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Effect of Environmental pH on Enzyme Activity and Growth of *Bacteroides gingivalis* W50

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Since the pH of the gingival crevice increases from below neutrality in health to above pH 8 in disease, we decided to investigate the effect of environmental pH on the growth and enzyme activity of *Bacteroides gingivalis* W50. Cells were grown in a chemostat under hemin-excess conditions over a range of pH values; stable growth was observed only between pH 6.7 and 8.3, with the maximum yields obtained between pH 7.0 and 8.0. The enzyme profile of cells varied markedly with pH. Enzymes with a specificity for gingival connective tissue (collagenase, hyaluronidase) were produced optimally at or below neutral pH, whereas trypsinlike activity increased with the growth pH and was maximal at pH 8.0. Chymotrypsinlike activity was generally low, although its activity was highest at the extremes of growth pH, i.e., at pH 6.7 and 8.3. Inhibitor studies provided evidence that the breakdown of collagen involved the concerted action of both a collagenase and the trypsinlike enzyme. The ratio of trypsin to collagenolytic activity rose from 1:1 during growth at neutral pH and below to almost 7:1 during growth at pH 8.3. Thus *B. gingivalis* appears to be uniquely adapted as a periodontopathic organism in that under environmental conditions likely to prevail during the initial stages of pocket development it produces maximally those enzymes with a tissue-damaging potential. Then, as the pH of the pocket rises during the host inflammatory response, the activity of the trypsinlike enzyme increases markedly, which may enable cells to inactivate key components of the host defenses such as immunoglobulins and complement.

Although diverse microbial communities have been recovered from sites with human periodontal disease, only a limited number of species are believed to play an active role in its pathogenesis. In particular, members of the black-pigmented *Bacteroides* spp. have been strongly implicated, and of these *B. gingivalis* has been isolated more frequently and in higher numbers from sites in adults with advanced periodontitis (17). Moreover, *B. gingivalis* is highly virulent in experimental animals; it is the only species of black-pigmented *Bacteroides* to consistently cause spreading, inflammatory monoinfections (26).

The virulence of *B. gingivalis* is associated with its ability to evade host defenses and produce a range of cytotoxins and enzymes with a tissue-damaging potential (18). The expression of such virulence components can be markedly influenced by environmental conditions. Since the pH of a periodontal pocket has been shown to increase with its depth and also with the severity of the inflammatory host response (1), and since pH can affect bacterial enzyme production, we have compared the growth and enzyme activity of a virulent strain of *B. gingivalis* cultured over a range of pH values likely to occur during disease.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *B. gingivalis* W50 was inoculated and grown in an LH 500 series II chemostat (LH Engineering, Stoke Poges, England) at 37°C at pH 7.5 ± 0.2 and at a dilution rate of 0.1 per h (this corresponds to a constant mean generation time of 6.9 h) in an atmosphere of 5% CO_2 in N_2, as described previously (11). The composition of the BM medium, modified as before (11), was adjusted to ensure conditions of hemin excess (final hemin concentration, 5.0 μg/ml). After growth had been established at pH 7.5, the pH was increased stepwise up to pH 8.5 or down to pH 6.5 by the automatic addition of 0.5 M NaOH or 0.5 M HCl, respectively. Repeat chemostat cultures were set up at each pH value for confirmation of the results obtained originally.

Growth parameters. The pH, turbidity, dry weight, and viable count of the cultures were determined regularly (11) when steady-state conditions were attained at each growth pH. Ten pot volume changes of medium were allowed to elapse before a culture was considered to have reached a steady state.

Enzyme activity. At each steady state, cultures taken on different days directly from the chemostat were tested for trypsinlike and chymotrypsinlike activity as well as for the ability to break down collagen and hyaluronic acid (HA). No attempt was made to discriminate between activities associated with vesicles and those that were cell bound. All assays were carried out at 37°C with an SP 1700 recording spectrophotometer (Pye-Unicam, Cambridge, England) over the pH range pH 6.0 to 9.0; all buffers were supplemented with 1 mM dithiothreitol (Sigma, Poole, England) to maintain reduced conditions. The assays for trypsinlike and chymotrypsinlike activities were based on those of Schwert and Takenaka (14) and involved measuring the rates of hydrolysis of 0.25 mM *N*-α-benzoyl-L-arginine ethyl ester at 253 nm and of 0.936 mM *N*-acetyl-L-tyrosine ethyl ester at 237 nm, respectively. Substrates were made up freshly at regular intervals in 0.067 M (for *N*-α-benzoyl-L-arginine ethyl ester) and 0.05 M (for *N*-acetyl-L-tyrosine ethyl ester) phosphate and Tris buffers and were stored at −20°C until required. Activities were measured after the addition of 0.1 ml of culture and, for control purposes, standard beef pancreas trypsin, soya bean

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trypsin inhibitor, and bovine pancreas chymotrypsin. One *N*-α-benzoyl-L-arginine ethyl ester or *N*-acetyl-L-tyrosine ethyl ester unit of activity caused a change in absorbance of 0.001 per min at 253 or 237 nm, respectively.

The assay for collagen breakdown was based on that of Mandl et al. (8), in which helical regions of the molecule are degraded, liberating peptides which are estimated spectrophotometrically as leucine. Type 1 insoluble collagen (1 g) from bovine achilles tendon was pretreated with 20 mg of pepsin in 100 ml of 0.1 M glycine hydrochloride buffer (pH 2) at 37°C for 16 h to remove contaminating proteins and denatured collagen (gelatin) (15) and freeze-dried. Culture samples (0.1 ml) were incubated anaerobically for 18 h with 25 mg of this enzymically pretreated collagen suspended in 5 ml of 0.05 M Tris or phosphate buffer containing 0.25 mg of calcium acetate. After incubation, duplicate 0.25-ml samples were removed, mixed with 1 ml of freshly prepared ninhydrin reagent, and heated for 20 min in a boiling water bath. When the mixture was cool, 5 ml of propan-1-ol–water (1:1, vol/vol) was added; after 15 min, the A_{600} was measured and compared with those of leucine standards and standard collagenase from *Clostridium histolyticum*. Heat-killed cells (100°C for 20 min) were used for control purposes. One unit of activity liberated 1 μmol of leucine from collagen in 18 h at 37°C.

The assay for HA breakdown was based on the turbidity reduction method of Dorfman (2). Highly polymerized HA from human umbilical cord (10 mg) was dissolved in 25 ml of 0.3 M phosphate buffer (pH 5.3) and incubated for 45 min at 37°C with 0.5-ml cultures in 1 ml of 0.02 M phosphate or Tris buffer containing 0.45% (wt/vol) NaCl and 0.1% (wt/vol) bovine serum albumin. Heat-killed cells were again used for control purposes. After incubation, any HA remaining was precipitated by rapid mixing with acid albumin reagent (pH 3.75) (2). After 5 min, the decrease in turbidity was measured at 600 nm and compared with that obtained with known dilutions of standard hyaluronidase (500 IU/mg) from ovine testes. Low (apparent) hyaluronidase activities (<1 U) were detected when HA was incubated with standard trypsin preparations (200 U). Therefore, the HA was enzymically pretreated in a way identical to that described for collagen to increase the specificity of the assay.

All reagents were of the purest grade and were obtained from Sigma or BDH, Eastleigh, England.

Statistical analysis. Initially, Bartlett's variance test was used to determine whether the data could be analyzed subsequently by the Kruskal-Wallis and Mann-Whitney *U* test or by a one-way analysis of variance and Student's *t* test.

RESULTS

Growth of *B. gingivalis* W50 at different pH values. *B. gingivalis* grew over the pH range 6.7 through 8.3 but could not be maintained stably at pH 6.5 or pH 8.5. There was little difference in the number of viable cells per milliliter of culture over the range pH 7.0 through 8.0, although the highest turbidity and dry weight were obtained during growth at pH 7.5 (Table 1). The optical density and dry weight of cultures grown at pH 6.7 and pH 8.3 were significantly lower (*P*, 0.05 to 0.001) than those grown at pH 7.0 and pH 7.5.

Protease activity. The addition of 1 mM dithiothreitol increased all enzyme activities by approximately 10%. Of the enzymes studied, the protease with the highest activity had a trypsinlike substrate specificity; this activity could be inhibited by soya bean trypsin inhibitor. The other major activity was associated with the breakdown of collagen. Pretreatment of the collagen with pepsin to remove contaminating proteins made the substrate almost totally resistant to breakdown by standard trypsin preparations. However, incubation of collagen with both standard collagenase (40 U) and trypsin enzymes (200 U) resulted in an increased (52 ± 9%) degradation of the molecule. Similarly, when bacterial cultures were incubated with collagen there was a 36 ± 9% reduction in the breakdown of the substrate in the presence of trypsin inhibitor, suggesting that *B. gingivalis* degrades collagen by the concerted action of both a specific collagenase and its trypsinlike protease.

TABLE 1. Effect of growth pH on the yield of *B. gingivalis* W50[a]

Growth pH	A_{540}	Viable count (10^9 cells/ml)	Dry wt (mg/ml)
6.5	US	US	US
6.7	2.1 ± 0.1 (12)	0.7 ± 0.3 (8)	0.82 ± 0.07 (12)
7.0	2.4 ± 0.05 (8)	2.1 ± 0.6 (13)	1.12 ± 0.04 (8)
7.5	2.7 ± 0.1 (8)	2.7 ± 1.3 (6)	1.15 ± 0.05 (7)
8.0	2.2 ± 0.1 (7)	2.7 ± 0.9 (6)	1.11 ± 0.06 (2)
8.3	1.9 ± 0.05 (9)	0.5 ± 0.3 (5)	0.82 ± 0.09 (5)
8.5	US	US	US

[a] Results are means ± standard errors, with the number of replications given within parentheses. US, Unstable growth (i.e., steady-state growth was not achieved).

Enzyme activities during growth at different pH values. The profiles of enzyme activity of *B. gingivalis* during growth at different pH values are presented graphically in Fig. 1. In Table 2, some of the data are replotted so that the assay pH for enzyme activity corresponds to the pH of growth. Statistical analysis of the data in Table 2 by using the Kruskal-Wallis test or, when appropriate, the more powerful analysis of variance showed that the pH of growth had a significant effect on trypsin activity and collagen breakdown but had no significant effect on chymotrypsin activity or on hyaluronic acid breakdown.

Cells of *B. gingivalis* W50 had a significantly higher (*P*, 0.05 through 0.01) trypsinlike activity when grown at alkaline pH (pH 7.5 through 8.3) compared with cells grown at pH 6.7 and 7.0 (Table 2). For example, cells grown at pH 8.0 had more than three times the trypsin activity of cells cultured at pH 6.7. Also the trypsinlike enzyme showed some adaptation to the change in the environment, in that the pH optimum for enzyme activity increased as the growth pH was raised. During growth over the range pH 6.7 through 7.5, the pH optimum was pH 7.5, with activities decreasing sharply as the assay pH became more alkaline (Fig. 1a through c), whereas the optimum was pH 8.0 during growth at pH 8.0 (Fig. 1d) and was extended over the range pH 7.5 through 8.5 during growth at pH 8.3 (Fig. 1e). In contrast, the breakdown of collagen was generally greatest during growth at the lower pH values (Table 2), but no clear pH optimum could be measured with cells grown at any pH value. There was little breakdown of HA under any conditions, except with cells grown at pH 7.0. Under these conditions, HA degradation was between 2.5- and 10-fold higher than with cells grown at any other pH. This change in pattern of activity was repeated with cells grown in fresh chemostat cultures. Chymotrypsin activity was also low and showed little response to changes in growth or assay pH. However, there was a trend for cells grown at the lower pH values to have higher activities at the lower assay pH values

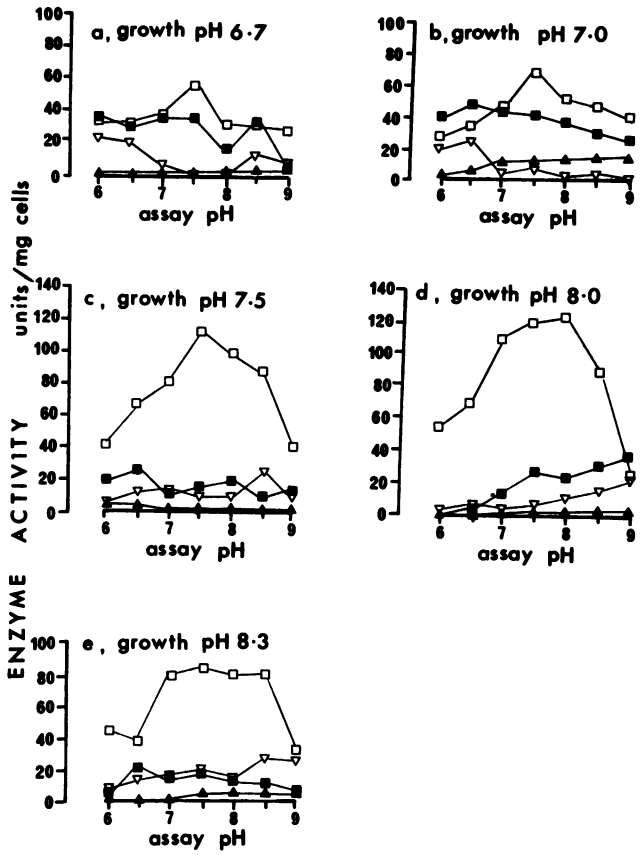

FIG. 1. Enzyme profile of *B. gingivalis* W50 during growth at (a) pH 6.7, (b) pH 7.0, (c) pH 7.5, (d) pH 8.0, and (e) pH 8.5. One unit of trypsin (□) or chymotrypsin (▽) activity caused a change in absorbance of *N*-α-benzoyl-L-arginine ethyl ester or *N*-acetyl-L-tyrosine ethyl ester of 0.001 per min per mg (dry weight) of cells at 253 or 237 nm, respectively. One unit of collagenolytic activity (■) liberated 1 μmol of leucine from enzymically pretreated collagen in 18 h at 37°C. Hyaluronidase activities (▲) were calculated from comparisons of the reduction in turbidity of HA incubated at 37°C for 45 min with the international standard enzyme from ovine testes. The results are the means of at least three assays made on different days with fresh cells.

(e.g., assay pH 6.5), whereas cells grown at alkaline pH had their maximum activities between assay pH 8.5 through 9.0.

The enzyme profile of *B. gingivalis* W50 changed markedly with the pH of the environment (Table 2). The ratio of trypsin activity to collagen breakdown activity was approximately 1:1 during growth at pH 6.7 and 7.0, whereas this ratio increased to 5:1 and nearly 7:1 during growth at pH 8.0 and 8.3, respectively. Chymotrypsin activity was higher during growth at pH 6.7 and 8.3, whereas HA breakdown was greatest when cells were grown at pH 7.0.

TABLE 2. Profile of the enzyme activity of *B. gingivalis* W50 at different growth pH values[a]

Growth pH	Mean enzyme activity (U/mg [dry wt] of cells ± SE)			
	Hydrolysis by:		Breakdown of:	
	Trypsin	Chymotrypsin	Collagen	HA
6.7	34 ± 4 (4)	23 ± 8 (3)	34 ± 12 (3)	1 ± 0.3 (3)
7.0	47 ± 8 (6)	4 ± 4 (3)	42 ± 4 (4)	10 ± 3 (5)
7.5	113 ± 10 (16)	10 ± 6 (4)	15 ± 2 (4)	1 ± 0.3 (4)
8.0	123 ± 21 (4)	12 ± 1 (2)	24 ± 12 (3)	3 ± 0.7 (4)
8.3	82 ± 8 (3)	26 ± 5 (4)	12 ± 4 (3)	4 ± 1 (4)

[a] The enzyme activities presented are those where the growth pH and the assay pH correspond; therefore, these are not necessarily the maximum enzyme activities (Fig. 1). The units of activity of each enzyme are described more fully in Materials and Methods.

DISCUSSION

In the pathogenesis of periodontal diseases, the normal gingival crevice enlarges into a periodontal pocket. This process involves the apical migration of the junctional epithelium with a subsequent loss of attachment fibers, followed by the degradation of connective tissue and resorption of alveolar bone. As the pocket develops, the host mounts an inflammatory response, which leads to an increased flow of crevicular fluid. Thus, for periodontopathic bacteria to survive in the pocket they must be capable of evading and inactivating components of the host defenses, including both antibodies and phagocytic cells. Although some of the tissue damage is an inevitable consequence of the inflammatory host response, bacteria are also believed to play a direct role. Many species produce acidic fermentation products that are toxic to a number of cell lines (25), whereas others are proteolytic and produce both general proteases (27) and enzymes such as hyaluronidase, collagenase, and chondroitinase with a specificity for gingival connective tissue (23, 24).

Of the bacteria that have been strongly implicated in chronic adult periodontitis, *B. gingivalis* has been shown to be the most virulent species in experimental infection studies in animals (26), with the most pathogenic of these strains being those which possess the greatest proteolytic (27) and collagenolytic (5, 9, 27) activity. One of the proteases produced by *B. gingivalis* has been shown to have a trypsinlike substrate specificity (16, 28). This enzyme may be important in obtaining nutrients for the cell; it might also interfere with the host defenses since *B. gingivalis* can degrade immunoglobulins (6, 13, 20), complement (19, 20), and host plasma proteinase inhibitors (12). We also report here low activities of a protease with a chymotrypsinlike substrate specificity. The possible role of this enzyme in the physiology of *B. gingivalis* or in the pathogenesis of periodontal disease is unclear at present. Our data provide support for the proposal of Mayrand and Grenier (9) that the breakdown of host macromolecules can involve the combined action of more than one bacterial protease. Degradation of collagen by our cultures of *B. gingivalis* was reduced in the presence of trypsin inhibitor, suggesting that once the collagen helices have been cleaved by a specific collagenase, general proteases are able to accelerate the breakdown of the substrate by partially digesting some of the peptides. Collagenolytic activity by our strain of *B. gingivalis* was detected during logarithmic growth at all pH values tested, which is in contrast to previous work with batch cultures (27). The lack of distinct pH optima for chymotrypsin activity or for the breakdown of collagen and HA might indicate that different enzyme complexes are being formed during growth at particular pH values.

During the development of a periodontal pocket, the local environmental conditions change. Inflammation and tissue damage result in an altered pattern of nutrients; when gingival fluid flow is increased and bleeding occurs, the availability of hemin (an essential growth factor for *B. gingivalis*) (3, 11) may increase, thereby facilitating the enrichment of this species in the subgingival microbial community. The pH of the developing pocket also changes;

recent studies have shown that in health the pH of the gingival sulcus is 6.90, whereas in disease the mean pH is 8.66 (1). Bacteria generally have a relatively narrow pH range for growth, and this may influence their intraoral distribution. Experimental studies have shown that a sudden drop in pH can shift markedly the balance of oral microbial communities, with bacteria found in periodontal pockets (e.g., *Fusobacterium nucleatum*, *Bacteroides intermedius*) growing poorly or not at all and acidophilic bacteria such as *Lactobacillus casei* and *Streptococcus mutans* predominating (10). These latter species also predominate at carious lesions (7) or in supragingival plaque at sites where the pH has been deliberately lowered (21). There is little information on the growth optimum of periodontopathic bacteria. It has been shown that *B. intermedius* has only a narrow pH range of growth (pH 6.0 through 7.3) in pure culture in a chemostat (I. R. Hamilton, G. H. Bowden, and A. S. McKee, J. Dent. Res. **65**:266, 1986). In our study, *B. gingivalis* W50 could not be maintained stably at a constant pH 6.5, and growth was only reproducible over the pH range 6.7 through 8.3. This implies that, as the severity of inflammation increases, the local environment (both in terms of pH and hemin availability) would favor and therefore tend to enrich for strains of *B. intermedius* and then *B. gingivalis*. Some evidence for such a succession has been obtained from studies of patients with early or late periodontitis (17).

Such a change in the local environmental pH will also result in a marked change in the enzyme profile and, therefore, pathogenic potential of *B. gingivalis*. During growth in the chemostat at pH 6.7 through 7.0 (values which correspond to a healthy gingival crevice [1]), the activities of enzymes capable of breaking down gingival connective tissues (hyaluronidase, collagenase) and therefore those that are necessary to initiate and cause progression of a periodontal lesion, were at their maximum (Fig. 1a and b, Table 2). This finding is in agreement with other published work on *B. gingivalis* (4) and on a *Peptostreptococcus* strain isolated from a periodontal pocket (22) but contrasts markedly with studies of similar enzymes of non-oral bacteria which have their highest activities at low pH values (4, 22). As the pH in the chemostat was increased to pH 8.0 and above (as is found during inflammation in periodontal pockets [1]), the specific activity of trypsin rose by between 240 and 360% and the ability to degrade collagen was reduced. Similarly, at neutral pH and below, the ratio of trypsin to collagen activities was 1:1, whereas during growth at alkaline pH this ratio was over 5:1 (Table 2). Significantly, perhaps, the activity of the trypsinlike enzyme was highest under pH conditions when the host inflammatory response is most severe (1), thereby providing *B. gingivalis* with the means to inactivate complement (19, 20) and degrade immunoglobulins (6, 13, 20) and thereby induce a local paralysis of the immune system (6).

It is clear from this study that the influence of pH on growth and enzyme production must be taken into account when attempting to relate in vitro enzyme activities to the disease potential of a bacterium, and that the profile of activity of an organism will respond to and change with any alteration or perturbation of the pocket environment. It is also apparent that *B. gingivalis* W50 is well adapted to its presumed periodontopathic role, in that the enzymes that are capable of either causing tissue damage or providing the bacterium with protection from host defenses were produced optimally under the environmental conditions that are most relevant to each stage of pocket development. More work is underway to see whether other periodontopathic bacteria (including other strains of *B. gingivalis*) respond to changes in environmental pH in a way similar to that reported here.

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