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The regulatory effect of fermentable sugar levels on the production of leukotoxin by *Actinobacillus actinomycetemcomitans*

Katsunori Mizoguchi [a], Hiroyuki Ohta [b,*], Atsushi Miyagi [b], Hidemi Kurihara [1,a], Shogo Takashiba [a], Keijiro Kato [b], Yoji Murayama [a], Kazuhiro Fukui [b]

[a] *Department of Periodontology and Endodontology, Okayama University Dental School, Shikata-cho 2-chome, Okayama 700, Japan*
[b] *Department of Microbiology, Okayama University Dental School, Shikata-cho 2-chome, Okayama 700, Japan*

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Abstract

The relationship between sugar availability and RTX (repeats in toxin) cytotoxin (leukotoxin) production in the periodontopathic bacterium, *Actinobacillus actinomycetemcomitans*, was investigated using a chemostat. *A. actinomycetemcomitans* 301-b produced significant amounts of leukotoxin in anaerobic fructose-limited chemostat cultures at a dilution rate of 0.15 h^{-1} and at pH 7.0. When the growth limitation was relieved by pulsing the cultures with 50 or 150 mM fructose (final concentrations), leukotoxin production immediately stopped and the amount of cellular leukotoxin decreased until the culture was returned to fructose-limited conditions. Leukotoxin synthesis was also repressed in the chemostat cultures by pulsing with glucose but not with the non-fermentable sugar analog, α-methyl-D-glucoside. Leukotoxin production was also repressed by fructose in chemostat cultures of ATCC 33384, which is generally recognized as a non-leukotoxin-producing or minimally leukotoxic strain.

Keywords: *Actinobacillus actinomycetemcomitans*; Leukotoxin; Chemostat culture; Sugar repression

1. Introduction

Actinobacillus actinomycetemcomitans is a Gram-negative, facultatively anaerobic, periodontopathic bacterium that produces a polypeptide cytotoxin (M_r 113 000–125 000) capable of specifically killing human polymorphonuclear leukocytes and monocytes [1]. The *A. actinomycetemcomitans* cytotoxin (leukotoxin) is a member of the RTX (repeats in toxin) family represented by *Escherichia coli* α-hemolysin [2]. Although the leukotoxin gene (*ltx*) appears to be present in all *A. actinomycetemcomitans* strains [3], individual strains vary in terms of leukotoxic potential [1,4]. The strains are roughly classified as highly (represented by strain JP2) and minimally leukotoxic (e.g., strain 652 and ATCC 33384) or variably toxic (ATCC 29523). The *ltx* promoters of the highly leukotoxic strain JP2 and the minimally toxic strain 652 were analyzed by Brogan et al. [5]. The nucleotide sequence of the strain 652 promoter was similar to that of the strain JP2 promoter but it contained a region of 530 bp that was not present

* Corresponding author. Tel.: +81 (86) 235 6656; Fax: +81 (86) 235 6658; e-mail: hohta@dent.okayama-u.ac.jp

[1] Present address: Departments of Periodontology and Endodontology, Hiroshima University School of Dentistry, Kasumi 1-chome, Minami-ku, Hiroshima 734, Japan.

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in JP2. Analysis of 15 additional strains of *A. actinomycetemcomitans* has indicated that high levels of leukotoxin expression correlate with the presence of the strain JP2-like promoter.

We reported that *A. actinomycetemcomitans* ATCC 29523 and ATCC 33384, which contain the 652-like promoter [5], produce measurable amounts of leukotoxin when grown in fructose-limited chemostat cultures [6]. Moreover, we also reported that leukotoxin production by a toxin-production-variable strain (301-b) stopped immediately when the growth mode was changed from chemostat to batch culture [7]. The essential difference between batch and chemostat growth is that bacteria in the latter are continuously starved of fructose, whereas in the former they grow at the maximal rate until fructose is exhausted. Therefore, in this study, we examined the effect of fructose availability on leukotoxin production in *A. actinomycetemcomitans* strains 301-b and ATCC 33384 under controlled conditions in a chemostat.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A. actinomycetemcomitans 301-b [8] and ATCC 33384 were grown in an anaerobic chemostat system (working volume 440 ml) as described [9]. The growth medium [9] was composed of 50 mM Tris-HCl, various mineral salts, and 0.2% (w/v) yeast extract (Difco); yeast extract is required for the stable growth of the organism [10]. For the growth of ATCC 33384, the KCl and yeast extract concentrations in the medium were increased from 0.3 to 6.0 g l^{-1} and from 0.2 to 0.3% (w/v), respectively, and the medium was supplemented with sodium bicarbonate (20 mM). Fructose was added at 10 mM (low-fructose medium) or 35 mM (high-fructose medium). The former medium was used for fructose-limited chemostat cultures [6,9]. Bacteria were grown at 37°C and at pH 7.0 with the automatic addition of 2 M NaOH or 2 M HCl. The optical density at 660 nm (OD_{660}) of cultures was measured in a 1-cm cuvette to determine the cell density by the averaged coefficient of the dry cell weight at OD_{660} (0.852 mg dry weight per ml per OD unit) [9].

2.2. Sugar-pulse studies

To instantaneously relieve the fructose limitation during growth in a chemostat, about 11 or 33 ml of sterilized fructose (360 g l^{-1}) was injected by means of a syringe into the chemostat (final concentrations, about 50 or 150 mM, respectively). In this experiment the dilution rate (D) of the chemostat culture was kept at 0.15 h^{-1}. Every 2–3 h for the initial 12 h following fructose addition, samples (15 ml) were withdrawn from the culture and 2 or 3 ml were rapidly passed through a membrane filter (pore size, 0.20 μm). The filtrate was stored at −30°C for later assays of residual fructose and fermentation products. Leukotoxin was determined in the rest of the sample. Glucose and α-methyl-D-glucoside were also tested as pulse substrates.

2.3. Extraction and determination of leukotoxin

Leukotoxin was extracted from whole cells essentially as described [6]. Briefly, bacterial cells sampled from the chemostat were incubated with a mixture of DNase I (100 U ml^{-1}) (Sigma) and RNase A (0.1 mg ml^{-1}) (Sigma) at 25°C for 30 min in 100 mM acetate buffer (pH 5.0) containing 150 mM NaCl and 5 mM $MgSO_4 \cdot 7H_2O$. After centrifugation at $10\,000 \times g$ for 10 min, the supernatant (nuclease digest) containing the toxin was collected. Leukotoxin was recovered from culture supernatants as described [6]. These samples were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotted against an anti-leukotoxin serum [11]. Proteins on the gel were visualized by silver staining and the gel was analyzed densitometrically using a Foto/Analyst Image Analysis System (FOTODYNE Inc., Hartland, WI) as described [7].

2.4. Chemical analysis

Acid products (formate, lactate, and succinate) and ethanol were determined by gas-liquid chromatography, and fructose by the enzymatic method as described [9]. Acetate was determined using an enzyme system composed of acetyl-CoA synthetase, citrate synthase, and malate dehydrogenase (Boehringer Mannheim).

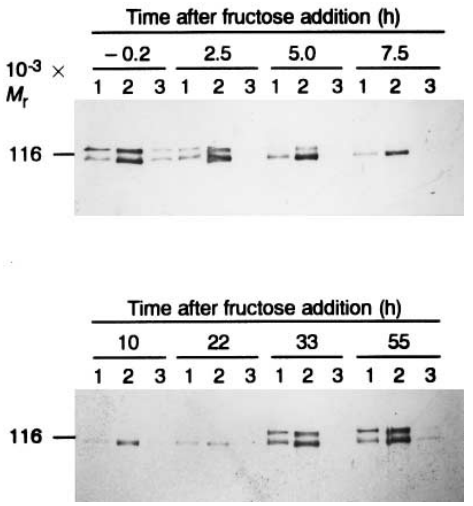

Fig. 1. Immunoblots of whole-cell suspensions (lanes 1), nuclease digests (lanes 2), and concentrated culture supernatants (lanes 3) prepared from anaerobic chemostat cultures ($D = 0.15$ h^{-1}, pH 7.0, 37°C) of *A. actinomycetemcomitans* 301-b pulsed with 150 mM fructose (final concentration). The low-fructose medium (10 mM fructose) was used for the chemostat culture and before fructose addition, the culture was in a steady state.

3. Results and discussion

A. actinomycetemcomitans 301-b grows with fructose, glucose, maltose, mannitol and mannose but not with cellobiose, galactose, lactose, raffinose, ribose, rhamnose, sucrose, trehalose and xylose [9]. In this study fructose was chosen as the growth-limiting nutrient since the growth rate of strain 301-b was higher with fructose than with any other sugar [9]. When the organism was grown in anaerobic fructose-limited chemostat cultures at $D = 0.15$ h^{-1}, the leukotoxin was found as a cluster of two polypeptides of M_r 113 000 and 120 000 and most of it was recovered in the nuclease digest (lane 2 at −0.2 h in Fig. 1). The estimated total amount of both polypeptides in the nuclease digest was 3.5 μg (mg dry weight)$^{-1}$. When the growth limitation was relieved by pulsing the culture with fructose (150 mM final concentration), the cellular leukotoxin level decreased instantaneously and gradually during the initial 22 h (4.8 generations), then returned to the original level (Fig. 1). No significant extracellular release of leukotoxin was caused by adding fructose (lanes 3 in Fig. 1). Hence, the total amount of both the 113 000 and 120 000 M_r polypeptides in the nuclease digest was estimated as the cellular content of leukotoxin. Fig. 2 shows changes in the cellular leukotoxin level and the amount of residual fructose in the culture after pulsing with fructose. If leukotoxin production stops immediately after the fructose addition, the cellular content of leukotoxin decreases with increasing cell mass. This theoretical change of the cellular leukotoxin level is shown in Fig. 2 (dashed line). The initial decreasing rate of leukotoxin level was estimated to be -0.24 h^{-1} and this value was 1.4-fold higher than the theoretical initial rate (-0.17 h^{-1}). When 50 mM fructose (final concentration) was added to the culture, a rapid decrease (-0.30 h^{-1}) in the cellular leukotoxin level was also initiated (Fig. 2). These decreases in the cellular leukotoxin level continued until the residual fructose in the culture decreased to less than the detection limit (0.02 mM) of the enzymatic assay. Pulsing the fructose-limited chemostat culture with glucose (150 mM) also immediately decreased the cellular leukotoxin level with an initial rate of -0.23 h^{-1}. However, the non-fermentable sugar analog, α-methyl-D-glucoside (50 mM), induced a small increase in the leukotoxin level (Fig. 2). These results indicated that leukotoxin production was repressed in the presence of excess fermentable sugars.

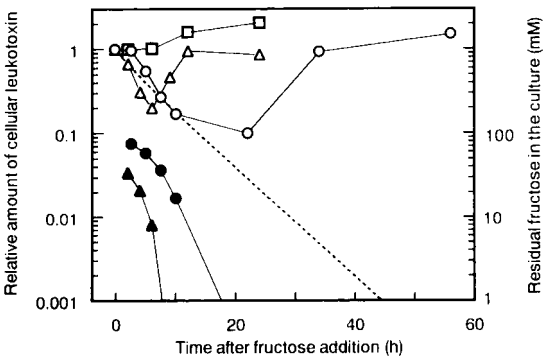

Fig. 2. Changes in the cellular leukotoxin levels (○, △, □) and the amounts of residual fructose (●, ▲) in chemostat cultures of *A. actinomycetemcomitans* 301-b after a pulse with 50 (△, ▲) and 150 mM (○, ●) fructose (final concentrations). The culture conditions were as described in the legend to Fig. 1. Culture samples were analyzed every 2-3 h for the initial 12 h following fructose addition. The dashed line indicates a theoretical change in the cellular leukotoxin level. The non-fermentable sugar analog, α-methyl-D-glucoside (50 mM final concentration) (□), was tested as the control substrate.

To address the difference between the catabolism under fructose-limited conditions and that under an excess, we analyzed the fermentation end products in the cultures. In *A. actinomycetemcomitans*, fructose is fermented primarily by the Embden-Meyerhof-Parnas pathway to phosphoenolpyruvate or pyruvate, then to a mixture of formate, acetate, ethanol, lactate, and succinate [9]. The fermentation products in the fructose-limited culture at $D = 0.15$ h^{-1} were formate, acetate, ethanol, and succinate (at −0.2 h in Fig. 3A). After 150 mM fructose was added to the culture, lactate was produced (at 2.5–22 h in Fig. 3A). This metabolic change might result from regulation of the enzyme activities (lactate dehydrogenase and pyruvate formate-lyase) involved in the conversion of pyruvate into fermentation products as found in oral streptococci [12]. In *Streptococcus mutans* JC2, intracellular levels of fructose-1,6-diphosphate and D-glyceraldehyde-3-phosphate are high under a glucose excess, which results in the activation of lactate dehydrogenase activity and the inhibition of pyruvate formate-lyase activity, respectively, thus influencing the type of fermentation products [13]. A similar mechanism would also be functioning in the sugar catabolism of *A. actinomycetemcomitans*. We also determined the fermentation products in cultures pulsed with α-methyl-D-glucoside (50 mM final concentration). This non-fermentable sugar analog did not cause a fermentation shift (Fig. 3B).

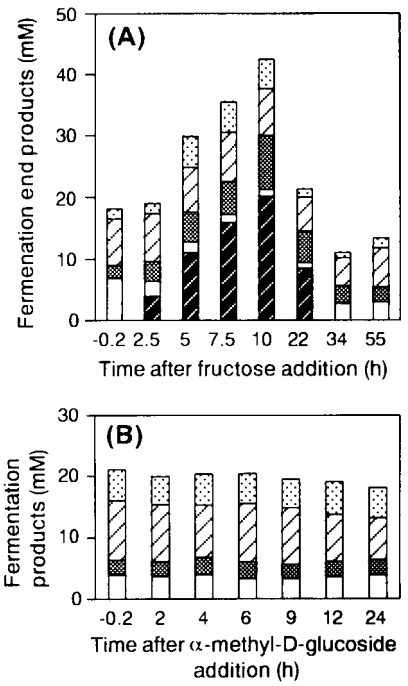

Fig. 3. Changes in the fermentation products of the chemostat culture of *A. actinomycetemcomitans* 301-b after a pulse with fructose (150 mM final concentration) (A) and α-methyl-D-glucoside (50 mM final concentration) (B). The culture conditions were the same as described in the legend to Fig. 1. Columns: stippled, formate; light-hatched, acetate; cross-hatched, ethanol; empty, succinate; dark-hatched, lactate.

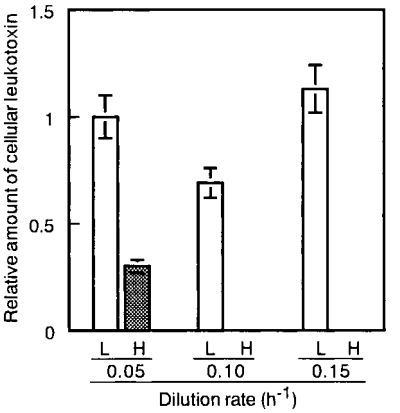

Fig. 4. Production of leukotoxin by *A. actinomycetemcomitans* 301-b grown in chemostat cultures with low- (L: 10 mM) and high-fructose (H: 35 mM) medium at different dilution rates. Cultures were maintained at pH 7.0 and at 37°C. Calculation of relative amount of cellular leukotoxin was based on the amount of leukotoxin in the nuclease extract prepared from the culture with low-fructose medium at $D = 0.05$ h^{-1}. Error bars indicate the 90% confidence interval.

The relationship between fructose availability and leukotoxin production was examined at different growth rates. When the anaerobic chemostat culture of strain 301-b was run at $D = 0.05$, 0.10, and 0.15 h^{-1} in low-fructose medium, leukotoxin was produced at all dilution rates examined (Fig. 4). The analysis of fermentation products in these cultures revealed the absence of lactate (Fig. 5). To establish a fructose excess, chemostat cultures were run in high-fructose medium. Although a small amount of leukotoxin was produced in the culture at $D = 0.05$ h^{-1}, it was not significant in those at $D = 0.10$ and 0.15 h^{-1} (Fig. 4). This result correlated with the profile of fermentation products; lactate was undetectable at $D = 0.05$ h^{-1} but found at $D = 0.10$ and 0.15 h^{-1} (Fig. 5). These results indicated that fructose

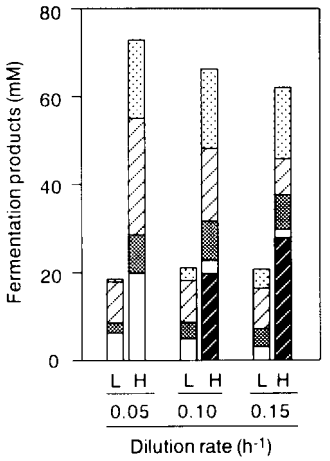

Fig. 5. Fermentation products of the chemostat cultures of *A. actinomycetemcomitans* 301-b grown on low- (L: 10 mM) and high-fructose (H: 35 mM) medium at various dilution rates. Chemostat cultures were maintained at pH 7.0 and at 37°C. Columns: stippled, formate; light-hatched, acetate; cross-hatched, ethanol; empty, succinate; dark-hatched, lactate.

limitation is a primary condition that induces leukotoxin synthesis.

We examined the relationship between fructose availability and leukotoxin production in *A. actinomycetemcomitans* ATCC 33384, which possesses the strain 652-like *ltx* promoter. This strain exhibited growth comparable to that of strain 301-b when the medium was supplemented with a relatively high concentration of KCl (6 g l^{-1}) and a small increase of yeast extract (from 2 to 3 g l^{-1}). When ATCC 33384 was grown in the chemostat with this modified medium under a fructose limitation at $D = 0.15$ h^{-1}, some amounts of leukotoxin were released extracellularly into the culture fluid. This was due to an increase in the ionic strength of the medium [6,7]. A pulse of 150 mM fructose to the culture decreased the amounts of both cellular and extracellular leukotoxin (Fig. 6). When the culture returned to fructose-limited conditions (residual fructose in the culture was less than 0.02 mM), leukotoxin production successively increased to the original level.

With respect to sugar concentrations in human periodontal pockets, one early study indicated that the glucose contents of the gingival exudates sampled from 29 individuals with some degree of gingival inflammation vary between 1.13 μg (mg exudate)$^{-1}$ (about 6.3 mM) and 7.09 μg (mg exudate)$^{-1}$ (about 39 mM) [14]. This glucose range covers both the sugar-limited (10 mM in low-fructose medium) and sugar-excess (35 mM in high-fructose medium) conditions in our growth experiments. Therefore, the leukotoxin production by *A. actinomycetemcomitans* in periodontal pockets is probably regulated by the exudate sugar level.

In conclusion, our results indicate that leukotoxin production by minimally leukotoxic (ATCC 33384) and variably toxic (301-b) groups of *A. actinomycetemcomitans* is down-regulated by growth under fermentable sugar-excess conditions. Therefore, batch culture, which contains an excess of all required nutrients, is not adequate to determine whether a strain of *A. actinomycetemcomitans* is classified as leukotoxin producing or not. In *E. coli*, the synthesis of heat-stable enterotoxin STa [15] and STb [16] is repressed in the presence of 0.2–1.0% (w/v) glucose and this was overcome by adding exogenous cyclic adenosine 3′,5′-monophosphate (cAMP). Recently, STb production was shown to be subject to catabolite repression [16]. To further characterize the leukotoxin production by *A. actinomycetemcomitans*, we are currently studying the relationship between the intra-

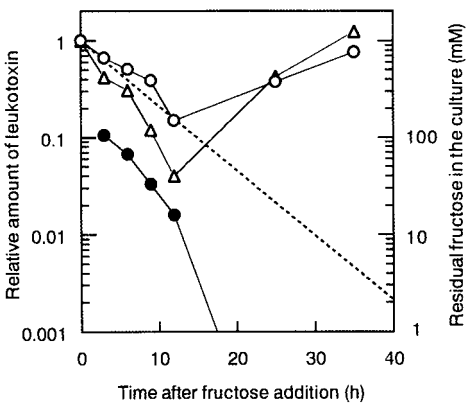

Fig. 6. Changes in the cellular (○) and extracellular (△) leukotoxin levels, and the amounts of residual fructose (●) in a chemostat culture of *A. actinomycetemcomitans* ATCC 33384 after a pulse with 150 mM fructose (final concentration). The culture was grown anaerobically at pH 7.0 and at $D = 0.15$ h^{-1}. The KCl and yeast extract concentrations in the medium were increased from 0.3 to 6.0 g l^{-1} and from 0.2 to 0.3% (w/v), respectively, and the medium was supplemented with sodium bicarbonate (20 mM). The low-fructose medium (10 mM fructose) was used for the chemostat culture and before fructose addition, the culture was in a steady state. The dashed line indicates a theoretical change in the cellular leukotoxin level.

cellular content of cAMP and leukotoxin production and analyzing the *ltx* promoter structure of strain 301-b.

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