Production of Cell-bound and Vesicle-associated Trypsin-like Protease, Alkaline Phosphatase and *N*-Acetyl- β -glucosaminidase by *Bacteroides gingivalis* Strain W50

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(Received 8 July 1988; revised 24 October 1988; accepted 15 November 1988)

Bacteroides gingivalis strain W50 was grown in batch and continuous culture on complex medium with haemin. In batch culture, cell-bound levels of trypsin-like protease (EC 3.4.21.4), alkaline phosphatase (EC 3.1.3.1) and *N*-acetyl- β -glucosaminidase (EC 3.2.1.30) increased during the exponential phase of growth. These enzyme activities were also detected in extracellular vesicles and in extracellular soluble forms in the supernatant fluid, but in lower amounts per unit biomass compared to cell-bound levels. In continuous culture, at high relative growth rates (0.7–0.9 μ_{rel}), the highest proportions of enzyme activities were cell-bound. In contrast, at low relative growth rates (0.1–0.2 μ_{rel}), highest enzyme levels were detected in the extracellular vesicle fraction. Levels of extracellular soluble enzymes were always low compared to cell-bound or extracellular vesicle levels, but were highest at low relative growth rates. All three enzymes appeared to be relatively stable in their soluble forms. Vesicle production appeared to be associated with actively growing cells but was influenced by growth rate. The results are consistent with the hypothesis that cell-bound ‘periplasmic’ enzymes are encapsulated into vesicles which are subsequently released by the cells. Therefore, levels of total extracellular enzyme (extracellular vesicle plus extracellular soluble) may depend on the rate of vesicle formation superimposed on the rates of production of ‘periplasmic’ enzymes in the cell.

INTRODUCTION

The black-pigmented asaccharolytic oral anaerobe *Bacteroides gingivalis* is present in subgingival dental plaque and has been implicated as an agent of severe periodontitis in humans (Slots, 1982; Slots & Genco, 1984; Slots & Dahlen, 1985). A feature of *B. gingivalis* which distinguishes it from other black-pigmented bacteroides is the production of a trypsin-like protease (Laughon *et al.*, 1982) which is possibly associated with the virulence of this organism (Slots & Genco, 1984; McKee *et al.*, 1986). Yoshimura *et al.* (1984) partially purified the trypsin-like enzyme from the envelope fraction of *B. gingivalis* cells. However, this enzyme has also been reported to occur in the supernatant fluid of *B. gingivalis* cultures (Fujimura & Nakamura, 1987).

It has been demonstrated by various workers (Laughon *et al.*, 1982; van Winkelhoff *et al.*, 1985) using API ZYM that *B. gingivalis* produces alkaline phosphatase and *N*-acetyl- β -glucosaminidase activities in addition to trypsin-like protease activity. No other hydrolytic enzyme activities were detected by these workers. The role and location of alkaline phosphatase and *N*-acetyl- β -glucosaminidase in the cell together with their significance as virulence factors are unknown.

Electron microscopy has been used to demonstrate that *B. gingivalis* strains are capable of producing and releasing ‘blebs’ or membrane vesicles into the medium (Shah *et al.*, 1976; Slots & Gibbons, 1978; Woo *et al.*, 1979; McKee *et al.*, 1986). Grenier & Mayrand (1987) and Smalley & Birss (1987) have shown that trypsin-like protease activity is associated with the membrane vesicles, suggesting that vesicle formation might be an important mechanism by which cells export this enzyme to the extracellular environment. The production of vesicles by *B. gingivalis*

grown in chemostat culture was shown to be influenced by the nature of the growth-limiting nutrient; more vesicles were formed when cultures were grown under haemin-limited conditions than when haemin was in excess (McKee *et al.*, 1986).

The aims of the present study were to measure the production of trypsin-like protease, alkaline phosphatase and *N*-acetyl- β -glucosaminidase during growth of *B. gingivalis* in stirred batch and continuous culture. Of particular interest was the association of the three enzymes with the cells, vesicles and the vesicle-free fraction of the culture fluid at different growth rates.

METHODS

Organism and media. Bacterial strain W50 (Shah *et al.*, 1976), classified as *Bacteroides gingivalis* (Coykendall *et al.*, 1980), was maintained as a stock culture in Robertson's Cooked Meat Medium (Difco). Samples of cultures taken during experiments and stock cultures were regularly tested for purity by plating on to blood agar plates (Oxoid) containing vitamin K₃ (1 mg l⁻¹). The plates were incubated in a cold catalyst anaerobic jar in an atmosphere of N₂/H₂/CO₂ (80:10:10, by vol.) at 37 °C for 7 d. A complex medium was used for batch and continuous culture studies. It consisted of brain heart infusion broth (Difco), 18.5 g l⁻¹ supplemented with L-cysteine hydrochloride (0.5 g l⁻¹), dithiothreitol (50 mg l⁻¹), haemin (10 mg l⁻¹) and vitamin K₃ (1 mg l⁻¹). Stock solutions of haemin and vitamin K₃ were sterilized by filtration and added aseptically to the bulk medium which had been previously sterilized by autoclaving.

Batch and continuous culture apparatus and conditions. *B. gingivalis* was grown in a one-litre culture vessel with control modules for temperature, pH, gas flow and stirrer rate (series 500, LH Engineering). The culture volume was 750 ml and the temperature of the culture was maintained at 37 °C (± 0.1 °C). The pH was maintained at 7.5 (± 0.1 unit) by the automatic addition of 2 M-NaOH or 2 M-H₂SO₄. Foaming was controlled by the addition of polypropylene glycol antifoam (BDH) when necessary. The impeller speed was 600 r.p.m. (± 10 r.p.m.) and the vessel was sparged with O₂-free N₂/CO₂ (90:10, v/v) at a flow rate of 100 ml min⁻¹ (± 5.0 ml min⁻¹) to maintain anaerobiosis. Batch and continuous culture experiments were carried out using the same conditions and apparatus. For batch culture the vessel was inoculated with a 10% (v/v) inoculum taken from a culture in the mid-growth phase. In continuous culture experiments the dilution rates were set to allow a range of relative growth rates (μ_{rel}) from 0.1 to 0.9 (Table 1).

Estimation of biomass. The biomass values were estimated from the OD₅₄₀ of culture samples, measured in an MSE spectrophotometer and related to the dry weight of cells by reference to a calibration curve. Direct determinations of biomass by dry weight were carried out using the method of Greenman *et al.* (1981).

Determination of maximum specific growth rate (μ_{max}). This was determined by the washout method of Tempest (1970) as described by Greenman *et al.* (1981). The dilution rate used for washout was 0.52 h⁻¹.

Culture fraction preparation. Samples taken during batch or continuous culture were centrifuged at 3000 *g* for 30 min in an MSE bench centrifuge. This produced a cell pellet which was separated from the remaining, slightly turbid or opalescent supernatant fraction. The supernatant fraction was centrifuged (MSE high speed 18 centrifuge) at 30000 *g* for 45 min at 10 °C to produce a pellet of vesicles and a clear supernatant fluid. Thus, three distinct fractions were produced: cells, vesicles and supernatant fluid. The enzyme activities measured in each fraction were denoted as cell-bound, extracellular vesicle, and extracellular soluble, respectively. No attempt was made to purify these fractions further. It was apparent from electron microscopy that some membrane vesicles were present in the cell pellet fraction; however, the vesicle fraction was relatively free of whole cells.

Enzyme assays. Trypsin-like protease activity (EC 3.4.21.4) was measured spectrophotometrically by the method of Yoshimura *et al.* (1984). The assay mixture was made up in a 10 mm light path spectrophotometric cuvette and consisted of 1.0 ml *N*- α -benzoyl-L-arginine-*p*-nitroanilide (0.2 mM); 1.7 ml Tris/HCl buffer (50 mM) at pH 7.5; 0.1 ml Triton X-100 (0.1%); 0.1 ml L-cysteine hydrochloride (10 mM) and 0.1 ml enzyme sample.

Alkaline phosphatase activity (EC 3.1.3.1) was measured using a method described for acid phosphatase (Holland *et al.*, 1979) but suitably modified. The assay mixture consisted of 2 ml CAPS buffer (0.1 M), pH 10.5; 0.5 ml *p*-nitrophenyl phosphate (0.5%) and 0.5 ml enzyme sample. Aliquots of assay mixture (1 ml) were removed at time zero (t_0) and after 10 min (t_{10}) and added to tubes containing 2 ml NaOH (0.2 M) to stop the reaction. The changes in absorbance readings ($t_{10}-t_0$) were taken at 420 nm.

N-Acetyl- β -glucosaminidase (EC 3.2.1.30) was assayed by the same method, but substituting *p*-nitrophenyl-*N*-acetyl- β -glucosaminide as the substrate and 0.1 M-MES buffer, pH 6.5. For this assay 1 ml aliquots were added to 2 ml sodium carbonate (0.2 M) to stop the reaction and develop the colour.

Units of enzyme activity. The units of enzyme activity (U) for bacterial cells or fractions derived from a known quantity of cells were expressed as μ mol end-product h⁻¹. Specific activities of enzymes were expressed as U (mg biomass)⁻¹ and production rates of enzyme activities were expressed as U (mg biomass)⁻¹ h⁻¹.

Enzyme stability. The stabilities of the enzymes in the extracellular soluble fraction were measured using the method described by Eaves *et al.* (1979), which gives a measure of the denaturation constants (λ -values) under

conditions very similar to those occurring during growth of the culture. Basically, volumes of culture (600–700 ml) were removed from the chemostat ($D = 0.2 \mu_{rel}$) and centrifuged at 30 000 g for 45 min. The cell-free, vesicle-free supernatant fraction was made up to 750 ml by the addition of an identically prepared batch of supernatant fluid (made previously and stored at -20°C) and put back into a chemostat vessel of an identical design as that used for the growth experiments (series 500, LH Engineering). The gas flow, stirrer speed, pH and temperature were set to the same values as those used to grow the cells. After temperature equilibrium had been reached, samples were taken at hourly intervals and enzyme levels determined by assay. Enzyme denaturation was calculated from the slope of the natural log plot of the results (Eaves *et al.*, 1979).

Steady state in continuous culture. Biomass and enzyme activities were determined on samples taken from the chemostat during steady-state growth conditions at the different relative growth rates that were selected. Only after a minimum of six culture-volume changes had occurred between particular conditions was a steady-state considered to have been achieved.

Statistical analysis. Linear regression analysis was used to determine the slope of the curves from both the enzyme denaturation experiments and the wash-out data for calculation of μ_{max} . For biomass and enzyme activity measurements, four (batch culture) or twelve (continuous culture) determinations were made for each fraction of each sample within single batch and continuous culture experiments and SEM was calculated.

Electron microscopy. Formvar-coated copper grids were briefly immersed into culture samples. Excess fluid was removed with blotting paper and the grids were then immersed in 1% (w/v) phosphotungstic acid (pH 7.5) for 30 s, blotted and allowed to dry. Specimens were examined with a Philips 300 electron microscope (80 kV).

Chemicals. All chemicals were supplied by Sigma.

RESULTS

Electron microscopy

Examination of the cell pellet fraction from samples taken from cultures growing at $0.3 \mu_{rel}$ in the chemostat revealed the presence of 'blebs' or vesicles in addition to the bacterial cells. However, the proportion of free vesicles to cells was relatively low. Where vesicles were observed they appeared to be 'cell surface associated', possibly in the process of formation. Observations on the harvested vesicle fraction showed that this fraction was essentially devoid of whole cells. The vesicles present ranged in size from about 30 nm to 100 nm in diameter. In whole culture samples, the ratio of free vesicles to cells appeared to be higher at $0.3 \mu_{rel}$ than at $0.9 \mu_{rel}$ although direct quantitative data were not recorded.

Batch culture of B. gingivalis

The growth of *B. gingivalis* strain W50 in stirred batch culture is shown in Fig. 1. For 3 h there was a period of fast growth ($\mu = 0.28 \text{ h}^{-1}$ approx.), as reflected by the increase in OD_{540} . The OD_{540} continued to increase but at a slower rate ($\mu = 0.10 \text{ h}^{-1}$ approx.) until 6 h post-inoculation, at which point growth ceased. From about 14 h onwards there was a gradual decrease in biomass. Comparison of the production of trypsin-like protease, alkaline phosphatase and *N*-acetylglucosaminidase (Fig. 1) showed that the cell-bound activities of these enzymes increased during the rapid phase of growth. For alkaline phosphatase, a further increase in the level of cell-bound enzyme activity occurred, reaching a maximum during the stationary phase. In general, levels of trypsin-like protease and alkaline phosphatase in extracellular vesicles roughly paralleled the level of cell-bound activity, but lower amounts of these enzymes were detected per unit biomass. For *N*-acetylglucosaminidase, extracellular vesicle activity increased slightly after 4 h and then remained approximately constant until 15 h post-inoculation when the activity dropped. The amounts of extracellular soluble enzyme activity produced were always considerably less than the levels measured in the cell-bound fraction and were usually less than the levels measured in the extracellular vesicle fraction.

Continuous culture of B. gingivalis

In continuous culture, cell biomass and hydrolytic enzyme production were measured during steady-state growth using a range of dilution rates (Table 1).

The biomass production rate increased with increasing growth rate (Table 1). Between 0.1 and $0.3 \mu_{rel}$ the biomass production rate increased proportionally with the increase in relative growth

Fig. 1. Growth of *Bacteroides gingivalis* in batch culture showing (a) the OD of the culture at 540 nm, and (b) trypsin-like protease, (c) alkaline phosphatase and (d) *N*-acetylglucosaminidase activities. Enzyme activities: ○, cell-bound; ●, extracellular vesicle; □, extracellular soluble. The error bars (shown only where larger than symbols) indicate SEM. Data are representative of four batch culture experiments.

rate. Thereafter (between 0.3 and $0.9 \mu_{rel}$), the increase in biomass production rate was less than the value predicted by a strictly proportional relationship. The cell yields in batch and continuous culture were comparable at approximately 0.25 g l^{-1} .

A comparison of total (cell-bound, extracellular vesicle plus extracellular soluble) enzyme production rates (Fig. 2) shows that the production rate of alkaline phosphatase increased with increasing relative growth rate. The production rate of trypsin-like protease also increased with increasing relative growth rate except at $0.9 \mu_{rel}$. The production rate of *N*-acetylglucosamini-

Fig. 2. (a) Effect of relative growth rate on total production rates of trypsin-like protease (○), alkaline phosphatase (●) and *N*-acetylglucosaminidase (□; NB values multiplied by 10 before plotting). (b–d) Effect of relative growth rate on levels of cell-bound (○), extracellular vesicle (●) and extracellular soluble (□) enzyme activities: (b) trypsin-like protease, (c) alkaline phosphatase and (d) *N*-acetylglucosaminidase. Error bars (shown only where larger than symbols) indicate SEM. Data are representative of two continuous culture experiments.

Table 1. Dilution rates, equivalent relative specific growth rates and steady-state biomass production rates for *B. gingivalis*

Steady-state dilution rate <i>D</i> (h ⁻¹)	Relative growth rate (μ_{rel})*	10 ² × Biomass production rate† (g l ⁻¹ h ⁻¹)
0.038	0.10	0.86 ± 0.04
0.076	0.20	1.91 ± 0.17
0.114	0.30	2.90 ± 0.10
0.190	0.50	4.23 ± 0.04
0.266	0.70	5.48 ± 0.27
0.342	0.90	6.45 ± 0.23

* μ_{max} (0.380 h⁻¹) was determined using a washout dilution rate of 0.52 h⁻¹.

† Results are means ± SEM of 12 determinations from each steady-state.

dase increased with increasing relative growth rate between 0.1 and 0.2 μ_{rel} , but thereafter the production rate remained constant with increasing relative growth rate.

In general, at high relative growth rates (0.7–0.9 μ_{rel}) the highest proportions (70–75%) of enzyme activities were cell-bound, intermediate proportions (25–30%) were extracellular vesicle and very little activity (<5%) was detected as extracellular soluble. However, at the lowest relative growth rate (0.1 μ_{rel}), the highest proportions of enzyme activities (55–60%) were found as extracellular vesicle, whilst intermediate proportions (30–40%) were measured as cell-bound. For extracellular soluble activities, the levels were always low (<12%) compared to the cell-bound or extracellular vesicle levels. Nevertheless, they were highest at low relative growth rates and lowest at high relative growth rates.

The results for extracellular soluble enzyme stabilities showed that all three enzymes were relatively stable. Alkaline phosphatase was the most stable ($\lambda = -0.015$; correlation coefficient = 0.932). Trypsin-like protease ($\lambda = -0.021$; correlation coefficient = 0.970) and *N*-acetylglucosaminidase ($\lambda = -0.028$; correlation coefficient = 0.835) were slightly less stable under the conditions (pH 7.5; 37 °C; anaerobic) employed in the experiment. The correction formula of Eaves *et al.* (1979) was not applied since the corrected production rates of enzyme activities were not significantly different from the measured values.

DISCUSSION

Although the nature of the growth-limiting nutrient in the complex medium used in this study is unknown, it is possible that the cells were limited by the supply of amino acids or peptides acting as carbon/energy sources for the cell. The levels of vitamin K₃ and haemin used in this study (1 mg l⁻¹ and 10 mg l⁻¹, respectively) are well above the levels which are thought to be limiting (McKee *et al.*, 1986). A synthetic medium suitable for continuous culture of *B. gingivalis* is not yet available.

It seems probable that *B. gingivalis* can synthesize hydrolytic enzymes and then export these enzymes across the cytoplasmic membrane into the periplasmic space, a common location for hydrolytic enzymes in many Gram-negative species of bacteria (Neu & Heppel, 1965; Cheng *et al.*, 1970; Silhavy *et al.*, 1983). In batch culture, the earliest detected activities and highest levels of enzymes were cell-bound, which suggests that enzyme synthesis was occurring during the rapid phase of growth. Levels of extracellular vesicle enzyme activity were also detected at an early stage, which suggests that vesicle production is a feature of growing cells rather than non-growing, ageing cells. The vesicles formed during the growth of *B. gingivalis* cells are thought to be composed of modified or unmodified outer membrane (Grenier & Mayrand, 1987). The vesicles are therefore likely to contain (in a bound or unbound form) a proportion of the periplasmic enzymes present in the region of, and at the time of, vesicle exocytosis. Assuming that the periplasmic enzymes are not preferentially directed into vesicles, the levels of extracellular vesicle enzyme activities will reflect firstly the underlying level of cell-bound activity and, secondly, the rate of vesicle formation. Therefore, during steady-state growth in continuous culture (where the production rates and decay rates of enzymes and vesicles are assumed to be constant) the ratio of extracellular vesicle to cell-bound enzyme levels can be used as an indicator or 'marker' of vesicle production. A high ratio suggests a high rate of vesicle production whilst a low ratio suggests a low rate of vesicle production. If this hypothesis is accepted, the results of this study show that vesicle production varies with growth rate, being highest at low growth rates.

In addition to cell-bound and extracellular vesicle enzyme activities, small amounts were detected in an extracellular soluble form. This may be due to vesicle breakdown and release of the enzyme into the culture supernatant fluid. Alternatively, it is possible that extracellular soluble enzymes may arise by other mechanisms, including release of the enzymes due to cell lysis or release due to 'leakage' from the cell at the points of vesicle exocytosis. For these reasons, in batch culture, extracellular soluble activity could occur at any point after the production of cell-bound activity, and not be restricted to the decline stage where cell or vesicle lysis may be occurring. In addition, cell-bound, extracellular vesicle and extracellular soluble levels of

enzyme activities may also be affected by the rate of enzyme degradation occurring. However, the extracellular forms of the three enzymes were all relatively stable. Although no attempt was made to measure the denaturation rates of extracellular vesicle enzyme activities, it might be expected that in this form they would be at least as stable as the enzymes in the extracellular soluble form.

The results of studying enzyme production using continuous culture suggest that alkaline phosphatase is a growth-linked enzyme, since it is produced in nearly constant amounts per unit biomass over the full range of relative growth rates achieved. The same is nearly true of the trypsin-like protease except at the highest relative growth rate employed. In contrast, *N*-acetylglucosaminidase activity is not growth-linked and must be subject to some form of control mechanism which allows significant rates of enzyme synthesis to occur at lower growth rates and enzyme repression to occur at higher relative growth rates. It is possible that non-growth-linked enzymes such as *N*-acetylglucosaminidase play a less important role for cellular growth than growth-linked enzymes. Strains of *B. gingivalis* have been isolated which do not possess *N*-acetylglucosaminidase activity (J. Greenman, unpublished data), suggesting that this enzyme does not play a central role in the organism's growth or survival in its natural habitat.

The results of changing specific growth rate by the dilution rate of the culture, with all other factors constant, demonstrates that specific growth rate is important with respect to the production of hydrolytic enzymes and vesicles. McKee *et al.* (1986) reported that *B. gingivalis* strain W50 grown under haemin limitation in continuous culture produced large numbers of cell-bound and extracellular vesicles and was less virulent in mice, whilst in haemin-excess conditions few extracellular vesicles were seen and the cells from the culture were virulent in mice. However, these workers did not use the same relative specific growth rates (Tempest, 1976) for comparing the haemin-limited and haemin-excess growth conditions, which would impose different specific growth rates on the organism. It is possible that haemin itself is neither a key factor in vesicle production nor in virulence but that growth rate is.

It is possible that the three hydrolytic enzymes may serve a nutritional role for the bacterial cells in supplying low-molecular-mass, cell-membrane-transportable nutrients. These originate in the natural environment from high-molecular-mass polymers or phosphorylated carbon sources. In addition, the cleavage of charged sugars from glycoproteins by *N*-acetylglucosaminidase activity or the cleavage of charged phosphate groups from phosphoserine residues present in phosphoproteins may make these proteins more susceptible to subsequent proteolytic breakdown by the trypsin-like protease.

The authors would like to thank Dr A. G. Schaffer for helpful suggestions and critical reading of the manuscript.

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