***Capnocytophaga gingivalis*: effects of glucose concentration on growth and hydrolytic enzyme production**

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In chemostat culture, the microaerophilic, CO₂ requiring, gingival-plaque-associated bacterium *Capnocytophaga gingivalis* responded to the addition of glucose (1–6 g l⁻¹) by doubling its growth rate and increasing its biomass yield fivefold. The data suggest that the glucose is catabolized by a fully aerobic route. Rather than repressing hydrolytic enzymes which might be associated with pathogenic properties, glucose enhanced the specific activity of aminopeptidase, trypsin-like protease, acid and alkaline phosphatase and α -glucosidase in comparison with a control culture grown in a tryptone/thiamin medium. Thus, the supply of glucose could be of importance in maximizing the pathogenic potential of this organism.

Keywords: *Capnocytophaga gingivalis*, continuous culture, glucose, hydrolytic enzymes, extracellular and cell-bound enzymes

INTRODUCTION

The genus *Capnocytophaga* was proposed by Leadbetter *et al.* (1979), who characterized non-flagellated, gliding, Gram-negative, fusiform bacteria isolated from the oral cavity. *Capnocytophaga* spp. have been reported to be involved in some forms of periodontal disease (Newman *et al.*, 1976; Slots, 1976) and a variety of non-dental clinical conditions (Forlenza *et al.*, 1980; Winn *et al.*, 1984; Parenti & Snyderman, 1985). The role of *Capnocytophaga* spp. in periodontal disease is still unclear. These micro-organisms have been shown to be a major component of subgingival plaque in juvenile periodontal disease (Newman *et al.*, 1976; Slots, 1976) and in patients with insulin-dependent diabetes mellitus (Mashimo *et al.*, 1983).

Capnocytophaga gingivalis has been shown to produce a range of hydrolytic enzymes (Shibata *et al.*, 1992; Poirier & Holt, 1983; Nakamura & Slots, 1982). The saccharolytic glycosidase enzymes include α - and β -glucosidases, *N*-acetylglucosaminidase and α - and β -galactosidases (Spratt, 1994). Enzymes which contribute to proteolytic activity include trypsin-like protease and aminopeptidase (Shibata *et al.*, 1992; Spratt *et al.*, 1995). Phosphatase activity is also apparent (Poirier & Holt, 1983). *C. gingivalis* has been reported to ferment glucose and other sugars (Socransky *et al.*, 1979). The effects of an easily available carbon/energy source, such as glucose, on the control of hydrolytic enzyme production have been investigated for other organisms of medical interest (e.g. *Propionibacterium*

acnes; Holland *et al.*, 1979) but not for *Capnocytophaga*. Of particular interest is whether glucose exerts differential control on the expression of enzymes involved in saccharolytic or proteolytic patterns of growth. The following study reports on the effects of glucose on the growth of *C. gingivalis* in continuous culture in tryptone/thiamin medium and on the production of a range of hydrolytic enzymes (trypsin-like protease, aminopeptidase, α -glucosidase and acid and alkaline phosphatase) by the organism under these conditions.

METHODS

Media and chemicals. Tryptone was obtained from Oxoid; Fastidious Anaerobe Agar was obtained from Lab M. All other chemicals were obtained from Sigma.

Organism. *Capnocytophaga gingivalis* strain ATCC 33624 was used in all experiments. Cultures were maintained on Fastidious Anaerobe Agar supplemented with defibrinated horse blood (5%, v/v). Plates were incubated in an anaerobic cabinet (MDH) at 37 °C, in an atmosphere of N₂/H₂/CO₂ (80:10:10, by vol.).

Continuous culture. *C. gingivalis* was grown in a 1 l chemostat vessel with control modules for temperature, pH and stirrer rate (series 500, LH Engineering). The culture volume was maintained at 750 ml. The temperature of the culture was maintained at 37 °C (± 0.1 °C) and the pH at 7.0 (± 0.05 units) by automatic addition of 2 M NaOH or 1 M H₂SO₄. The impeller speed was 500 r.p.m. (± 10 r.p.m.). The vessel was sparged with N₂, CO₂ and air to give an N₂/O₂/CO₂ mix of 85:10:5 (by vol.). Basal medium consisted of tryptone (10 g l⁻¹) and thiamin (0.5 mg l⁻¹).

Previous experiments have shown that thiamin is an essential B-vitamin for this species (Spratt, 1994). Solutions of sterile glucose were added to the basal medium to give a range of glucose concentrations from 0 to 6 g l⁻¹.

Maximum specific growth rates. The maximum specific growth rate ($\mu_{\max}$) for *C. gingivalis* was determined for each growth condition using a washout method (Tempest, 1970). The dilution rate (D) was set to a point beyond that expected for $\mu_{\max}$ and the concentration of cells (measured by taking OD₅₄₀ readings with a Jenway 6100 spectrophotometer) was measured against time. A graph of the washout of cells was constructed and $\mu_{\max}$ was calculated from the slope of the linear decline in the natural log (of optical density) with time, since $\mu_{\max} = D + \text{slope}$ of the washout curve.

Biomass estimations. Dry weight was estimated by the method of Holland *et al.* (1979). Cells were deposited by negative pressure filtration on to pre-dried and weighed membrane filters (Millipore; 25 mm, 0.45 µm pore size). The membrane plus deposited cells was dried to constant weight under an infrared lamp (domestic, 275 W at 15 cm distance) and the dry weight of the cells was calculated by taking the difference between the two weights. Culture OD₅₄₀ readings were related to cell dry weight by reference to a calibration curve.

Enzyme assays. Samples of *C. gingivalis* cultures were separated into two distinct fractions, cells and supernatant, by centrifugation at 2000 g for 20 min at 4 °C. Assays for aminopeptidase (Spratt *et al.*, 1995), trypsin-like protease (Yoshimura *et al.*, 1984), acid phosphatase (pH 5.5) and alkaline phosphatase (pH 9.0) (Bessey *et al.*, 1946) were carried out on both extracellular and cell-bound culture fractions. α -Glucosidase activity was assayed in a similar manner to that of phosphatase but using *p*-nitrophenyl α -D-glucopyranoside (0.5%, w/v) as the substrate and 0.1 M MOPS at pH 7.5 as the buffer. The assay stop solution was 0.2 M sodium carbonate.

Steady states. $\mu_{\max}$ values were determined for the control (zero glucose) and for each level of glucose used. From these results, the dilution rates were set to give steady-state growth rates of 0.3 $\mu_{\max}$ (i.e. 0.3 μ_{rel}) for each respective condition. Biomass and enzyme activities were determined on samples taken from the chemostat during steady-state growth conditions. Only after a minimum of six culture-volume changes had occurred between particular conditions and readings for enzymes and biomass were constant was steady state considered to have been achieved.

RESULTS AND DISCUSSION

Effects of glucose on growth and hydrolytic enzymes

Glucose (1–6 g l⁻¹) was added to the basal medium as the sole carbohydrate carbon/energy source for continuous culture studies. Biomass yields were compared for cultures grown at steady state at 0.3 μ_{rel} . From the results presented in Table 1, it can be seen that the biomass increased 3.5-fold with the addition of the first increment of glucose (1 g l⁻¹). The biomass increased to a maximum level at 4 g l⁻¹, where over a fourfold increase in biomass was measured with respect to the basal medium. The biomass yield then decreased with higher concentrations of glucose.

The $\mu_{\max}$ of the cells with respect to the various glucose concentrations is shown in Table 1. This shows a 1.8-fold increase in $\mu_{\max}$ following the addition of the first increment of glucose (1 g l⁻¹) to the basal medium, then a

Table 1. Effect of glucose concentration on the biomass and $\mu_{\max}$ of *C. gingivalis*

The medium used was tryptone/thiamin at a temperature of 37 °C.

Glucose concn (g l ⁻¹)	$\mu_{\max}$ (h ⁻¹)	Biomass yield* (g dry wt l ⁻¹)
0	0.13	0.24 (0.01)
1	0.24	0.84 (0.02)
2	0.27	0.94 (0.01)
3	0.30	0.95 (0.01)
4	0.31	0.99 (0.01)
5	0.32	0.91 (0.01)
6	0.28	0.86 (0.02)

* Biomass yields were measured at 0.3 μ_{rel} . Figures in parentheses represent the SEM of nine determinations.

gradual increase in $\mu_{\max}$ with increasing glucose concentration to 5 g l⁻¹, which gave a 2.5-fold increase in $\mu_{\max}$ with respect to the basal medium. Near maximal growth rates were attained at 3 and 4 g glucose l⁻¹. A further increase in glucose concentration (to 6 g l⁻¹) reduced the $\mu_{\max}$ with respect to the 5 g l⁻¹ level.

The tryptone/thiamin basal medium without glucose could therefore support growth, although both the culture biomass and $\mu_{\max}$ were relatively low. This establishes that peptides and amino acids can be utilized as both carbon/energy and nitrogen sources for the cell but that the efficiency of the system is low.

C. gingivalis is reported to be able to utilize carbohydrates, including glucose, as carbon/energy sources as well as amino acids and peptides (Socransky *et al.*, 1979). The increase of the culture biomass and $\mu_{\max}$ of the cells after the change from zero glucose to 1 g glucose l⁻¹ is substantial. This shows that cells can use glucose as an energy source, thus sparing the peptides and amino acids contained in the tryptone for use as cellular carbon and nitrogen sources. Increments of glucose greater than 1 g l⁻¹ caused only small additional increases in biomass. The yield on glucose is approximately 0.6 g cells (g glucose)⁻¹, which is similar to that of a prototrophic aerobic or facultatively anaerobic organism (e.g. *Escherichia coli*) when grown under fully aerobic conditions (Payne, 1970). Taken together, the biomass and $\mu_{\max}$ data suggest that growth is carbon/energy limited in the range of 4–5 g glucose l⁻¹ and in carbon/energy excess at glucose levels higher than this.

In general, the enzyme specific activities (Table 2) increased with the first increment of glucose, rose to a peak at intermediate glucose concentrations (2–4 g l⁻¹) and decreased at higher glucose levels. The aminopeptidase specific activity was very high compared to the other enzymes.

The levels of total enzyme activity for each of the hydrolytic enzymes tested at a glucose concentration of

Table 2. Effect of glucose concentration on hydrolytic enzyme production, expressed as specific activity [$\mu\text{mol product formed h}^{-1} (\text{mg cell dry wt})^{-1}$]

Figures in parentheses represent the SEM of 12 determinations. CB, cell-bound activity; EC, extracellular activity; Apase, aminopeptidase; TLP, trypsin-like protease; Acid P, acid phosphatase; Alk P, alkaline phosphatase; α -Gluc, α -glucosidase.

Glucose concn (g l^{-1})	Specific activity									
	Apase		TLP		Acid P		Alk P		α -Gluc	
	CB	EC	CB	EC	CB	EC	CB	EC	CB	EC
0	172 (7)	23 (0.42)	0.4 (0.07)	0.00	11 (0.14)	4.5 (0.07)	6.4 (0.24)	1.5 (0.15)	0.04 (0.01)	0.00
1	250 (13)	11 (0.45)	0.4 (0.10)	0.00	6.8 (0.68)	2.6 (0.06)	6.9 (0.2)	0.7 (0.08)	1.2 (0.02)	0.05 (0.001)
2	320 (19)	15 (0.70)	0.4 (0.02)	0.00	8.4 (0.30)	2.3 (0.03)	10.0 (0.18)	0.8 (0.05)	1.6 (0.06)	0.04 (0.001)
3	255 (14)	10 (0.36)	0.8 (0.10)	0.00	21 (0.47)	2.0 (0.07)	10.1 (0.18)	1.3 (0.03)	1.4 (0.10)	0.04 (0.001)
4	280 (30)	10 (0.36)	0.14 (0.10)	0.00	44 (0.60)	3.6 (0.01)	12.6 (0.20)	1.0 (0.01)	1.3 (0.04)	0.03 (0.001)
5	114 (6)	4 (0.12)	0.13 (0.03)	0.00	13 (0.60)	1.3 (0.03)	7.6 (0.40)	0.5 (0.03)	0.8 (0.01)	0.03 (0.001)
6	106 (6)	7 (0.10)	0.05 (0.01)	0.00	10 (0.36)	0.6 (0.09)	5.3 (0.1)	0.4 (0.02)	0.4 (0.01)	0.001 (0)

6 g l^{-1} (glucose excess) are significantly lower at the $P < 0.001$ level (ANOVA), than the levels at 3 g glucose l^{-1} , where glucose was considered to be limiting growth. A relative comparison of activity shows that cell-bound activity peaked at 3–4 g glucose l^{-1} for all the hydrolytic enzymes, with the exception of aminopeptidase, which peaked at 2 g l^{-1} . In contrast, the maximum relative extracellular activity was highest in the basal medium for aminopeptidase and acid and alkaline phosphatase, and highest at 1 g glucose l^{-1} for α -glucosidase. For trypsin-like protease, the extracellular activity was below the detection limit of the assay. The absence of extracellular trypsin-like protease activity when cells were grown on glucose was also noted by Shibata *et al.* (1992). For α -glucosidase activity, extracellular activity was undetectable in cultures grown on basal medium (zero glucose). In general, however, for all measured enzymes, the extracellular activity progressively decreased from a maximum at low or no glucose to a minimum at glucose concentrations of 5–6 g l^{-1} .

The trend shown by both the cell-bound and extracellular enzyme activities may indicate that so-called catabolite repression (Magasanik, 1961) was occurring, whereby a freely available carbon/energy source (glucose in this case) represses the synthesis of enzymes now redundant since they are concerned with acquiring more carbon/energy. This type of repression also allows conservation of nitrogen (amino acids) needed for more important cellular functions, which under conditions of excess glucose may be the growth-limiting nutrient. The use of glucose as a source of carbon/energy to study the effects of carbon/energy limitation on enzyme production may

not reflect the nutritional changes likely to be encountered *in vivo*, where amino acids, peptides and host proteins are more likely to be the predominant carbon/energy sources. Nevertheless, glucose is present in the oral environment. Sources include that present in gingival crevicular fluid (Parker *et al.*, 1993), the glucose which may be present in food and drink (dietary sources), and glucose which may be liberated from the enzymic hydrolysis of dextran, glycogen and glycoproteins (e.g. mucin) within dental plaque. It is of interest to note that *C. gingivalis* has been associated with the periodontal disease occurring in patients with insulin-dependent diabetes mellitus (Mashimo *et al.*, 1983), a condition where the glucose levels within tissues and in crevicular fluid may be higher than that in subjects without this condition. In patients with insulin-dependent diabetes mellitus, a relationship has been demonstrated between the degree of periodontal breakdown and both elevated blood-glucose levels and levels of glycosylated haemoglobin, which occur as a consequence of high blood glucose (Ainamo *et al.*, 1990; Safkan-Seppälä & Ainamo, 1992). It is possible that *Capnocytophaga* glucosidase activity is capable of removing the glucose moieties from glycosylated haemoglobin, thus providing the cells with an additional source of glucose.

This study suggests that α -glucosidase is induced or derepressed by glucose. In general, if cells are adapted to growing on peptides or amino acids in the absence of glycosides or carbohydrates, then α -glucosidase production would be redundant for the cell and therefore not expressed, thus allowing ATP to be conserved for the cell.

Aminopeptidase and trypsin-like protease are repressed by excess glucose, which suggests that their role for the

cell is (in the absence of glucose) to obtain cell-permeable peptides or amino acids as carbon/energy sources rather than as nitrogen sources. With regard to phosphatase activity, no obvious pattern or trend is observed in response to glucose levels and therefore no special role associated with a saccharolytic or proteolytic pattern of growth can be ascribed to the production of these enzymes.

REFERENCES

- Ainamo, J., Lahtinen, A. & Uitto, V.-J. (1990). Rapid periodontal destruction in adult humans with poorly controlled diabetes. *J Clin Periodontol* **17**, 22–28.
- Bessey, O. A., Lowry, O. H. & Brock, M. J. (1946). A method for the rapid determination of alkaline phosphatase with five cubic millilitres of serum. *J Biol Chem* **164**, 321–329.
- Forlenza, S. W., Newman, M. G., Lipsey, A. I., Siegel, S. E. & Blachman, U. (1980). *Capnocytophaga* sepsis: a newly recognized clinical entity in granulocytopenic patients. *Lancet* **1**, 567–568.
- Holland, K. T., Greenman, J. & Cunliffe, W. J. (1979). Growth of propionibacteria on synthetic medium; growth yields and exo-enzyme production. *J Appl Bacteriol* **47**, 383–394.
- Leadbetter, E. R., Holt, S. C. & Socransky, S. S. (1979). *Capnocytophaga*: new genus of Gram-negative gliding bacteria. I. General characteristics, taxonomic considerations and significance. *Arch Microbiol* **122**, 9–16.
- Magasanik, B. (1961). Cellular regulatory mechanisms. *Cold Spring Harbor Symp Quant Biol* **26**, 249–256.
- Mashimo, P. A., Yamamoto, Y., Slots, J., Park, B. H. & Genco, R. J. (1983). The periodontal microflora of juvenile diabetics: culture, immunofluorescence and serum antibody studies. *J Periodontol* **54**, 420–430.
- Nakamura, M. & Slots, J. (1982). Aminopeptidase activity of *Capnocytophaga*. *J Periodontol Res* **17**, 597–603.
- Newman, M. G., Socransky, S. S., Savitt, E. D. & Crawford, A. (1976). Studies in the microbiology of periodontitis. *J Periodontol* **47**, 373–379.
- Parenti, D. M. & Snyderman, D. R. (1985). *Capnocytophaga* species: infections in non-immunocompromised and immunocompromised hosts. *J Infect Dis* **151**, 140–147.
- Parker, R. C., Rapley, J. W., Isley, W., Spencer, P. & Killoy, W. J. (1993). Gingival crevicular blood for the assessment of blood-glucose in diabetic patients. *J Periodontol* **64**, 666–672.
- Payne, W. J. (1970). Energy yields and the growth of heterotrophs. *Annu Rev Microbiol* **24**, 17–52.
- Poirier, T. P. & Holt, C. S. (1983). Acid and alkaline phosphatases of *Capnocytophaga* species. I. Production and cytological localization of enzymes. *Can J Microbiol* **29**, 1350–1360.
- Safkan-Seppälä, B. & Ainamo, J. (1992). Periodontal conditions in insulin-dependent diabetes mellitus. *J Clin Periodontol* **19**, 24–29.
- Shibata, Y., Fujimura, S. & Nakamura, T. (1992). Isolation and characterization of enzymes hydrolyzing chymotrypsin synthetic substrate (enzyme 1) and trypsin synthetic substrate (enzyme 2) from the envelope fraction of *Capnocytophaga gingivalis*. *Med Microbiol Immunol* **181**, 107–115.
- Slots, J. (1976). The predominant cultivable organisms in juvenile periodontitis. *Scand J Dent Res* **84**, 1–10.
- Socransky, S. S., Holt, S. C., Leadbetter, E. R., Tanner, A. C. R., Savitt, E. & Hammond, B. F. (1979). *Capnocytophaga*: new genus of Gram-negative gliding bacteria. III. Physiological characterization. *Arch Microbiol* **122**, 29–33.
- Spratt, D. A. (1994). *A study of aminopeptidase and other hydrolytic enzymes produced by Capnocytophaga gingivalis*. PhD thesis, University of the West of England, Bristol, UK.
- Spratt, D. A., Greenman, J. & Schaffer, A. G. (1995). *Capnocytophaga gingivalis* aminopeptidase: a potential virulence factor. *Microbiology* **141**, 3087–3093.
- Tempest, D. W. (1970). The continuous cultivation of micro-organisms. I. Theory of the chemostat. *Methods Microbiol* **2**, 259–276.
- Winn, R. E., Chase, W. F., Lauderdale, P. W. & McCleskey, F. K. (1984). Septic arthritis involving *Capnocytophaga ochracea*. *J Clin Microbiol* **19**, 583.
- Yoshimura, F., Nishikata, M., Suzuki, T., Hoover, C. I. & Newbrun, E. (1984). Characterisation of a trypsin-like protease from the bacterium *Bacteroides gingivalis* isolated from human dental plaque. *Arch Oral Biol* **29**, 559–564.

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