Immunological specificity of oral *Eubacterium* species

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Antigens of *Eubacterium* species including *E. alactolyticum*, *E. brachy*, *E. nodatum*, *E. saburreum*, *E. timidum*, *E. yurii* subsp. *yurii* and *E. yurii* subsp. *margaretiae*, which have been isolated frequently from periodontal pockets and associated with periodontal diseases, were extracted by ultrasonication from whole bacterial cells. Antigens were also prepared from *E. aerofaciens*, *E. lentum* and *E. rectale*, which have been found in intestinal tracts and infected abscesses in human oral cavities. The antigens of the oral *Eubacterium* species were compared with antigens from *E. limosum*, the type species of the genus *Eubacterium*, by using SDS-PAGE and Western immunoblot assays. SDS-PAGE gels stained with Coomassie brilliant blue indicated that no major peptide bands were common among the *Eubacterium* species examined. The protein profile patterns were distinctly different from each other. Western immunoblotting reactions with rabbit antisera showed that the *Eubacterium* species could be clearly distinguished serologically, and that the species-specific antigens were peptide components of ultrasonic extracts from the whole bacterial cells. The present study demonstrates that these *Eubacterium* species show great heterogeneity in their peptide components and immunological reactions, which may be useful for identification of the *Eubacterium* species from human oral specimens.

Introduction

Micro-organisms belonging to the genus *Eubacterium* (non-sporing, Gram-positive, obligately anaerobic rods) have been isolated from human oral specimens including those taken from periodontal pockets (Moore *et al.*, 1982*a*, 1985; Uematsu & Hoshino, 1992), dental pulps (Hoshino *et al.*, 1992*a*) and carious dentines (Ando *et al.*, 1990; Edwardsson, 1974; Hoshino, 1985). Within the past decade, several new species of *Eubacterium*, such as *E. brachy*, *E. nodatum*, *E. timidum* and *E. yurii*, have been found frequently from the subgingival area associated with various periodontal diseases (Holdeman *et al.*, 1980; Hoshino *et al.*, 1992*b*). Some species of oral *Eubacterium* have also been reported to be more numerous in subgingival sites with moderate and severe periodontitis than in supragingival sites or subgingival sites of healthy subjects (Moore *et al.*, 1982*b*, 1983, 1985; Han *et al.*, 1991; Tanner *et al.*, 1984; Wade *et al.*, 1992). However, *Eubacterium* species still remain unfamiliar to many investigators because of their poor growth, tiny colony formation, fastidious nutritional requirements or strict anaerobic conditions for growth.

It is known that oral *Eubacterium* species are associated with enhanced aggregation of several pathogenic

bacteria associated with periodontal diseases, bone resorption in the foetal rat and destruction of periodontal tissues (Mashimo *et al.*, 1981; Vincent *et al.*, 1984). It has been also reported that titres of immunoglobulin (Ig) G and IgA against *Eubacterium* species are significantly increased in patients with periodontitis (Gunsolley *et al.*, 1990; Martin *et al.*, 1986; Sandholm & Tolo, 1986; Tew *et al.*, 1985; Tolo & Jorkjend, 1990; Tolo & Schenk, 1985). These findings suggest that *Eubacterium* species may play an important role in the pathogenesis of periodontal disease.

Although serological reactions of antigenic polysaccharide isolated from strains of *E. saburreum* have been well documented in recent literature (Kondo *et al.*, 1983; Nakazawa *et al.*, 1987; Nakazawa & Hoshino, 1992; Skaug & Hofstad, 1983), no comprehensive study of the immunological relationships among the various species of the genus *Eubacterium* has been published. This study was designed to assess the serological relationship of oral *Eubacterium* species by using ultrasonic extracts from whole bacterial cells with SDS-PAGE and Western immunoblot techniques.

Methods

Bacterial strains and culture conditions. Ten species and two subspecies of the genus *Eubacterium* including type species, several of which are often found in human oral cavities (Hoshino *et al.*, 1992*b*), were selected for the present study. ATCC strains including *Eubac-*

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terium aerofaciens ATCC 25986^T (^T = type strain), *E. alactolyticum* ATCC 23263^T, *E. brachy* ATCC 33089^T, *E. lentum* ATCC 25559^T, *E. limosum* ATCC 8486^T, *E. nodatum* ATCC 33099^T, *E. rectale* ATCC 33656^T, *E. saburreum* ATCC 33318, *E. timidum* ATCC 33092, *E. yurii* subsp. *yurii* ATCC 43714^T and *E. yurii* subsp. *margaretiae* ATCC 43715^T were used as representatives of these species in this study. All bacterial strains were cultured in brain heart infusion broth supplemented with bovine serum, haemin and vitamin K₁ (Holdeman *et al.*, 1977). Cultures were incubated at 37 °C for 3 d in an anaerobic chamber (Hirasawa Works, Tokyo, Japan) containing an atmosphere of 80% N₂, 10% H₂ and 10% CO₂. The bacterial cells were harvested by centrifugation at 10000 *g* for 20 min at 4 °C and washed with 10 mM-sodium phosphate-buffered saline, pH 7.2 (PBS).

Production of polyclonal antiserum. Whole bacterial cells to be used as immunogens were suspended at a concentration of 10 mg (wet wt) ml⁻¹ in sterile saline, and the suspensions (0.1–1.0 ml) were intravenously injected into 10-week-old female rabbits via the marginal ear vein twice a week for 3 or 4 weeks. The antisera were stored in small portions at –80 °C until used.

Preparation of antigens. Bacterial ultrasonic extracts were utilized as antigens for serological assays in this study. Whole bacterial cells (100 mg wet wt) were resuspended in 15 ml of PBS and ultrasonicated with cooling until more than 95% of the cells had been disrupted, as determined by phase contrast microscopy. The extracts were centrifuged at 12000 *g* for 30 min at 4 °C, and the supernatants were dialysed against distilled water, and lyophilized as antigens.

SDS-PAGE and Western immunoblot analysis. SDS-PAGE was carried out by the method of Laemmli (1970) in 7.5% (w/v) polyacrylamide gels (1 mm thick), using a Micro Slab Gel Electrophoresis System KS-8010 (Marysol). Antigens (50 µg) were solubilized in 0.125 M-Tris/HCl buffer, pH 6.8, containing 4% (w/v) SDS, 20% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol for 10 min at 100 °C. Electrophoresis was conducted at constant 20 mA at room temperature. After electrophoresis, gels were stained with Coomassie brilliant blue R-250 or periodic acid–Schiff (PAS) base reagent, or used for Western transfer. The electrophoretic transfer of the antigens from the gel to nitrocellulose membrane (pore size, 0.45 µm; Bio-Rad) was performed by using the transfer buffer system described by Burnette (1981) in conjunction with the Trans Blot System (Marysol) at a constant current of 350 mA for 3 h with cooling. After Western transfer, the nitrocellulose membrane with the antigens was soaked for 1 h in 20 mM-Tris/HCl-buffered saline (TBS; pH 7.5), containing 1% (w/v) bovine serum albumin (BSA), and then incubated with diluted rabbit antiserum (1:1000) as the first antibody for 2 h with mild agitation at room temperature. After washing with TBS containing 0.05% Tween 20, the nitrocellulose membrane was soaked in TBS containing 1% BSA and incubated with goat anti-rabbit IgG conjugated with peroxidase (1:1000 dilution; Cooper Biomedical) as the second antibody for 2 h. Following washing, the membrane was placed in a mixture of 4-chloro-1-naphthol (40 mg) dissolved in 5 ml ethanol and 95 ml TBS containing 0.015% hydrogen peroxide, for 10 min, rinsed in distilled water and dried for storage.

Chemical analysis. Carbohydrate was quantified by the phenol–sulphuric acid reaction using glucose as a standard (Hodge & Hofreiter, 1962). Protein was measured by the procedure of Lowry with BSA as standard.

Double-immunodiffusion assay. Immunodiffusion tests were carried out in 1.2% (w/v) agar gel in PBS containing 0.02% sodium azide. Twenty microlitres of antigens (5 mg ml⁻¹ in PBS) and undiluted antisera were applied to each well.

Mild periodate oxidation. Periodate oxidation can distinguish between antibodies directed against carbohydrate and against peptide

antigenic determinants, the latter being unaffected by the oxidation. In the present study, antigens on the nitrocellulose membrane were subjected to mild periodate oxidation by the method of Faye & Chrispeels (1985). Briefly, after Western transfers, the nitrocellulose membrane with antigens was incubated for 2 h at room temperature in the dark with 0.1 M-sodium acetate buffer, pH 4.5, containing 10 mM-sodium metaperiodate for mild oxidation. At the end of the oxidation, each blot was soaked for 30 min with 50 mM-sodium borohydride in PBS. After these treatments, the membrane was rinsed with TBS and treated with the same procedures for Western immunoblot analysis as described above.

Results

The antigens (ultrasonic extracts) from each of the *Eubacterium* species contained 72.8–80.3% protein and widely different concentrations of carbohydrate, ranging from 0.9–19.4%. The antigens from *E. aerofaciens*, *E. alactolyticum*, *E. brachy*, *E. lentum*, *E. nodatum* and *E. saburreum* were examined with the corresponding rabbit antisera in double-immunodiffusion assays. This preliminary study demonstrated that each antigen produced immunoprecipitin bands only with the homologous antiserum indicating that each *Eubacterium* species might induce species-specific antibodies, and that the specific immunological determinant might be contained in the ultrasonic extracts (Fig. 1). The relationships of serological reactions within the ten *Eubacterium* species and two subspecies were studied further using Western immunoblot assay following SDS-PAGE.

The SDS-PAGE assay of the 11 ultrasonic extract antigen preparations showed that all of them had a large

Fig. 1. Double-immunodiffusion reactions of antigens from six species of *Eubacterium*. Rabbit antisera, anti-*E. aerofaciens* ATCC 25986^T (A), anti-*E. alactolyticum* ATCC 23263^T (B), anti-*E. brachy* ATCC 33089^T (C), anti-*E. lentum* ATCC 25559^T (D), anti-*E. nodatum* ATCC 33099^T (E), and anti-*E. saburreum* ATCC 33318 (F) sera, were undiluted. Antigens of *E. aerofaciens* ATCC 25986^T (1), *E. alactolyticum* ATCC 23263^T (2), *E. brachy* ATCC 33089^T (3), *E. lentum* ATCC 25559^T (4), *E. nodatum* ATCC 33099^T (5), and *E. saburreum* ATCC 33318 (6) were prepared as described in the text.

Fig. 2. SDS-PAGE profiles of the antigens from *Eubacterium* species. Lanes: 1, *E. aerofaciens* ATCC 25986^T; 2, *E. alactolyticum* ATCC 23263^T; 3, *E. limosum* ATCC 8486^T; 4, *E. rectale* ATCC 33656^T; 5, *E. saburreum* ATCC 33318; 6, *E. brachy* ATCC 33089^T; 7, *E. lentum* ATCC 25559^T; 8, *E. nodatum* ATCC 33099^T; 9, *E. yurii* subsp. *yurii* ATCC 43714^T; 10, *E. yurii* subsp. *margaretae* ATCC 43715^T; 11, *E. timidum* ATCC 33092; S, protein standards.

number of bands in the region between about 20 kDa and 110 kDa (Fig. 2). Except for *E. yurii* subsp. *yurii* and *E. yurii* subsp. *margaretae*, the protein profile patterns were quite distinct from each other, and no major bands were shared among these antigens from the 10 species of *Eubacterium* (Fig. 2). No bands were shown from any antigen preparations when the gels were stained with PAS base reagent (data not shown).

The Western immunoblot assays with anti-rabbit IgG as conjugate revealed that only their homologous antigens reacted strongly with anti-*E. aerofaciens* (Fig. 3a), anti-*E. alactolyticum* (Fig. 3b), anti-*E. saburreum* (Fig. 3e), anti-*E. nodatum* (Fig. 3h), anti-*E. yurii* subsp. *margaretae* (Fig. 3j) and anti-*E. timidum* (Fig. 3k) rabbit sera. However, antisera against *E. brachy* (Fig. 3f), *E. lentum* (Fig. 3g), *E. limosum* (Fig. 3c), *E. rectale* (Fig. 3d) and *E. yurii* subsp. *yurii* (Fig. 3i) also showed weak or positive cross-reactions with antigens from several different species. Antigens from *E. aerofaciens*, *E. alactolyticum*, *E. rectale*, *E. brachy*, *E. lentum* and *E. timidum* reacted weakly with anti-*E. limosum* rabbit serum. The antigen of *E. yurii* subsp. *yurii* reacted weakly with anti-*E. rectale* serum, and that of *E. timidum* reacted weakly with anti-*E. brachy* serum. Antigens from *E. rectale* and *E. limosum* showed weak cross-reaction with anti-*E. lentum* serum. Anti-*E. yurii* subsp. *yurii* serum reacted with the antigen of *E. yurii* subsp. *margaretae*, but anti-*E. yurii* subsp. *margaretae* serum did not react with the antigen of *E. yurii* subsp. *yurii*. The serological reactions within these *Eubacterium* species shown by Western immunoblot assays are summarized in Table 1.

Mild periodate oxidation of the antigens on nitrocellulose membranes was used to determine whether

carbohydrate moieties of the antigens acted as the immunodeterminants that reacted with the antiserum. The periodate oxidation at acidic pH specifically cleaves carbohydrate vicinal hydroxyl groups without altering the structure of polypeptide chains. In this study, mild oxidation did not change any serological reactions of the antigens from these *Eubacterium* species in the Western immunoblot assay indicating that it is the peptide moieties of the antigens that react with the antisera (data not shown).

Discussion

In the present study, the ultrasonic extracts of whole bacterial cells were used for SDS-PAGE and Western immunoblot assay as antigens because they may include more components that have immunological activity than materials extracted by specific methods such as extraction by organic solvents, surfactants or enzymes. There were no detectable bands in the SDS-PAGE gel stained with PAS base reagents although sensitivity of PAS staining is lower than that of Coomassie brilliant blue. It indicated that the extracts were mainly composed of peptides, which was in agreement with the results of chemical analysis.

The SDS-PAGE analysis with Coomassie brilliant blue revealed that the 10 species of *Eubacterium* tested in this study displayed distinct differences of protein profile patterns apart from the antigens from *E. yurii* subsp. *yurii* and *E. yurii* subsp. *margaretae* which showed a more similar profile pattern (Fig. 2, lanes 9 and 10). It was reported by Margaret & Krywolap (1986, 1988) that SDS-PAGE of cell lysates of the three subspecies, *E. yurii* subsp. *yurii*, *E. yurii* subsp. *margaretae* and *E. yurii* subsp. *schtitka*, isolated from human dental plaque, showed some common protein bands but their profile patterns were distinct from each other. In this study, major peptide bands shared by all species were not detected in the SDS-PAGE gel although it was possible that minor bands might be shared. These results of SDS-PAGE analysis demonstrated that *Eubacterium* species showed great heterogeneity in protein components including structural proteins of whole bacterial cells.

Major bands on nitrocellulose membrane, which were visualized with homologous antisera in Western immunoblot assay, did not react with antisera raised against different species. There were no common cross-reactive antigenic bands shared over the whole range of species, although a few cross-reactions were observed. The serological reactions among 10 species of *Eubacterium* examined in the present study thus demonstrated considerable species specificity.

Margaret & Krywolap (1988) reported that antisera to *E. yurii* subsp. *yurii* and *E. yurii* subsp. *margaretae*

Fig. 3. Western immuno-blotting reactions of the antigens from *Eubacterium* species, which were recognized by their rabbit antisera. Lanes correspond to those in Fig. 2. Antisera: panels (a) anti-*E. aerofaciens* ATCC 25986^T; (b) anti-*E. alactolyticum* ATCC 23263^T; (c) anti-*E. limosum* ATCC 8486^T; (d) anti-*E. rectale* ATCC 33656^T; (e) anti-*E. saburreum* ATCC 33318^T; (f) anti-*E. brachy* ATCC 33089^T; (g) anti-*E. lentum* ATCC 25559^T; (h) anti-*E. nodatum* ATCC 33099^T; (i) anti-*E. yurii* subsp. *yurii* ATCC 43714^T; (j) anti-*E. yurii* subsp. *margaretiae* ATCC 43715^T; and (k) anti-*E. timidum* ATCC 33092.

reacted only with their homologous subspecies in serological tests using immunodiffusion in agar. In the present study, anti-*E. yurii* subsp. *margaretiae* serum did

not react with the antigen of *E. yurii* subsp. *yurii*, but anti-*E. yurii* subsp. *yurii* serum reacted with the antigen of *E. yurii* subsp. *margaretiae*. This discrepancy between

Table 1. Western immuno-blotting reactions of the antigens from *Eubacterium* species

Antigens (a-k) correspond to the bacteria that are indicated in the column for antisera (A-K).

Antiserum	Antigen*										
	a	b	c	d	e	f	g	h	i	j	k
A <i>E. aerofaciens</i> ATCC 25986 ^T	+	-	-	-	-	-	-	-	-	-	-
B <i>E. alactolyticum</i> ATCC 23263 ^T	-	+	-	-	-	-	-	-	-	-	-
C <i>E. limosum</i> ATCC 8486 ^T	w	w	+	w	-	w	w	-	-	-	+
D <i>E. rectale</i> ATCC 33656 ^T	-	-	-	+	-	-	-	-	w	-	-
E <i>E. saburreum</i> ATCC 33318	-	-	-	-	+	-	-	-	-	-	-
F <i>E. brachy</i> ATCC 33089 ^T	-	-	-	-	-	+	-	-	-	-	+
G <i>E. lentum</i> ATCC 25559 ^T	-	-	+	w	-	-	+	-	-	-	-
H <i>E. nodatum</i> ATCC 33099 ^T	-	-	-	-	-	-	-	+	-	-	-
I <i>E. yurii</i> subsp. <i>yurii</i> ATCC 43714 ^T	-	-	-	-	-	-	-	-	+	w	-
J <i>E. yurii</i> subsp. <i>margaretiae</i> ATCC 43715 ^T	-	-	-	-	-	-	-	-	-	+	-
K <i>E. timidum</i> ATCC 33092	-	-	-	-	-	-	-	-	-	-	+

* +, Positive reaction; -, negative reaction; w, weakly positive reaction.

two studies may be due to the method of antigen preparation or the sensitivity of the assay.

When goat anti-rabbit IgM instead of IgG as conjugate for Western immunoblot assay was applied, there were no visible bands in any ultrasonic extract antigen preparations from these *Eubacterium* species. Although we have no clear explanation for this result, it may be due to the period of stimulation for immunization. Also, it may be related to the fact that the antigens consist mainly of proteins, as shown by SDS-PAGE stained with PAS, chemical analysis and mild periodate oxidation which indicates that the immunodeterminants of the antigens are peptide moieties.

Recently numerous strains belonging to genus *Eubacterium* have been isolated from periodontal regions, including periodontal pocket, supragingival plaque and various sites affected by periodontitis (Theilade *et al.*, 1982). These oral *Eubacterium* species have been suggested to be potential etiologic periodonto-pathogens in many bacteriological studies (Margaret & Krywolap, 1988; Martin *et al.*, 1986; Sandholm, 1985, 1987; Sandholm & Tolo, 1986; Vincent *et al.*, 1984). Immunological studies (Gunsolley *et al.*, 1990; Sandholm, 1987; Tew *et al.*, 1985; Vincent *et al.*, 1985) have also indicated that certain species of *Eubacterium* may play important immunological roles in the progression of periodontal disease.

Uematsu & Hoshino (1992) and Wade *et al.* (1991) reported that there were many unnamed *Eubacterium* species in the periodontal regions. At the present time, classification of the genus *Eubacterium* is based on elimination of other anaerobic genera (Moore & Moore, 1986). The asaccharolytic members of the genus *Eubacterium* have proved difficult to identify because these micro-organisms show little reactivity in most biochemi-

cal tests (Hill *et al.*, 1987; Holdeman *et al.*, 1980; Uematsu & Hoshino, 1992). Therefore, it has been suggested that the genus *Eubacterium* may include some species that are phylogenetically distant from each other. Indeed, although more than 40 species have been recognized in *Bergey's Manual of Systematic Bacteriology*, these species may exhibit a variety of cellular morphologies, and are biochemically and physiologically extremely heterogeneous (Moore & Moore, 1986). The recent studies of antigens from strains of *E. saburreum* and *E. brachy* may indicate great heterogeneity of chemical and antigenic properties among these micro-organisms (Kondo *et al.*, 1983; Nakazawa, 1985; Nakazawa & Hoshino, 1992; Skaug & Hofstad, 1983; Vincent *et al.*, 1984).

The present study has demonstrated that the oral *Eubacterium* species tested could be clearly distinguished serologically by species-specific bands of antigens, which indicates immunological heterogeneity within *Eubacterium* species, and they also showed great heterogeneity in structural peptide components of whole bacterial cells. These results may be useful in identification of *Eubacterium* species, and may suggest the necessity for reclassification of the genus *Eubacterium*.

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