Comparison of peptidase, glycosidase and esterase activities of oral and non-oral *Treponema* species

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The enzyme profiles of 20 oral and non-oral *Treponema* strains were investigated using an API ZYM Complete Research kit. The test included 10 2-naphthyl derivatives of fatty acids, 20 *p*-nitrophenol derivatives of carbohydrates and 60 2-naphthylamide derivatives of amino acids and peptides. The oral *Treponema* species investigated were *T. denticola*, *T. vincentii* and *T. pectinovorum*. The non-oral species examined were *T. phagedenis*, *T. hyodysenteriae* and intestinal spirochaetes of human and chicken origin. Esterase activities on C₅ to C₁₀ fatty acids were common among different *Treponema* species. Glycosidase activities were infrequently observed in *T. vincentii*, *T. pectinovorum* and *T. phagedenis* Reiter strain. Arabinosidase, lactosidase and xylosidase activity was observed in the *T. hyodysenteriae* strains but α -L-fucosidase activity was found only in *T. denticola* and *T. phagedenis*. More exo- and endo-peptidase activities were found in *T. denticola* than in other species. The enteropathogenic *T. hyodysenteriae* isolates had a very low proteolytic profile. Dipeptidyl prolyl amidase activity was observed in all species except in the *T. phagedenis* Reiter strain and the avian intestinal spirochaetes. The enzyme profiles did not discriminate between oral and non-oral *Treponema* species.

Introduction

Spirochaetes are helical-shaped Gram-negative organisms which are prominent in subgingival plaque of inflamed periodontal tissue and in the microflora associated with the mucosa of the intestinal tract of man and animals with severe forms of diarrhoea (Harris *et al.*, 1976; Listgarten & Hellden, 1978; Sanna *et al.*, 1982). Oral spirochaetes have been observed in gingival lesions in and between gingival epithelial cells (Listgarten, 1965; Mikx *et al.*, 1990). Similar lesions have been described for *T. hyodysenteriae* in swine and chicken intestinal mucosa (Kennedy *et al.*, 1973; Davelaar *et al.*, 1986). The pathogenicity of *T. hyodysenteriae* is generally accepted. The pathogenicity of oral spirochaetes is still under discussion. Several oral treponemas can attach to epithelial cells and suppress cell proliferation, and have proteolytic enzymes which are considered of pathogenic importance (Olson 1984; Reijntjens *et al.*, 1986; Uitto *et al.*, 1986).

In order to compare the enzymic profiles of distinct pathogenic and other treponemas, 20 isolates were tested for the hydrolysis of a large number of synthetic substrates.

Methods

Oral Treponema strains. *T. denticola* ATCC 33520, Ny375 (a subgingival isolate; ter Steeg *et al.*, 1988), B11 and B12 (isolated from periodontal biopsies; Mikx *et al.*, 1989) and Ny576 (a subgingival isolate; this laboratory) were cultured anaerobically in BM broth (Gibbons & MacDonald, 1960) with 25 mg thiamin pyrophosphate l⁻¹. *T. vincentii* ATCC 35580, LA1 (a subgingival isolate; obtained from R. Taichman, Philadelphia, USA), CH3 (oral isolate; obtained from E. Chan, Montreal, Canada), Ny535 (isolated from gingival biopsy; this laboratory) and *T. pectinovorum* ATCC 337768 were cultured anaerobically in TYGVS broth (Laughton *et al.*, 1981) with 0.8 g l⁻¹ galacturonic acid.

Non-oral Treponema strains. *T. phagedenis* Reiter (human isolate; RIVM, Bilthoven, The Netherlands) was cultured in Todd Hewitt broth (Difco). *T. hyodysenteriae* ATCC 271664, ATCC 31212 and T50 and T47, isolated from swine intestine (obtained from R.P.A.M. Segers, Utrecht, The Netherlands), chicken intestine isolates 1380 and 4742 (obtained from H. F. Smit, Doorn, The Netherlands) and human intestine isolates HRM4, HRM6, HRM13 and HRM14 (obtained from G. Dettori, Rome, Italy) were cultured anaerobically in TSBS broth (Sanna *et al.*, 1982).

Enzyme assays. Bacterial cultures, 4–5 d old, were harvested by centrifugation and resuspended in 10 ml 0.05 M-HEPES buffer, pH 7.2, to give an OD₅₅₀ of at least 1.5. According to the instructions of the manufacturer, drops of the suspension were added to cupules of the API ZYM Complete Research kit and incubated aerobically for 4 h at

Table 1. *Glycosidase activities of different Treponema species and intestinal spirochaetes of human and avian origin*

The explanation of scores is given in Methods. The number of strains tested is given in parentheses.

<i>p</i> -Nitrophenol derivative	<i>T. denticola</i> (4)	<i>T. vincentii</i> (4)	<i>T. pectinovorum</i> (1)	<i>T. phagedenis</i> (1)	<i>T. hyodysenteriae</i> (4)	Intestinal spirochaetes	
						Human (4)	Avian (2)
α -D-Galactose	3	0	0	0	0	1	1
β -D-Galactose	1	0*	0	0	3	2	1
α -L-Arabinose	0	0	0	0	1	0	0
α -D-Glucose	3	0	0	0	0	0	0
β -D-Glucose	3	0	0	0	1	0†	1
β -Maltose	0	0	0	0	0	0†	0
<i>N</i> -Acetyl- β -D-glucosamine	1	2	2	1	1	1	1
α -L-Fucose	1	0	0	2	0	0	0
β -D-Fucose	3	0*	0	0	2	2	1
β -D-Lactose	0	0	0	0	1	0†	0
β -D-Xylose	0	0	0	0	1	0	0

* Strain LA1 score 2.

† Strain HRM6 score 2.

Table 2. *Esterase activities of different Treponema species and intestinal spirochaetes of human and avian origin*

The explanation of scores is given in Methods. The number of strains tested is given in parentheses.

2-Naphthyl derivative	<i>T. denticola</i> (4)	<i>T. vincentii</i> (4)	<i>T. pectinovorum</i> (1)	<i>T. phagedenis</i> (1)	<i>T. hyodysenteriae</i> (4)	Intestinal spirochaetes	
						Human (4)	Avian (2)
Butyrate C ₄	1	0	1	0	1	1	0
Valerate C ₅	1	1	1	1	1	1	1
Caproate C ₆	3	2	2	2	3	3	2
Caprylate C ₈	3	2	2	2	3	3	2
Nonanoate C ₉	1	2	1	3	2	2	1
Caprate C ₁₀	2	0	1	0	1	2	0
Laurate C ₁₂	1	0	1	1	1	0	0
Myristate C ₁₄	1	0	0	0	0	0	0
Palmitate C ₁₆	1	0	0	0	0	0	0
Stearate C ₁₈	1	2	1	0	1	1	0

37 °C. The API ZYM Complete Research kit allows the simultaneous study of 90 different enzymes: 10 esterases using 2-naphthyl derivatives of fatty acids, 20 glycosidases using *p*-nitrophenol (PNP) derivatives of carbohydrates and 60 peptidases using 2-naphthylamide derivatives of amino acids and peptides (API bio Mérieux, Den Bosch, The Netherlands). Hydrolysis of the PNP derivatives gave a yellow colour (liberated PNP). Release of naphthylamide was estimated with the Fast blue reagents ZYM A and ZYM B. The reactions were read by the use of the API ZYM colour chart. According to the instructions of the manufacturer the scores 1 to 5 are related to 5 to 40 nmol substrate hydrolysed. The median scores are presented in the Tables.

Results

The glycosidase activities of the different strains are presented in Table 1. *T. denticola* showed a larger number and higher levels of activities than the other oral strains. Only for *N*-acetyl- β -D-glucosaminidase was the

activity higher in *T. vincentii*. The α -L-fucosidase activity in *T. denticola* and *T. phagedenis* Reiter strain was particularly noteworthy. The swine *T. hyodysenteriae* strains showed some arabinosidase, lactosidase and xylosidase activity. Atypical results for *T. vincentii* β -D-galactosidase and β -D-fucosidase activities were (score 2) found with one strain, LA1. This strain also gave different scores in the arylamidase tests (Tables 3 and 4). An atypical result was also found among the human intestinal spirochaetes with strain HRM6 (score 2).

The esterase activities are shown in Table 2. The strongest reactions were found using the derivatives of the C₆ to C₁₀ fatty acids.

The results of the peptidase activities are grouped according to the number of peptide bonds and the carboxy-terminal amino acid. Amino arylamidase activities were found in *T. denticola* but less so in other species

Table 3. Amino arylamidase activities of different *Treponema* species and intestinal spirochaetes of human and avian origin

The explanation of scores is given in Methods. The number of strains tested is given in parentheses.

2-Naphthylamide derivative	<i>T. denticola</i> (4)	<i>T. vincentii</i> (4)	<i>T. pectinovorum</i> (1)	<i>T. phagedenis</i> (1)	<i>T. hyodysenteriae</i> (4)	Intestinal spirochaetes	
						Human (4)	Avian (2)
L-Ala	1	0	0	0	0	2	0
L-Gly	0	0	0	0	0	0	0
L-Pro	3	0	0	0	0	0	0
L-hPro	1	0	0	0	0	0	0
L-Ser	1	0	0	0	0	0	0
L-Thr	1	0	0	0	0	0	0
L-Phe	1	0	0	0	0	0	0
L-Tyr	1	0	0	0	0	0	0
L-Trp	1	0	0	0	0	0	0
L-Arg	2	0*	0	2	0	2	0
L-Lys	1	0*	0	2	0	1	0
L-His	1	0*	0	2	0	0	0
L-Gln	2	1	0	1	0	0	0
L-Asp	1	0	0	0	0	0	0
L-Glu	1	0	0	0	0	0	0
DL-Met	1	0	0	1	1	2	0
L-Pyr	3	0	0	2	0	0	0

* Strain LA1 score 3.

Table 4. Dipeptidyl arylamidase activities of different *Treponema* species and intestinal spirochaetes of human and avian origin

The explanation of scores is given in Methods. The number of strains tested is given in parentheses.

2-Naphthylamide derivative	<i>T. denticola</i> (4)	<i>T. vincentii</i> (4)	<i>T. pectinovorum</i> (1)	<i>T. phagedenis</i> (1)	<i>T. hyodysenteriae</i> (4)	Intestinal spirochaetes	
						Human (4)	Avian (2)
L-Asp-L-Ala	1	1	0	1	0	0	0
Gly-L-Ala	1	0	0	0	0	0	0
L-Leu-L-Ala	1	0	0	0	0	0	0
L-Lys-L-Ala	1	0	0	0	0	0	0
Gly-Gly	1	0	0	0	0	0	0
L-Leu-Gly	1	0	0	0	0	0	0
Gly-L-Pro	2	1	1	0	1	0†	0
L-Phe-L-Pro	3	1	1	0	1	1	0
L-His-L-Ser	1	0	0	0	0	0	0
Gly-L-Phe	2	0	0	0	0	0	0
L-His-L-Phe	1	0	0	0	0	0	0
L-Ser-L-Tyr	1	0	0	1	0	0	0
Gly-L-Trp	1	0	0	1	0	0	0
L-Ala-L-Arg	2	0*	0	0	0	0	0
L-Asp-L-Arg	1	0*	0	1	0	0†	0
Gly-L-Arg	2	0*	0	1	0	0	0
L-Phe-L-Arg	2	0*	0	0	0	0	0
L-Arg-L-Arg	2	0*	0	0	0	0	0
L-Pro-L-Arg	2	0*	0	0	0	0	0
L-Lys-L-Lys	2	0*	0	0	0	0	0
L-Gln-L-His	0	0*	0	1	0	0	0
L-Gln-L-Glu	0	0	0	1	0	0	0
L-Ser-L-Met	1	0	0	2	0	0	0

* Strain LA1 score 5.

† Strain HRM6 score 2.

Table 5. Oligopeptidyl arylamidase activities of different *Treponema* species and intestinal spirochaetes of human and avian origin

The explanation of scores is given in Methods. The number of strains tested is given in parentheses.

2-Naphthylamide derivative*	<i>T. denticola</i> (4)	<i>T. vincentii</i> (4)	<i>T. pectinovorum</i> (1)	<i>T. phagedenis</i> (1)	<i>T. hyodysenteriae</i> (4)	Intestinal spirochaetes	
						Human (4)	Avian (2)
L-Ala-L-Phe-L-Pro-L-Ala	2	0	0	0	0	0	0
L-Phe-L-Pro-L-Ala	2	0	0	1	0	0	0
L-Ala-L-Phe-L-Pro	3	0	0	0	0	1	0
L-Leu-L-Leu-L-Val-L-Tyr-L-Ser	1	0	0	0	0	1	0
L-Val-L-Tyr-L-Ser	1	0	0	1	0	0	0
N-CBZ-Gly-Gly-L-Arg	3	0	0	0	0	0	0
N-CBZ-Gly-Gly-L-Arg	3	0	0	0	0	0	0
N-Acetyl-Gly-L-Lys	2	0	0	0	0	0	0
L-His-L-Leu-L-His	1	0	0	0	0	0	0

* CBZ, carbobenzoxy.

Table 6. Amino-, di- and oligo-peptidase activities of different *Treponema* species and intestinal spirochaetes of human and avian origin

The number of strains tested is given in Tables 1-5. Activities of aminopeptidase (A), dipeptidase (D) or oligopeptidase (O) were either present (+) or absent (-). v, Variable, with strain LA1 positive and the other strains negative. NT, not tested.

C-terminal amino acid in 2-naphthylamide derivative																Intestinal spirochaetes					
	<i>T. denticola</i>			<i>T. vincentii</i>			<i>T. pectinovorum</i>			<i>T. phagedenis</i>			<i>T. hyodysenteriae</i>			Human			Avian		
	A	D	O	A	D	O	A	D	O	A	D	O	A	D	O	A	D	O	A	D	O
Alanine	+	+	+	—	—	—	—	—	—	—	—	+	—	—	—	+	—	—	—	—	—
Proline	+	+	+	—	+	—	—	+	—	—	—	—	—	+	—	—	+	+	—	—	—
Serine	+	+	+	—	—	—	—	—	—	—	—	+	—	—	—	—	—	+	—	—	—
Tyrosine	+	+	NT	—	—	NT	—	—	—	—	+	NT	—	—	NT	—	—	NT	—	—	NT
Arginine	+	+	+	V	V	—	—	—	—	+	+	—	—	—	—	+	—	—	—	—	—

(Table 3). Arginyl amidase activity was observed in *T. denticola*, *T. phagedenis* and in the human strains of *T. hyodysenteriae*. Pyrrolidonyl amidase activity (L-Pyr) was only found in *T. denticola* and *T. phagedenis*. Dipeptidyl arylamidase activities were mainly found in *T. denticola* (Table 4). Dipeptidyl prolyl activities were observed in *T. hyodysenteriae* and in all the oral and human intestinal isolates. *T. denticola* showed oligopeptidyl arylamidase activities for all the substrates tested (Table 5).

The amino-, di- and oligopeptidase activities are summarized in Table 6. The grouping of the activities towards 2-naphthylamide derivatives according to the carboxy-terminal amino acid showed a consistent pattern. The most frequently observed activities among the 20 *Treponema* strains investigated were prolyl and arginyl amidases. *T. denticola* showed the widest variety of activities, followed by *T. phagedenis* Reiter strain and the human intestinal strains.

Discussion

The enzymic profiles of different pathogenic and non-pathogenic strains of *Treponema* were compared in order to look for indications of common putative pathogenic activities. Care was taken to test and score the twenty isolates uniformly. However, the different species had to be grown in different media, which might have influenced the presence of inducible enzymes. There was, however, no indication of a medium effect when the results of the API ZYM Complete Research kit were compared with those of the API, ZYM or Rap ID-ANA systems using other culture media (Hunter & Wood, 1979; Syed *et al.*, 1988; Fukumoto *et al.*, 1987).

Most glycosidase activities detected in *T. denticola* were also present in the intestinal spirochaetes. In addition, the *T. hyodysenteriae* isolates showed arabinosidase, lactosidase and xylosidase activities. These activities might be of nutritional value in the swine gut, or, as is

possible for α -L-fucosidase in *T. denticola*, in contact with salivary glycoproteins. The glycosidase profiles of *T. vincentii*, *T. pectinovorum* and *T. phagedenis* resemble those of the small spirochaetes investigated by Fiehn (1986) and indicate that these species probably depend on substrates as carbon and energy source which differ from those used by *T. denticola* and the intestinal spirochaetes.

Esterase activities were common among all the *Treponema* species investigated. Esterases play a role in nutrition and probably, in conjunction with phospholipase activities, in tissue destruction. Phospholipases have not, however, been found in the enteropathogenic *T. hyodysenteriae* isolates (Trevathan *et al.*, 1982; Sibo *et al.*, 1989).

In addition to the present extended comparative study, previous work on the enteropathogenic species *T. hyodysenteriae* has given no indication of a high proteolytic activity nor of a common peptidase activity among the swine, human and chicken intestinal strains. The human intestinal spirochaetes, the swine *T. hyodysenteriae* strains and also the oral *Treponema* species showed a dipeptidyl-prolyl amidase activity. This activity may indicate a requirement for proline peptides, as has been shown in other anaerobic bacteria (Wahren & Holme, 1973).

The observed enzymic activities for the 60 different amino acid and peptide naphthylamide derivatives could be grouped according to the carboxy-terminal amino acid. Arylamidases of *T. denticola* specific for alanine, proline, tyrosine or basic residues have also been found in other oral bacteria. Fukasawa *et al.* (1982) suggested that these enzymes play a role in the degradation of certain salivary proteins and in the provision of nitrogenous growth substrates. Others have indicated that the proteases of *Bacteroides gingivalis* (recently named *Porphyromonas gingivalis*) and oral spirochaetes mediate tissue destruction and perturbation of the host defences during the pathogenic process of periodontal disease (Ohta *et al.*, 1986; Mikx & de Jong, 1987; Carlsson *et al.*, 1986). Initiation of periodontal lesions by oral spirochaetes has also been postulated, although in periodontal disease a mixed microbial infection is involved (Maltha *et al.*, 1985). Recently Grenier *et al.* (1990) demonstrated a chymotrypsin-like protease of *T. denticola* on the outside of the cell and suggested a function of this enzyme in invasion and destruction of the basement membrane. However, a model infection system for oral spirochaetes is lacking, and the spirochaetes found in and between junctional epithelial cells are probably not *T. denticola* (Mikx *et al.*, 1990). ter Steeg *et al.* (1988) showed that *T. denticola* can be enriched on human serum and can take part in consortia associated with periodontal destruction. The proteolytic activities of *T.*

denticola might be of importance for its participation and the concerted action of the consortium in periodontal pockets and surrounding tissues (ter Steeg & van der Hoeven, 1990).

In conclusion, the enzyme profiles of the 20 oral and non-oral *Treponema* strains showed some common esterase and *N*-acetyl- β -D-glucosaminidase activities. The profiles did not discriminate between oral and non-oral treponemas. With the exception of *T. denticola*, pathogenic and non-pathogenic *Treponema* species showed a low proteolytic profile. Dipeptidyl prolyl amidase activity was observed in all species except *T. phagedenis* strain Reiter and the avian intestinal spirochaetes.

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