Oral Microbiology Immunology 2008: 23: 234–238
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

Effect of glucose on *Treponema denticola* cell behavior

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Ruby JD, Lux R, Shi W, Charon NW, Dasanayake A. Effect of glucose on Treponema denticola *cell behavior.*
Oral Microbiol Immunol 2008: 23: 234–238. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

Introduction: *Treponema denticola* inhabits the oral subgingival environment and is part of a proteolytic benzoyl-DL-arginine-naphthylamide-positive 'red complex' associated with active periodontal disease. Spirochetes have a unique form of chemotactic motility that may contribute to their virulence. Chemotaxis is essential for efficient nutrient-directed translocation.
Methods: We examined the effect of glucose on *T. denticola* cell velocity, expression of periplasmic flagella proteins, and chemotaxis, e.g. translocation into capillary tubes.
Results: The presence of glucose did not significantly effect *T. denticola* cell velocity in high viscosity conditions nor did it alter periplasmic flagella protein expression. The addition of glucose to capillary tubes resulted in greater numbers of *T. denticola* cells in tubes containing glucose. A non-motile mutant did not migrate into capillary tubes containing glucose.
Conclusion: These results are consistent with a chemotactic response to glucose that is motility dependent.

Key words: glucose chemotaxis; motility; oral spirochetes; periodontal diseases; *Treponema denticola*

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Accepted for publication August 15, 2007

The transition from a healthy to an inflamed periodontium parallels a microbial shift from a gram-positive/non-motile flora to a gram-negative/motile flora within the subgingival sulcus or periodontal pocket (19, 27, 41). As inflammation intensifies and continues, enhanced gingival crevicular fluid (GCF) flow across the sulcular epithelium provides a rich milieu of serum components that are selective for proteolytic, anaerobic bacteria (28, 50, 52). This secluded anaerobic environment, enriched by serum proteins, favors motile organisms; the ability to translocate by flagellar movement would enable motile organisms to chemotactically seek out nutrient sources where gradient concentrations are optimal for growth (33). Spirochetes are found in close juxtaposition to the apical epithelial lining of the sulcus or pocket (20, 39), the source of GCF.

Spirochetes are the quintessential motile microbes of the subgingival environment and their numbers correlate well with the severity of periodontal diseases (9, 29, 37, 46). *Treponema denticola*, a cultivable small spirochete, is a component of a highly proteolytic trio of subgingival microorganisms (*T. denticola*, *Tannerella forsythia*, and *Porphyromonas gingivalis*) capable of digesting benzoyl-DL-arginine-naphthylamide (BANA) that is collectively referred to as the 'red complex' associated with advanced periodontal disease (30, 49). Their unique spiral motility enables them to penetrate sulcular epithelium and bore into the periodontium (12, 26, 45). *T. denticola* strains that are mutant for chemotaxis and motility show diminished penetration of epithelial cell layers (31). Motility and chemotaxis, therefore, may be considered important spirochete virulence factors; the translocating organism uses chemotaxis to navigate toward the desired source of chemical attractant (5–7, 10). However, limited studies are available on the chemotactic responses of oral spirochetes (18, 24, 34, 35, 42, 51). In this study we investigate glucose chemotaxis of *T. denticola* cells using capillary tube assays, and the effect of glucose on *T. denticola* cell velocity and the expression of periplasmic flagella proteins.

Materials and methods

Bacterial strain and culture conditions

Cultures of *T. denticola* 33520 (American Type Culture Collection, Rockville, MD) and its spontaneous non-motile mutant (JR1) (44) were grown anaerobically (80% N_2, 10% H_2, 10% CO_2) at 35°C in modified NOS medium (12.5 g/l brain–heart infusion broth; 10 g/l trypticase; 2.5 g/l yeast extract; 0.5 g/l sodium thioglycolate; 1 g/l L-cysteine; 0.25 g/l L-asparagine; 0.2% sodium bicarbonate; 10% heat-inactivated rabbit serum; 0.0006% thiamine pyrophosphate; pH 7.4) (23). *T. denticola* 35405 (American Type Culture Collection, Rockville, MD)

and its non-motile insertional mutant construct HL51 (25) were grown anaerobically (85% N_2, 10% H_2, 5% CO_2) at 35°C in TYGVS broth [10 g/l tryptone; 5 g/l veal heart infusion broth; 10 g/l yeast extract; 10 g/l gelatin; 0.5 g/l $(NH_4)_2SO_4$; 0.1 g/l $MgSO_4 \bullet 7\ H_2O$; 1.13 g/l K_2HPO_4; 0.9 g/l KH_2PO_4; 1 g/l NaCl; 1 g/l glucose; 1 g/l cysteine hydrochloride; 0.0125 g/l thiamine pyrophosphate; 0.25 g/l sodium pyruvate; 0.27 ml acetic acid; 0.10 ml propionic acid; 0.064 ml *n*-butyric acid; 0.016 ml *n*-valeric acid; 0.016 ml isobutyric acid; 0.016 ml isovaleric acid; 0.016 ml DL-methylbutyric acid; 10% heat-inactivated rabbit serum] (40). *T. denticola* wild-type strains 33520 and 35405 have identical phenotypes and their respective non-motile mutants JR1 and HL51 lack periplasmic flagella.

Videomicroscopy

Cell velocity measurements using videomicroscopy were performed using previously described methods (43). A *T. denticola* 33520 clone was selected from the edge of a colony swarm to ensure selection of rapidly translocating cells. In addition, cell velocity was assessed with non-motile mutant JR1. All cells used in the motility assays were from mid-exponential phase growth in NOS medium either without glucose or with 100 mM glucose. One milliliter of cells was centrifuged at 12,000 ***g*** for 10 min under anaerobic conditions and the pellet was suspended in 200 μl fresh NOS medium with 10% rabbit serum. An equal volume of methylcellulose (MC_{15} or MC_{4000}) (Fisher Scientific, Pittsburgh, PA) in NOS medium (MC-NOS) was added and allowed to equilibrate for 1 h anaerobically. MC-NOS was prepared by incubating NOS medium without rabbit serum with MC overnight at 5°C. The final viscosity of the MC-NOS medium was measured in centipoise (cP) as determined by a Brookfield DV III rheometer (Brookfield Engineering, Stoughton, MA). To determine cell velocity, 5 μl of cells were placed on a glass slide under anaerobic conditions and covered with a 22-mm^2 glass cover-slip with silicone grease along the apposing edges. *T. denticola* cells were observed under dark-field illumination with a 200-Watt mercury arc lamp with a heat barrier filter and were video-recorded under the Leitz microscope with either a ×54 or ×100 objective, DAGE-MTI model 72 CCD camera, and a Panasonic SVHS UP-910 video recorder. To avoid surface effects in motility determinations, the plane of focus was restricted to the central regions of the preparations (2). A video recording of a stage micrometer calibrated the distance cells moved. Speed was determined by recording translocating cells and measuring the distance traveled across the screen over 2- to 10-s intervals. Thirty cells were analyzed per condition at 25°C and the results were expressed as the mean ± standard deviation; the effect of 100 mM glucose on the mean velocities of *T. denticola* cells were compared using *t*-test (Table 1).

Gel electrophoresis and Western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting of periplasmic flagella were carried out as previously described (44). The 0.75-mm resolving gel contained 10% polyacrylamide. Approximately 5 μg of periplasmic flagella protein from *T. denticola* 33520, isolated and purified as previously described (44), and 10^8 *T. denticola* 33520 cells grown in NOS medium with 0, 10, 25, 50, or 100 mM glucose to mid-exponential phase underwent electrophoresis for 40 min at 30 mA and were stained with Coomassie brilliant blue-250. The *T. denticola* cell lysates and periplasmic flagella proteins separated by SDS–PAGE were electrophoretically transferred to polyvinyl difluoride (PVDF) membrane (Millipore Corp., Bedford, MA) for 1 h at 200 mA in electroblot buffer (3.3 g Tris–HCl, 1.14 g glycine, in 800 ml H_2O and 200 ml methanol) at 5°C. The PVDF membrane was blocked with 5% bovine serum albumin (Sigma, St Louis, MO) in 20 mM Tris–HCl, 500 mM NaCl, pH 7.5 and incubated for 2 h in a 1 : 500 dilution of antisera directed to the 37-kDa FlaA protein of *T. pallidum* or a 1 : 200 dilution of antisera directed to the 33-kDa FlaB2 protein of *T. pallidum*. Blots were developed by using a horseradish peroxidase second antibody with 4-chloronaphthol and hydrogen peroxide.

Chemotaxis assays

Capillary tube assays (36) were adapted to the anaerobic conditions required for *T. denticola* 35405 wild-type and its non-motile mutant HL51 (25). Approximately 10^8 cells/ml were suspended in chemotaxis buffer (0.15 M NaCl, 10 mM NaH_2PO_4, pH 7.6) containing 0.5% methylcellulose to enhance *T. denticola* translocation and assessed for chemotactic responses to glucose. Capillary tubes (0.025 mm internal diameter) were filled with 0.1 mM or 1.0 mM glucose in chemotaxis buffer and reduced in an anaerobic chamber overnight before being inserted into the cell suspension. Capillary tubes filled with chemotaxis buffer were used as a background control. After a 2-h anaerobic incubation at 37°C, the contents of the capillary tubes were transferred to a Petroff–Hausser bacterial counting chamber and cells were enumerated. Results were based on averages of triplicate assays and the mean values of wild-type cells under different glucose conditions were compared using analysis of variance (ANOVA; Table 2).

Results

Cell velocity

We examined the translocational motility of *T. denticola* cells ($n = 30$) using dark-field videomicroscopy to determine the effect of glucose on velocity. Methylcellulose was added to the cell preparations because *T. denticola* does not translocate

Table 1. Effect of glucose on the velocity of *Treponema denticola* 33520 cells at 25°C

Conditions	Viscosity (cP)	Velocity (μm/s)
NOS medium with 1.9% MC_{15}	9.2	2.53 ± 0.34
NOS medium with 1.9% MC_{15} and 100 mM glucose	9.2	2.06 ± 0.53
NOS medium with 0.9% MC_{4000}	155	4.20 ± 1.16
NOS medium with 0.9% MC_{4000} and 100 mM glucose	155	4.70 ± 1.57

Results are expressed as mean ± standard deviation ($n = 30$).
MC, methylcellulose; cP, centipoise (measurement of viscosity).

Table 2. Capillary tube assay of *Treponema denticola* 35405 chemotactic responses to glucose

Conditions	Wild-type (number of cells)[1]	Non-motile mutant (number of cells)[1]
Chemotaxis buffer (control)	651 ± 249	231 ± 58
Chemotaxis buffer and 1.0 mM glucose	2845 ± 863	201 ± 78
Chemotaxis buffer and 0.1 mM glucose	26,644 ± 10,178	366 ± 73

Results are expressed and mean ± standard deviation, ($n = 3$).
[1]Total cell count within each capillary tube.

in liquid broth and requires a viscous environment for its movement (43). Cells grown in NOS medium and cells grown in NOS medium with the addition of 100 mM glucose were observed at viscosities of 9.2 and 155 cP, respectively. The translocating *T. denticola* cells at 9.2 cP grown without the addition of glucose had an average velocity of 2.53 ± 0.34 μm/s while cells adapted to 100 mM glucose had an average velocity of 2.06 ± 0.53 μm/s. Increasing the viscosity of the translational medium to 155 cP resulted in an increase in average cell velocity to 4.20 ± 1.16 μm/s for cells grown in NOS medium and 4.70 ± 1.57 μm/s for cells adapted to 100 mM glucose in NOS medium. Cell velocities doubled as the viscosity increased from 9.2 to 155 cP. The addition of glucose to cells at a viscosity of 155 cP did not have a significant effect on cell velocity (4.20 ± 1.16 μm/s vs. 4.70 ± 1.57 μm/s, $P > 0.05$); however, at a viscosity of 9.2 cP the cell velocities were significantly different with the addition of glucose (2.53 ± 0.34 μm/s vs. 2.06 ± 0.53 μm/s, $P < 0.05$) (Table 1). The non-motile *T. denticola* mutant JR1 did not exhibit translocational movement in any of the test conditions presented in Table 1 (data not shown).

Periplasmic flagella proteins

The effect of glucose on the expression of periplasmic flagella proteins was examined using SDS–PAGE and Western blotting to determine if the synthesis of *T. denticola* periplasmic flagella is negatively regulated by cyclic adenosine monophosphate-mediated glucose catabolite repression, as has been reported for *Escherichia coli* (1, 53). *T. denticola* cell lysates from cultures grown in NOS with varying concentrations of glucose were analyzed for the expression of major periplasmic flagella proteins, e.g. FlaA (38 kDa) outer sheath protein and FlaB (35 kDa) core protein (Fig. 1). Lane 1 was isolated periplasmic flagella while lanes 2–6 were cell lysates of *T. denticola* grown in NOS medium with 0, 10, 25, 50, or 100 mM glucose. No differences were observed in the amount of FlaA or FlaB protein in cell lysates separated by SDS–PAGE as glucose concentrations increased. Western blotting of *T. denticola* cell lysates and isolated periplasmic flagella proteins with polyclonal antibody to the FlaA and FlaB major periplasmic flagella proteins of *T. pallidum* verified that expression of *T. denticola* FlaA and FlaB was not affected in glucose-adapted cells (Fig. 2). Taken together these

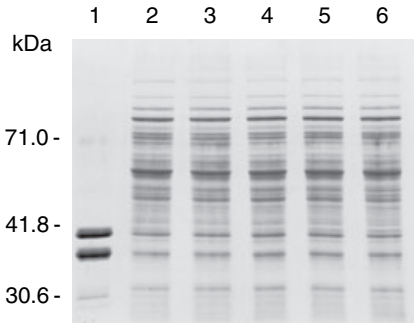

Fig. 1. SDS-PAGE of *Treponema denticola* 33520 isolated PFs, and cell lysates grown in NOS medium with varying concentrations of added glucose. Lane 1, PFs; lane 2, no glucose; lane 3, 10 mM glucose; lane 4, 25 mM glucose; lane 5, 50 mM glucose; lane 6, 100 mM glucose.

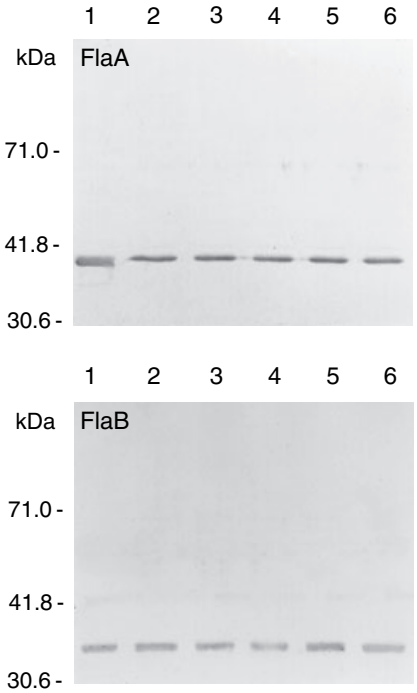

Fig. 2. Western blots of *Treponema denticola* 33520 isolated PFs, and cell lysates grown in NOS medium with varying glucose concentrations. FlaA was blotted with antisera to the 37 kDa PF sheath protein (FlaA) of *Treponema pallidum*, and FlaB was blotted with antisera to the 33 kDa PF core protein (FlaB2) of *T. pallidum*. Lane 1, PFs; lane 2, no glucose; lane 3, 10 mM glucose; lane 4, 25 mM glucose; lane 5, 50 mM glucose; lane 6, 100 mM glucose.

results indicate that synthesis of periplasmic flagella proteins is not affected by glucose concentration.

Chemotaxis assays

Capillary tube assays were performed to assess the chemotactic responses of *T. denticola* to glucose. Glucose is metabolized by *T. denticola* (4, 16) and, therefore, would serve as a substrate and potential chemoattractant. Total direct counts of *T. denticola* 35405 within capillary tubes containing 0.1 mM and 1.0 mM glucose had a significantly greater number of cells compared to the chemotaxis buffer control ($P < 0.05$). There was approximately a 10-fold increase in the number of cells in tubes containing 0.1 mM glucose compared with 1.0 mM glucose. HL51 non-motile mutant cells were present in low numbers in all capillary tubes compared to the number of wild-type cells in the chemotaxis buffer control (TABLE 2). These observations indicate that *T. denticola* 35405 is chemotactically attracted to glucose; the cell migration into capillary tubes being gradient and motility dependent.

Discussion

An increase in viscosity of the medium from 9.2 to 155 cP enhanced the velocity of *T. denticola* cells. Increases in translocation rates of spirochete cells as the medium viscosity increases have been reported for *Leptospira interrogans* (17), *Borrelia burgdorferi* (21), *T. denticola* (22, 43), and *Brachyspira pilosicoli* (38). However, the addition of 100 mM glucose to the medium has no appreciable affect on cell velocities when compared to medium without added glucose (Table 1). These results indicate that periplasmic flagella are functional in the presence of glucose, suggesting that glucose catabolite repression of the flagellar regulon does not occur in *T. denticola*, as has been reported for *E. coli* (1, 53).

Both SDS–PAGE and Western blotting of *T. denticola* cell lysates indicated that the expression of the major periplasmic flagella proteins FlaA and FlaB was not affected in glucose-adapted *T. denticola* cells (Figs 1 and 2). These results are in agreement with the previous observations that the presence of glucose has no affect on cell motility (Table 1). Since motility and periplasmic flagella expression are not affected by varying concentrations of glucose, the chemotactic behavior of *T. denticola* is primarily the result of cellular responses to glucose gradients and not of changes in cell velocity.

The *T. denticola* genome contains the necessary genes for a complete chemotaxis signaling pathway (14, 18, 48). Previous studies of chemotaxis in *T. denticola* have reported that TYGVS medium, along with rabbit serum, albumin, and ‘undefined

nutrients', are chemoattractants (18, 24, 34, 51). Glucose chemotaxis has not been studied extensively in *T. denticola* (35, 42). Chemotactic responses to glucose were observed with *T. denticola* cells using capillary tube assays (Table 2). Although *T. denticola* prefers amino acids as a fermentative substrate, glucose is transported and catabolized to pyruvate through an Embden–Myerhoff glycolytic pathway (4, 16). Coding genes for parts of the phosphotransferase system (PTS) are located within the genome, but *T. denticola* lacks a PTS transporter complex (47). However, the presence of genes for a glucose/galactose ABC transporter (family of membrane transport proteins) and of enzymes associated with glycogen synthesis indicates that glucose is an important catabolite (47).

Capillary tube assays require the addition of 0.5% methylcellulose to the chemotaxis buffer because high-viscosity 'gel-like' conditions are necessary for *T. denticola* translocation – cells in low-viscosity liquids have active flexing and spinning movement, but do not translocate (43). Chemotactic responses toward glucose were observed for *T. denticola*: compared to the background control more than four times the number of cells accumulated in capillaries containing 1 mM glucose whereas at 0.1 mM this value increased to 40 times the spirochetes found in capillaries filled with 0.5% methylcellulose in chemotaxis buffer only (Table 2). Glucose chemotaxis has also been studied in the aquatic spirochete, *Spirochaeta aurantia*, using capillary tube assays and swarm plates (3, 5, 13).

T. denticola inhabits the subgingival sulcus/periodontal pocket, an environment that is continuously enriched by GCF (8). Human GCF contains approximately 87 mg% (4.8 mM) glucose and these levels decrease with periodontal inflammation (15); GCF glucose levels from diabetics are elevated to 170 mg% (9.4 mM) (11). GCF glucose could direct *T. denticola* cells toward glucose gradients of higher concentration emanating from the gingival sulcular epithelium – the source of GCF. Their potential for epithelial penetration is motility driven and chemotaxis dependent; therefore, glucose could serve as an important chemoattractant for spirochete orientation leading to invasion of the periodontium (31–33).

Acknowledgments

The authors wish to thank Pravin Khandare, School of Engineering, West Virginia University, Morgantown, WV for his assistance with the viscosity determinations; Steven Norris, Department of Pathology and Laboratory Medicine, Medical School, University of Texas Health Sciences Center at Houston, Houston, TX for kindly providing antisera for the Western blotting; and Gary Xie, Oralgen, Los Alamos National Laboratory, Los Alamos, NM for his valuable support in accessing the *T. denticola* genome. We thank Mr David Fisher, Medical Education and Design Services, The University of Alabama at Birmingham, Birmingham, AL for his valuable assistance in the technical production of the figures for this manuscript. This research was funded by research grants K11-DE000257, R03-DE016040, DE01246, and AI29743.

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