The relationships between leukotoxin production, growth rate and the bicarbonate concentration in a toxin-production-variable strain of *Actinobacillus actinomycetemcomitans*

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*Actinobacillus actinomycetemcomitans*, a Gram-negative periodontopathic bacterium, produces a leukotoxin belonging to the RTX family. The production of leukotoxin varies greatly among different strains of this species. In this paper the effects of growth rate and bicarbonate on the leukotoxin production by a toxin-production-variable strain (301-b) during growth in a chemostat were examined. When the bacterium was grown in anaerobic fructose-limited chemostat cultures (pH 7.0 and 37 °C) at dilution rates (D) ranging from 0.04 to 0.20 h⁻¹ in the absence and presence of 10 mM bicarbonate, it produced leukotoxin as a cluster of two polypeptides (M, 113 000 and 120 000) and complexed with nucleic acids on the bacterial cell surface. The relationship between leukotoxin production and specific growth rate was analysed by plotting the specific rate of leukotoxin production [q_LT, in μg (mg dry wt)⁻¹ h⁻¹] against D. The plots were approximated to the linear relationships q_LT = 2.7D – 0.058 and q_LT = 9.3D – 0.407 without and with bicarbonate, respectively. These relationships suggest that the apparent leukotoxin production is a result of both growth-rate-dependent production and growth-rate-independent decomposition. The cellular leukotoxin level was also followed after the change from chemostat to batch culture in the same fermenter. In batch culture leukotoxin production stopped immediately and the cellular toxin level rapidly decreased, suggesting toxin decomposition. From the slopes of the approximated linear relationships between q_LT and D, a theoretical maximum leukotoxin yield (Y_LT) was estimated as 2.7 and 9.3 μg (mg dry wt)⁻¹ in the absence and presence of 10 mM bicarbonate, respectively. The increased Y_LT value in the cultures containing bicarbonate indicated that the addition stimulated the efficiency of leukotoxin synthesis up to about threefold. Further increases of bicarbonate concentration to between 20 and 40 mM had no effect on the total leukotoxin production, but the amount of extracellular leukotoxin increased with higher bicarbonate concentrations.

**Keywords**: *Actinobacillus actinomycetemcomitans*, leukotoxin, chemostat culture, bicarbonate effect

**INTRODUCTION**

*Actinobacillus actinomycetemcomitans* is a non-enteric, Gram-negative, non-motile, facultatively anaerobic bacterium which is associated with progressive periodontal disease in children, adolescents and adults (Zambon, 1985; Slots & Schonfeld, 1991). A major virulence factor in the pathogenicity of this bacterium is its ability to produce a polypeptide cytotoxin (M, 113 000–125 000) capable of killing specifically human polymorphonuclear leukocytes.
and monocytes (Tsai et al., 1979, 1984; DiRienzo et al., 1985; Ohta et al., 1991). Molecular genetic studies have indicated that the \textit{A. actinomycetemcomitans} cytotoxin (leukotoxin) belongs to the group of RTX cytotoxins that are characterized by the presence of a repeat domain in the C-terminal half of the proteins and by a genetic organization consisting of four contiguous genes designated \textit{ltxC}, \textit{ltxA}, \textit{ltxB} and \textit{ltxD}. \textit{ltxA} is the toxin structural gene and \textit{ltxC} is involved in the activation of \textit{ltxA} product, whereas \textit{ltxB} and \textit{ltxD} encode a secretion function (Lally et al., 1989; Strathdee & Low, 1989; Guthmiller et al., 1990a, b; Kraig et al., 1990).

Although the \textit{ltx} operon appears to be present in all \textit{A. actinomycetemcomitans} strains (Poulsen et al., 1994), the level of toxin expression varies considerably between different strains. Analysis of several non-leukotoxic strains (e.g. ATCC 33384) showed that they possessed the \textit{ltx} genes but contained low steady-state levels of \textit{ltx} mRNAs relative to a highly leukotoxic strain JP2 (Spitznagel et al., 1991). Recently, the \textit{ltx} promoters of JP2 and a minimally toxic strain (652) of \textit{A. actinomycetemcomitans} were analysed by Brogan et al. (1994). The nucleotide sequence of the 652 promoter was similar to that of the JP2 promoter but contained a region of 530 bp that was not present in JP2. Analysis of 15 additional strains of \textit{A. actinomycetemcomitans} has indicated that high levels of leukotoxin expression correlate with the presence of the JP2-like promoter. However, we found that ATCC 33384 (NCTC 9710), containing the 652-like promoter, produced a significant amount of leukotoxin when it was grown in a fructose-limited chemostat culture (Ohta et al., 1993). Therefore, not only the promoter structure but also some physiological factors must be involved in the regulation of leukotoxin expression.

Spitznagel et al. (1991) reported that the highly toxic \textit{A. actinomycetemcomitans} JP2 produced high levels of toxin activity during the mid- to late-exponential phase of growth and then production decreased dramatically in the stationary phase. Growth phase-dependence was also reported in the expression of \textit{Escherichia coli} haemolysin, a prototype of the RTX cytotoxins (Nicaud et al., 1985). Generally, in batch culture, the processes of growth cause the environment to change progressively and the organisms adapt continuously to these changes. Accordingly, probable environmental changes such as exhaustion of growth substrate, the accumulation of fermentation products, and environmental acidification, all may be factors leading to the cessation of toxin expression. Alternatively, the regulation of leukotoxin expression may be controlled primarily by the growth rate of the cell. This notion is supported in part by our preliminary studies of chemostat cultures of a clinical isolate (301-b) of \textit{A. actinomycetemcomitans} (Ohta et al., 1993). Fructose (quantities as indicated in Results and Discussion) was autoclaved separately and added to the medium aseptically. The organism was grown in an anaerobic chemostat system as described by Ohta et al. (1989). In brief, the system was kept under a stream (200–300 ml h\(^{-1}\)) of \(N_2\) which was freed of traces of oxygen by passage over a gas purifying column (Gas Clean GC-RX, Nikka Seiko). The temperature of culture was set at 37 \(^\circ\)C and the pH was maintained at 7.0 with 2 M NaOH or 2 M HCl. Anaerobic conditions was checked by monitoring culture redox potential using a platinum electrode with an Ag/AgCl reference cell. In this system the culture redox potential (without bicarbonate, pH 7.0) was maintained below \(-400 \text{ mV}\) (Ohta et al., 1991). The \(OD_{660}\) (LKB Novaspec 4049 spectrophotometer) of cultures was measured in a 1-cm-light-path cuvette to determine the cell densities by the averaged coefficient of the cell dry weight at \(OD_{660} [0.852 \text{ mg dry wt cells ml}^{-1} (\text{OD}_{660} \text{ unit})^{-1}]\): the relationship is linear up to \(OD_{660} 0.6\) (Ohta et al., 1989).

**Extraction of leukotoxin from whole cells.** As described previously (Ohta et al., 1991, 1993), the leukotoxin produced is associated with nucleic acids on the cell surface when the organism is grown in fructose-limited chemostat cultures. Therefore, the leukotoxin was extracted by incubating whole cells with nuclease. In brief, bacterial cultures removed from the chemostat were centrifuged at 10000 \(g\) for 10 min at 4 \(^\circ\)C. The cell pellets were suspended at 17 mg dry wt cells ml\(^{-1}\) in 100 mM acetate buffer (pH 5.0) containing 150 mM NaCl and 5 mM MgSO\(_4\).7H\(_2\)O. The cell suspensions were incubated with a mixture of DNase I (100 \(U\) ml\(^{-1}\)) (Sigma) and RNase A (0.1 mg ml\(^{-1}\)) (Sigma) at 25 \(^\circ\)C for 30 min. After centrifugation at 10000 \(g\) for 10 min, the supernatant (nuclease digest) containing the toxin was collected.

**Recovery of leukotoxin from culture supernatants.** The culture supernatants resulting from the centrifugation of bacterial cultures were passed through filters (pore size 0.22 \(\mu\)m). The filtrate was dialysed against deionized water, lyophilized and dissolved in distilled water (1/20th of the original culture volume). The leukotoxin was detected by immunoblotting with an anti-leukotoxin serum prepared previously (Ohta et al., 1991).

**SDS-PAGE and immunoblotting.** SDS-PAGE was performed with 7.5% (w/v) acrylamide gels essentially as described by Laemmli (1970). Each sample (11 \(\mu\)l) from the nuclease digest, concentrated culture supernatants, or whole-cell suspensions (3-4 mg dry wt cells ml\(^{-1}\)) was mixed with 9 \(\mu\)l SDS-containing sample buffer and boiled for 3 min. Proteins on the gel were visualized by silver staining with a commercial kit (Daiichi). The \(M_r\) of the leukotoxin was determined by comparison with...
known standards. Immunoblotting was carried out by the methanol/Tris/glycine method (Towbin et al., 1979). Reactivity with the anti-leukotoxin serum was detected with swine antirabbit immunoglobulin G conjugated to peroxidase and then visualized by adding 4-methoxy-1-naphthol and hydrogen peroxide. Leukotoxin in the samples was quantified by densitometric analysis of the SDS-polyacrylamide gel using a Beckman DU-8 spectrophotometer installed with a Beckman slab gel scanning system at a wavelength of 660 nm ($A_{440}$). To construct a standard curve of leukotoxin concentration, serial dilutions of the purified leukotoxin (0–100 μg ml$^{-1}$) (Ohta et al., 1991) were processed essentially as above and analysed together with the samples on the same gel. The relationship between leukotoxin concentration and the $A_{440}$ value was linear up to $A_{440}$ 0.6. The amount of leukotoxin produced was expressed in μg (mg dry wt cells)$^{-1}$.

Decomposition of leukotoxin in resting cell suspension. A batch culture (100 ml medium with 10 mM fructose) was incubated at 37 °C in an anaerobic glove box (Bainbox ANK-1, Hirasawa Works) filled with 80% (v/v) $N_2$, 10% (v/v) $H_2$ and 10% (v/v) CO$_2$. At the mid-exponential growth phase bacterial cells were collected by centrifugation at 10000 g for 10 min at 4 °C, washed once with the growth medium without fructose and suspended in the fructose-free growth medium. The cell suspensions (29 mg dry wt $^{-1}$) were incubated at 37 °C for 16.5 h without and with the purified leukotoxin (110 μg ml$^{-1}$) in the anaerobic glove box. A leukotoxin solution without bacterial cells was incubated in the same way as a control. Aliquots of the cell suspensions were sampled at 0, 1, 4, 10 and 16.5 h and each of them was centrifuged at 10000 g for 5 min and the supernatant was analysed by immunoblotting as described above.

Chemical analysis. Fructose concentrations in the reservoir medium and culture supernatants were determined by using the enzyme system composed of hexokinase, glucose-6-phosphate dehydrogenase and glucose-6-phosphate isomerase (Boehringer Mannheim). Bicarbonate was determined by GLC as described previously (Ohta et al., 1989).

Protein N-terminal analysis. The separated proteins on the SDS-polyacrylamide gel were electrophoretically transferred to a PVDF membrane by the methanol/CAPS buffer method (Matsudaira, 1987). The membrane was stained with 0.1% Coomassie Blue R-250, then destained with 50% (v/v) methanol and washed with distilled water. The band of interest was cut out with a scalpel and subjected to automated Edman degradation (Hunkapiller et al., 1983) on a Perkin Elmer (Applied Biosystems) 476A protein sequencer.

RESULTS AND DISCUSSION

Leukotoxin production during growth in chemostat culture

In general, a chemostat culture is assumed to be in a steady state after growth for a minimum of five volume changes (about seven generations) under unaltered circumstances. Therefore, to examine whether or not the leukotoxin production is stabilized after five volume changes, A. actinomycetemcomitans 301-b was grown in a chemostat culture over an extended period and variation in the leukotoxin production during growth was followed. The growth of 301-b in the chemostat in a medium containing less than 10 mM fructose was limited by fructose at $D$ values between 0.04 and 0.20 h$^{-1}$ (Ohta et al., 1989). The initial fructose-limited chemostat culture (7 mM fructose in the inflowing medium) without bicarbonate was maintained at $D = 0.04$ h$^{-1}$ for 7.6 volume changes. $D$ was then increased to 0.10 h$^{-1}$ and culture samples were taken after 1.7, 5.0 and 7.5 volume changes. Fig. 1 shows immunoblots of the whole-cell suspensions, nuclease digests and concentrated culture supernatants prepared from the anaerobic fructose-limited chemostat cultures (pH 7.0, 37 °C) of A. actinomycetemcomitans 301-b after a shift from $D = 0.04$ to 0.10 h$^{-1}$. Culture samples were taken at 0, 1.7, 5.0 and 7.5 volume changes after the shift. Lanes: 1, whole-cell suspension; 2, nuclease digest; 3, concentrated culture supernatant.

![Fig. 1. Immunoblots of whole-cell suspensions, nuclease digests and concentrated culture supernatants prepared from the anaerobic fructose-limited chemostat cultures (pH 7.0, 37 °C) of A. actinomycetemcomitans 301-b after a shift from $D = 0.04$ to 0.10 h$^{-1}$](image)
Volume change

![Leukotoxin Production](image)

**Fig. 2.** Production of leukotoxin (○) by *A. actinomycetemcomitans* 301-b in an anaerobic fructose-limited chemostat culture (pH 7.0, 37°C) shifted from *D* = 0.04 to 0.10 h⁻¹, from *D* = 0.10 to 0.04 h⁻¹ and finally from *D* = 0.04 to 0.06 h⁻¹. The dashed line indicates changes in *D*. Error bars indicate the 90% confidence interval.

reached a steady state. A constant *D* was maintained (0.20 h⁻¹). In these bicarbonate-added cultures the growth was still limited by fructose (Table 1). The addition of 10 mM bicarbonate resulted in a 1.1-fold increase of the molar growth yield [g dry wt (mol fructose)⁻¹], but a further increase of bicarbonate concentration from 10 to 40 mM had no effect (Table 1). Fig. 3 shows immunoblots of the whole-cell suspensions, nuclease digests and concentrated culture supernatants from cells cultured with different levels of bicarbonate. Two important characteristics of leukotoxin production during growth in the presence of bicarbonate were recognized. The cellular content of leukotoxin (*C*₁₁₀ᵏ plus *C*₁₂₀ᵏ) increased with increasing bicarbonate concentration, with an apparent optimum at 10 or 17 mM bicarbonate (lanes 2 in Fig. 3). When the bicarbonate concentration was increased to 28 mM, then to 40 mM, the leukotoxin (*M*<sub>r</sub> 113000 and 120000 polypeptides) was detected in the culture supernatant fraction (lanes 3 in Fig. 3). These two characteristics of leukotoxin production were reproducibly observed in the culture run at a lower *D* (0.15 h⁻¹) (data not shown).

The concentration of leukotoxin in the culture supernatant was measured by SDS-PAGE and densitometry and expressed in μg (mg dry wt cells)⁻¹ (*S*₁₁₀ᵏ and *S*₁₂₀ᵏ). Then, the total production of leukotoxin (*P*<sub>LT</sub>) was calculated by the following equation:

\[
P_{LT} = C_{110k} + C_{120k} + S_{110k} + S_{120k}
\]

Variations of these parameters by increasing the additional amount of bicarbonate are shown in Fig. 4. The addition of 10 and 17 mM sodium bicarbonate resulted in a 2.5- to 2.7-fold increase in the level of cell-associated toxin (*C*₁₁₀ᵏ plus *C*₁₂₀ᵏ). This stimulation was not due to the increase of sodium ion concentration in the culture because the additions of 25 and 49 mM sodium chloride resulted in only small increases (1.3- to 1.5-fold) of leukotoxin production (Fig. 4). With the further increase of bicarbonate concentration to 28 and 40 mM the cellular toxin level decreased in a dose-dependent manner, but in compensation, the amount of extracellular toxin (*S*₁₁₀ᵏ plus *S*₁₂₀ᵏ) increased. As a result, the total production of leukotoxin was fairly constant at a bicarbonate concentration ranging from 10 to 40 mM. This indicated that

**Table 1.** Effect of bicarbonate on the growth of *A. actinomycetemcomitans* 301-b in anaerobic fructose-limited chemostat cultures (*D* = 0.20 h⁻¹, pH 7.0, 37°C)

<table>
<thead>
<tr>
<th>Input (mM)</th>
<th>Residual (mM)</th>
<th>Yield of cells (mg dry wt ml⁻¹)</th>
<th><em>Y</em>&lt;sub&gt;fructose&lt;/sub&gt; (g cells mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicarbonate</td>
<td>Bicarbonate</td>
<td>Fructose</td>
<td>Fructose</td>
</tr>
<tr>
<td>0</td>
<td>6.8</td>
<td>ND</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>9.6</td>
<td>6.7</td>
<td>6.5</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>17.2</td>
<td>6.6</td>
<td>11.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>28.4</td>
<td>7.0</td>
<td>19.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>40.4</td>
<td>6.8</td>
<td>28.6</td>
<td>&lt; 0.1</td>
</tr>
</tbody>
</table>

ND, Not determined.
The extracellular release was due to an increase in the ionic strength of the medium rather than the bicarbonate itself. To examine this, extra sodium chloride (51 mM) was added to a fructose-limited chemostat culture run at $D = 0.20$ h$^{-1}$ in the presence of 10 mM bicarbonate and the parameters $C_{113k}$, $C_{120k}$, $S_{113k}$, and $S_{120k}$ of a steady-state culture were calculated. Under these conditions, the percentage of $S_{113k}$ plus $S_{120k}$ to $P_{LT}$ increased from 2 to 77% (Fig. 4).

**Relation between specific growth rate and leukotoxin production**

To examine the effect of the specific growth rate on leukotoxin production, the anaerobic chemostat culture was run at various $D$ values between 0.04 and 0.20 h$^{-1}$ in the absence and presence of 10 mM bicarbonate. In each steady-state culture, the specific rate of leukotoxin production [$q_{LT}$, in µg leukotoxin (mg dry wt)$^{-1}$ h$^{-1}$] was calculated from the following equation:

$$ q_{LT} = P_{LT} D $$

When $q_{LT}$ was plotted against the corresponding $D$, the plots were approximately linear (Fig. 5). This indicated that $q_{LT}$ increased with increasing specific growth rate. Assuming that the leukotoxin production is partly growth-linked and partly independent of growth rate, the relationship between $q_{LT}$ and $D$ is expressed as follows (Pirt, 1975):

$$ q_{LT} = Y_{LT} D + m_{LT} $$

where $Y_{LT}$ is the theoretical maximum leukotoxin yield referred to biomass formed [µg leukotoxin (mg dry wt)$^{-1}$] and $m_{LT}$ is termed the maintenance rate of leukotoxin production [µg leukotoxin (mg dry wt)$^{-1}$ h$^{-1}$]. The plots of $q_{LT}$ against $D$ in Fig. 5 can be approximated to the following equations:

- For the cultures without bicarbonate,
  $$ q_{LT} = 2.7D - 0.058 \quad (r = 0.815) $$

  and for those with 10 mM bicarbonate,
  $$ q_{LT} = 9.3D - 0.407 \quad (r = 0.923) $$

These equations contain minus values of $m_{LT}$ and therefore it can be expected that apparent leukotoxin production is a result of both growth-rate-dependent production and growth-rate-independent decomposition. Assuming the occurrence of the toxin decomposition, the 'critical' growth rate where the production rate is equal to the decomposition rate, i.e. the extrapolated intercept on the abscissa, is calculated to be 0.021 and 0.044 h$^{-1}$ in the absence and the presence of bicarbonate, respectively. The $Y_{LT}$ value was estimated to be 2.7 and 9.3 µg leukotoxin (mg dry wt)$^{-1}$ without and with bicarbonate, respectively. This parameter implies the efficiency of leukotoxin synthesis in the growing cells and thus it can be concluded that bicarbonate stimulates leukotoxin synthesis up to about threefold. Previously, we estimated several growth parameters of anaerobic fructose-limited chemostat cultures with and without bicarbonate and

larger amounts of leukotoxin were released extracellularly into the culture fluid with higher bicarbonate concentrations. Considering the fact that the leukotoxin (pI approximately 8.8) binds ionically to nucleic acids on the bacterial cell surface (Ohta et al., 1993), we assumed that
showed that the addition of bicarbonate (48 mM) resulted in a 1.1-fold increase in the efficiency of ATP generation in fructose fermentation and a 1.3-fold increase of the production by bicarbonate is not simply a result of increased efficiency of total biosynthesis.

With respect to the bicarbonate effect, it was reported that the production of virulence factors by *Streptococcus pyogenes* and *Bacillus anthracis* was stimulated at the transcriptional level by bicarbonate, which was dependent on a trans-acting regulator (Bartkus & Leppia, 1989; Perez-Casal et al., 1991; Caparon et al., 1992; Uchida et al., 1993; Koehler et al., 1994; Sirard et al., 1994). Such transcriptional regulation may be expected for the leukotoxin production by *A. actinomyctetemcomitans*.

**Decomposition of leukotoxin in batch culture and resting cell suspension**

We examined the effect of a culture change from chemostat to batch on the production of leukotoxin. After a fructose-limited chemostat culture run at *D* = 0.10 h⁻¹ in the presence of 10 mM bicarbonate reached a steady state, the batch culture was begun by removing about 80% of the culture liquid from the fermenter and by immediately replacing it with the inflowing medium. The regulation of temperature, pH and stirring speed was the same as in the chemostat. Fig. 6 shows changes in cell density and the relative amount of cellular leukotoxin in the nuclease digest. If leukotoxin production stops immediately after the growth mode change, the cellular content of leukotoxin decreases with the increase of cell mass. This theoretical change in the cellular leukotoxin level is shown also in Fig. 6 (dashed line). The actual decreasing rate of leukotoxin level (−0.40 h⁻¹) was twice as high as the theoretical value (−0.20 h⁻¹), suggesting that the toxin is decomposed in batch culture. During the batch culture, the leukotoxin was not detected in the culture supernatant (data not shown). The toxin decomposition was also observed when the purified toxin was incubated anaerobically in a resting cell suspension. As shown in Fig. 7, the extracellular fraction of the suspension sampled after 10 h incubation contained a no longer detectable amount of toxin, although the amount of toxin in the control without cells did not change significantly during 16.5 h incubation. In the 10 h incubation sample a partial cell lysis was detected by SDS-PAGE (data not shown) and therefore the leukotoxin might be decomposed by cellular protease.

**N-terminal sequences of the *M*, 113000 and 120000 polypeptides**

To investigate the biochemical relationship between the two polypeptides, N-terminal amino acid sequences of 26 and 25 residues were determined for the *M*, 113000 and 120000 polypeptides, respectively. The results are listed in Table 2 along with the corresponding sequence deduced from the DNA sequence of the *ltxA* gene of *A. actinomyctetemcomitans* JP2 (Kraig et al., 1990). The N-terminal 10 amino acid sequence of the *M*, 113000 polypeptide was identical to the sequence between amino acid residues 16 and 25 of the *M*, 120000 polypeptide. This result suggests that the cleavage of the first N-terminal 15 residues from the *M*, 120000 polypeptide occurs and thus yields the *M*, 113000 polypeptide. However, the calculated *M*, 1617 of the 15 cleaved amino acid residues does not appear to compensate the difference between the apparent values of both the...
polypeptides. Other unknown processing of the \( M_r \) 120000 polypeptide might occur in the formation of the \( M_r \) 113000 polypeptide. One may assume that 301-b cells contain multi copies of the leukotoxin gene. However, in our Southern blot experiments (T. Tada & A. Miyagi, unpublished), a single hybridization signal was detected for a \( S_{al}I \) digest of 301-b DNA with a 1.6 kb probe encoding the N-terminal half of leukotoxin. When the N-terminal 25 amino acid sequence of the \( M_r \) 120000 polypeptide was compared with that of the predicted LtxA polypeptide of strain JP2, both the polypeptides were almost identical to each other except for the N-terminal methionine residue and a proline residue at amino acid position 7 in the LtxA polypeptide.

With respect to the production of \( M_r \) 120000 polypeptide, two characteristics can be noted from the present chemostat experiments. First, the relative amount of \( M_r \) 120000 polypeptide increased with higher specific growth rates. For example, this is seen by comparing the amount of the polypeptide at 0 volume change (\( D = 0.04 \) h\(^{-1}\)) with that at 7-5 volume changes (0.10 h\(^{-1}\)) in the first experiment (Fig. 1). Second, the relative amount of the polypeptide increased in response to the increase of bicarbonate concentration in the culture (Fig. 4). In the culture at \( D = 0.20 \) h\(^{-1}\), the percentage of \( C_{120k}^{120k} \) plus \( S_{120k} \) to \( P_{LT} \) was 21 % in the absence of bicarbonate, but increased to 52 % with the addition of 40 mM bicarbonate. These observations indicate that the possible processing of the \( M_r \) 120000 polypeptide is also dependent on environmental conditions.

In conclusion, we have described several characteristics of the leukotoxin production by \( A. \) actinomycetemcomitans 301-b. Our results show that the production is dependent on the growth rate and stimulated by bicarbonate, and also suggest the toxin decomposition in growing organisms and the cessation of toxin production by the change from chemostat to batch culture. These findings will contribute to a better understanding of the in vivo pathogenesis of \( A. \) actinomycetemcomitans. Genetic analysis of the regulatory effects of growth rate and bicarbonate remain to be further explored.

**ACKNOWLEDGEMENTS**

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**Table 2. N-terminal sequences of the A. actinomycetemcomitans leukotoxin**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Protein ( M_r )</th>
<th>N-terminal sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>JP2*</td>
<td>LktA 120000</td>
<td>MATTLPLNTKQQAAQFAANSVADRKENIDAAKEQLQKALDKL</td>
</tr>
<tr>
<td>301-b</td>
<td>( M_r ) 120000</td>
<td>ATTLLLNNTKQQAAQFAANSVADRKE ( \rightarrow ) unsequenced</td>
</tr>
<tr>
<td>301-b</td>
<td>( M_r ) 113000</td>
<td>ANSVADRKENIDAAKEQLQKALDKL</td>
</tr>
</tbody>
</table>

* Data from Kraig et al. (1990).


