterium* cultures were monitored by HPLC analysis of fluorescent isoindole derivatives (White et al., 1989). After incubation of cultures supplied with racemic amino acids the residual amino acid concentration was close to either 0, 50 or 100% of the initial concentration, indicating complete, partial or insignificant utilization of each enantiomer. The third group included aromatic amino acids (phenylalanine, *threo*-phenylserine, tyrosine and tryptophan) and amino acids with nonpolar, aliphatic side chains (alanine, 2-aminobutanoic acid, 3-aminobutanoic acid, isoleucine, leucine, norleucine, L-norvaline, and valine); these were not reduced in concentration after approximately 42-h incubations with each organism. The concentrations of several amino acids with acidic, basic or

polar side chains (2-aminoadipic acid, asparargine, aspartic acid, citrulline, homoserine, methionine, ornithine, 5-hydroxy-4-oxo-L-norvaline and threonine) remained at more than 80% of the initial concentration, while the concentrations of five others decreased significantly. Whereas these decreases occurred during incubation with either *F. nucleatum* or *F. varium* (Table 1), the arginine concentration declined in only the *F. varium* culture.

Whether an approximately 50% residual concentration (Table 1) represented selective utilization of one component in the racemic mixture was examined in more detail. HPLC separations of diastereomeric, fluorescent isoindole derivatives prepared from *o*-phthalaldehyde and *N*-acetylcysteine (Lam, 1986; White et al., 1994) provided the enantiomeric composition of amino acids in culture fluids. In *F. nucleatum* cultures the residual glutamate and serine were more than 95% D isomer, and only D-arginine remained in the *F. varium* culture. Subsequent incubations of the individual stereoisomers demonstrated that L-glutamate (*F. nucleatum*), L-serine (*F. nucleatum*), and L-histidine (*F. varium*) were utilized within the first 30 h, and that utilizations of L-arginine and L-glutamine by *F. varium* were slower. Parallel cultures containing the D isomers showed no decrease in amino acid concentration after 50 h of incubation.

On the other hand, enantiomeric analysis by HPLC demonstrated that both isomers of serine were utilized by *F. varium*. Incubations of the individual isomers confirmed that the approximately 50% residual concentrations of DL-lysine and DL-serine (Table 1) were due to the partial utilization of both isomers by *F. varium*. The utilization of other D-amino acids (D-glutamate by *F. varium*; D-glutamine, D-histidine and D-lysine by *F. nucleatum*) was indicated by low (<20%) residual concentrations of the racemic amino acids, and was confirmed by separate incubations of the D and L enantiomers (Table 1). With the exception of serine, the L-amino acids were utilized without a signifi-

Table 1. Residual concentrations of amino acids after approximately 42 h of incubation with *Fusobacterium* species (expressed as a percentage of the initial concentration)

Amino acid[a]	*F. nucleatum*			*F. varium*		
	DL	D	L	DL	D	L
Arginine	101			38[b,c]	99	24
Glutamic acid[d]	45[b]	96	0	0	0	0
Glutamine	0	52[e]	0	42	89	18
Histidine	0[d]	0	0	64[d]	99	0
Lysine	15[d]	5	3	66[d]	4	3
Serine	48[b,f]	102	1	54[f,g]	0	0

[a] 10 mM initial concentration of either the DL-, D-, or L-amino acid as indicated. [b] Utilization of the L isomer detected by HPLC. [c] Accumulation of ornithine detected by HPLC. [d] 17 mM initial concentration. [e] Accumulation of D-Glutamate detected by HPLC. [f] 20 mM initial concentration. [g] Utilization of both enantiomers detected by HPLC.

cant lag period and at a slightly faster rate than their enantiomers (Fig. 1). Except in the utilization of D-glutamine by *F. nucleatum*, the D-amino acids were removed from culture supernatants within approximately 40 h. The difference between the rates of D- and L-lysine utilization decreased at a higher initial concentration (20 mM) of the racemate.

No new amino acids accumulated in cultures of *F. nucleatum* during utilization of L-glutamate, L-glutamine, DL-histidine, DL-lysine and L-serine, and in cultures of *F. varium* during utilization of DL-glutamate, L-glutamine, L-histidine, DL-lysine and DL-serine, but a chromatographic peak corresponding to D-glutamate was detected by HPLC in the *F. nucleatum* culture supplemented with D-glutamine. HPLC analysis after 20 h of incubation showed that the original glutamate in the peptone medium had been depleted; as the incubation continued, the fall in glutamate concentration was reversed. The rise in glutamate concentration corresponded to the decreased concentration of glutamine. Under identical conditions, little or no D-glutamine hydrolyzed in the *F. varium* culture (Table 1), excluding a nonenzymatic process in the *F. nucleatum* culture. An accumulation of ornithine was also detected by HPLC in *F. varium* cultures supplemented with either DL- or L-arginine.

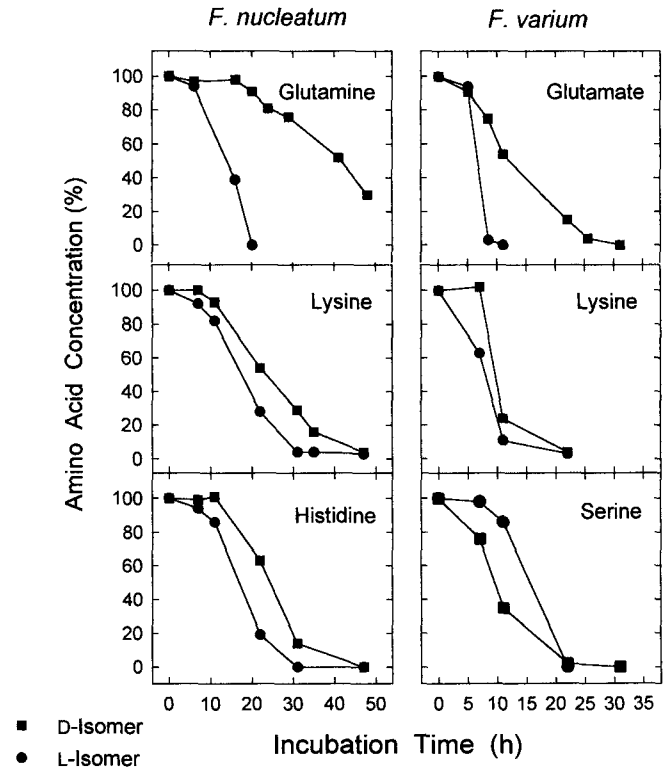

Fig. 1. Utilization of D- and L-amino acids. Each amino acid stereoisomer was incubated separately with *F. nucleatum* and *F. varium*. Except for glutamate at 17 mM, the initial concentration was 10 mM

Discussion

Of the 27 amino acids investigated, six with acidic, basic or polar side-chain substituents (Table 1) were utilized most rapidly by the two *Fusobacterium* species. While L-arginine was a substrate only for *F. varium*, the L isomers of the other five amino acids were utilized by both species. D-Lysine was utilized by each organism, but complementary preferences were exhibited towards other D-amino acids, *i.e.*, D-glutamine and D-histidine were substrates for *F. nucleatum*, and *F. varium* utilized D-glutamate and D-serine. Whenever a D-amino acid was utilized the corresponding L isomer was also catabolized. Therefore it is inappropriate to assume that only L-amino acids are utilized by fusobacteria, and investigations of D-amino acid catabolism are warranted when the corresponding L isomer is utilized. Examples of the selective utilization of L- over D-amino acids were observed, this stereoselective catabolism of certain L-amino acids suggests that *F. nucleatum* and *F. varium* could be employed to prepare D-amino acids from the corresponding racemic mixtures.

The utilization of L-arginine by *F. varium*, and of five other L-amino acids (glutamate, glutamine, histidine, lysine and serine) by both *Fusobacterium* species, is consistent with the major preferences demonstrated in previous investigations (Bakken et al., 1989; Dzink and Socransky, 1990; Gharbia and Shah, 1989, 1991; Gharbia et al., 1989; Jackins and Barker, 1951; Loesche and Gibbons, 1968; Rogers et al., 1991). As the uptake of amino acids from culture medium by *Fusobacterium* species is influenced by peptides and other amino acids (Gharbia and Shah, 1991; Gharbia et al., 1989; Shah et al., 1993), the somewhat narrower range of amino acids utilized in our investigation is attributed to the rich medium employed. The different preferences of *F. nucleatum* and *F. varium* towards D-amino acids and L-arginine reflect genetic differences between these species, which map to different branches of the phylogenetic tree (Lawson et al., 1991); these characteristics may be useful for taxonomic purposes.

Most pathways and many of the enzymes involved in the catabolism of L-amino acids by anaerobic bacteria have been characterized (Barker, 1981; McInerney, 1988), and the information available suggests that D-amino acids are either degraded by a separate pathway or converted to the corresponding L isomer before degradation. Conversion of serine to pyruvate is catalyzed by serine dehydratase; enzymes specific for D- or L-serine (EC 4.2.1.14 and EC 4.2.1.13, respectively) have been characterized (McFall and Newman, 1966). A higher level of D-serine dehydratase activity in *F. varium* would account for the faster utilization of D-serine (Fig. 1). Distinct routes have been proposed for the catabolism of D- and L-lysine in *Clostridium* species (Stadtman, 1973), and the degradation of ^{14}C-labelled DL-lysine by *F. nucleatum* has been interpreted in terms of two different pathways (Barker et al., 1982). However, lysine racemase activity has also been reported (Stadtman, 1973).

Two distinct pathways for the degradation of glutamate have been documented in different species of anaerobic bacteria (Barker, 1981). L-Glutamate is degraded by the hydroxyglutarate pathway in *F. nucleatum* (White et al.,

1995) and by the methylaspartate pathway in *F. varium* (Ramezani, 1996). A partially purified glutamate racemase from *F. varium* (Ramezani, 1996) is probably involved in the catabolism of D-glutamate. Glutamate has been suggested as an intermediate of histidine catabolism in anaerobic bacteria (Barker, 1961). However, the lack of D-glutamate utilization in *F. nucleatum* is not consistent with D-glutamate as an intermediate of D-histidine catabolism. In *Salmonella typhimurium*, a D-amino acid dehydrogenase is involved in a two-step conversion of D- to L-histidine (Hecht et al., 1996). Whether oxidative deamination or another route operates for D-histidine catabolism in *F. nucleatum* has not been determined. The slower utilization of D-glutamate and D-histidine (Fig. 1) is consistent with racemization as a slow first step in each catabolic pathway.

Catabolism of arginine and glutamine proceeds by hydrolytic processes. The accumulation of ornithine in arginine-supplemented cultures is consistent with the arginine deiminase pathway, which generates ammonia, carbon dioxide, and 1 mol of ATP from each mole of arginine degraded (Abdelal, 1979). Whether glutamine hydrolysis is catalyzed by specific enzymes or by cell-bound exopeptidases (Rogers et al., 1991) is not known.

In summary, both D- and L-amino acids are utilized by *F. nucleatum* and *F. varium*, and further investigations are needed to define the pathways of D-amino acid catabolism. Since D-amino acids are provided by diet and the breakdown of bacterial cell walls, the ability to utilize D-amino acids provides a competitive advantage to fusobacteria, which inhabit the oral cavity and the intestine together with many other species of bacteria.

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Authors' address: Dr. Robert L. White, Department of Chemistry, Dalhousie University, Halifax, Nova Scotia, B3H 4J3, Canada, E-mail: RLWHITE@Chem1.Chem.Dal.Ca

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