Microbial Pathogenesis 53 (2012) 49–55

Contents lists available at SciVerse ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Endogenous CO_2 may inhibit bacterial growth and induce virulence gene expression in enteropathogenic *Escherichia coli*

Haydee Martínez [a], Thomas Buhse [b], Marco Rivera [a], P. Parmananda [c], Guadalupe Ayala [d], Joaquín Sánchez [e,*]

[a] Facultad de Ciencias, UAEM, Cuernavaca, Morelos, Mexico
[b] Centro de Investigaciones Químicas, UAEM, Cuernavaca, Morelos, Mexico
[c] Department of Physics, IIT, Powai 400076, Mumbai, India
[d] Centro de Investigación sobre Enfermedades Infecciosas, INSP, Cuernavaca, Morelos, Mexico
[e] Facultad de Medicina, Universidad Autónoma del Estado de Morelos UAEM, Av. Universidad 1001, 62209 Cuernavaca, Morelos, Mexico

ARTICLE INFO

Article history:
Received 18 January 2012
Received in revised form
2 April 2012
Accepted 9 April 2012
Available online 20 April 2012

Keywords:
Bacterial growth kinetics
Bacterial growth auto-inhibition
Carbon dioxide inhibited growth
Culture surface-to-volume ratio
Volatile bacterial growth inhibitor
Carbon dioxide-induced virulence

ABSTRACT

Analysis of the growth kinetics of enteropathogenic *Escherichia coli* (EPEC) revealed that growth was directly proportional to the ratio between the exposed surface area and the liquid culture volume (SA/V). It was hypothesized that this bacterial behavior was caused by the accumulation of an endogenous volatile growth inhibitor metabolite whose escape from the medium directly depended on the SA/V. The results of this work support the theory that an inhibitor is produced and indicate that it is CO_2. We also report that concomitant to the accumulation of CO_2, there is secretion of the virulence-related EspB and EspC proteins from EPEC. We therefore postulate that endogenous CO_2 may have an effect on both bacterial growth and virulence.

1. Introduction

In liquid cultures of *Escherichia coli* and other enteric bacteria, there is an exponential phase wherein bacterial numbers typically double every 20–30 min when incubated at 37 °C. This phase lasts for only a few generations and is usually followed by a stationary phase during which bacterial numbers remain mostly stable. The end of the exponential phase may be induced by nutrient depletion and/or waste-product accumulation [1,2]. However, under otherwise standard conditions, we detected variations in the growth kinetics of enteropathogenic *E. coli* (EPEC). Specifically, we observed growth after the exponential phase (i.e., growth during transition to the stationary phase) [3]. We showed that the rate of post-exponential growth was directly proportional to the ratio between the exposed surface area and the culture volume (SA/V): the larger the exposed surface area, the greater the growth and vice versa. This effect cannot be simply explained by the rate of oxygen uptake from the surrounding air [3]. A plausible model to explain this bacterial behavior proposes that under low SA/V, there is an accumulation of a volatile inhibitor metabolite that suppresses bacterial growth. Alternatively, under high SA/V, the inhibitor escapes from the medium and allows for growth to continue. We present experiments that support this model and suggest that the volatile inhibitor is CO_2. Although CO_2 can be incorporated into the bacterial cell via its conversion into bicarbonate [4], CO_2 may also inhibit growth [5–7]. In addition, we report that concomitant to the accumulation of CO_2, there was increased secretion of virulence-associated EPEC proteins.

The strain of EPEC studied in this work is a human pathogen that causes diarrhea. Its infectious process begins with the colonization of the small intestine, which is aided by the production of an attachment and effacing lesion (A/E) that is characterized by the loss of enterocyte microvilli. The A/E lesion is linked to the formation of a pedestal or cup-shaped cellular structure produced by a rearrangement of the host cell's cytoskeleton. Induction of the A/E lesion depends on a genetic region known as the locus of enterocyte effacement (LEE). This region encodes proteins that assemble into a complex needle-like structure through which

* Corresponding author. Tel.: +1 52 777 3184797.
E-mail address: joaquin.sanchez@microbio.gu.se (J. Sánchez).

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doi:10.1016/j.micpath.2012.04.002

bacterial effector proteins can be translocated to the cytoplasm of the enterocyte [8–11]. This structure has been generically called the Type III Secretion System (T3SS). The LEE encoding the T3SS comprises five polycistronic operons that are organized in three domains. One domain consists of the operons LEE1, LEE2 and LEE3, which encode a majority of the proteins needed for the assembly of the needle-like structure. Another domain is the LEE5 operon that encodes the translocated intimin receptor (Tir) and proteins involved in the attachment of EPEC to the target cell (intimin) [12–14]. The third domain is LEE4, which encodes EPEC-secreted proteins (Esp proteins) that either form part of the needle-like structure (EspA) or form a pore (EspB and EspD) in the target cell through which effectors are translocated. LEE1 contains the main regulator called the LEE-encoded regulator (Ler), which positively regulates numerous genes within LEE [15] as well as genes outside of LEE, such as *espC*, which is located in the IE5 pathogenicity island [15–17]. *espC* codes for the serine-protease autotransporter EspC, which cleaves actin-binding fodrin protein in the target cell [18,19]. In this work, we report an increase in the extracellular levels of the EspB and EspC proteins concomitant with the accumulation of endogenous CO_2.

2. Results

2.1. The effect of SA/V on EPEC growth under a helium atmosphere supports the presence of a volatile growth inhibitor

The effect of SA/V on EPEC growth was revealed while continuously analyzing EPEC growth in spectrophotometer cuvettes [3]. A representative result is shown in which EPEC was grown under a helium atmosphere at two different SA/V ratios (Fig. 1A). The use of a helium atmosphere allowed us to discount the potential enhancement of bacterial growth by increased oxygen uptake due to the larger exposed surface. Similar to the cultures in spectrophotometer cuvettes, we also observed a direct effect of SA/V on bacterial growth when the cultures were maintained in static Erlenmeyer flasks (Fig. 1B) under a helium atmosphere. These results support the hypothesis that an inhibitor was lost from the culture medium. To further test our hypothesis, cultures were grown in orbitally shaken flasks because shaking the cultures should enhance growth more than increases in SA/V alone due to the improved volatilization of the inhibitor resulting from mechanical agitation. We found that orbitally shaken cultures grew better (Fig. 1C) than unshaken cultures (Fig. 1B) in a helium atmosphere. These results supported the presence of a volatile inhibitor because in the absence of air, growth enhancement by shaking could not be attributed to the improved oxygenation of cultures. Identification of the proposed volatile growth inhibitor in the headspace of EPEC cultures has remained elusive because preliminary analysis by gas chromatography and mass spectra (GC–MS) showed weak indistinctive signals, except for an m/z peak indicative of CO_2. Additionally, initial experiments had indicated that the inhibitor was accumulating during growth. We therefore considered the possibility that metabolic CO_2 could be the inhibitor molecule, which led to investigation of the kinetics of dissolved CO_2 during EPEC growth.

2.2. The kinetics of dissolved CO_2 in the culture medium indicates that CO_2 could be the postulated growth inhibitor

Follow-up of the kinetics of dissolved CO_2 in EPEC cultures under different SA/V ratios in static flasks demonstrated the accumulation of CO_2 followed by a decrease in CO_2 that was directly affected by the SA/V (Fig. 2). In an EPEC culture with a SA/V of 0.47 cm^{-1}, the CO_2 remained essentially constant after 4.5 h (Fig. 2C), and growth was inhibited in comparison to the culture with a SA/V of 2.5 cm^{-1}, in which CO_2 did not remain constant but decreased progressively (Fig. 2A). The response was intermediate with a SA/V of 1.0 cm^{-1} (Fig. 2B). The association between the retention of accumulated CO_2 and the inhibition of bacterial growth, as well as the opposing effect of continued growth when accumulated CO_2 decreased in the medium, supported the hypothesis that CO_2 could be the postulated volatile growth inhibitor.

2.3. Demonstration of inhibition of EPEC growth by exogenous CO_2

To determine if CO_2 could inhibit EPEC growth under our culturing conditions, the amount of dissolved CO_2 was artificially increased in the medium by replacing air with a CO_2 atmosphere.

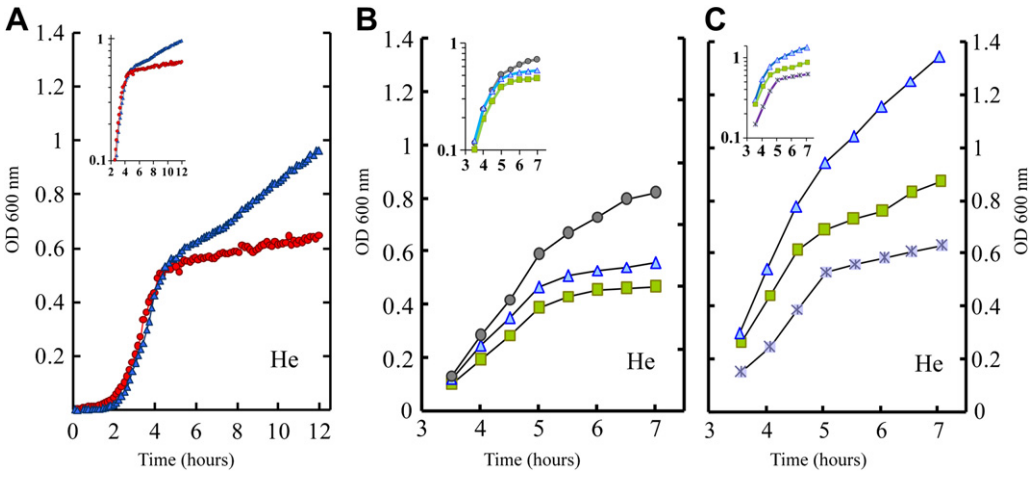

Fig. 1. The direct relationship between the SA/V and EPEC growth under a helium atmosphere in spectrophotometer cuvettes and in static or shaken flasks. In all panels, the *y*-axis shows the OD_{600}, and the *x*-axis shows time in hours. Insets show growth curves in semi-log style with *y*-axis values expressed as $\log_{10}$. (A) Cultures in spectrophotometer cuvettes under helium at SA/V = 1 cm^{-1} (blue triangles) or SA/V = 0.5 cm^{-1} (red circles). (B) Growth in static flasks under helium and at different SA/V ratios. Curves correspond to SA/V = 2.5 cm^{-1} (gray circles), SA/V = 1.0 cm^{-1} (blue triangles) and SA/V = 0.48 cm^{-1} (green squares). (C) Growth under helium and in orbitally shaken flasks at different SA/V ratios. Curves correspond to SA/V = 1.0 cm^{-1} (blue triangles), SA/V = 0.48 cm^{-1} (green squares) and SA/V = 0.14 cm^{-1} (asterisks). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

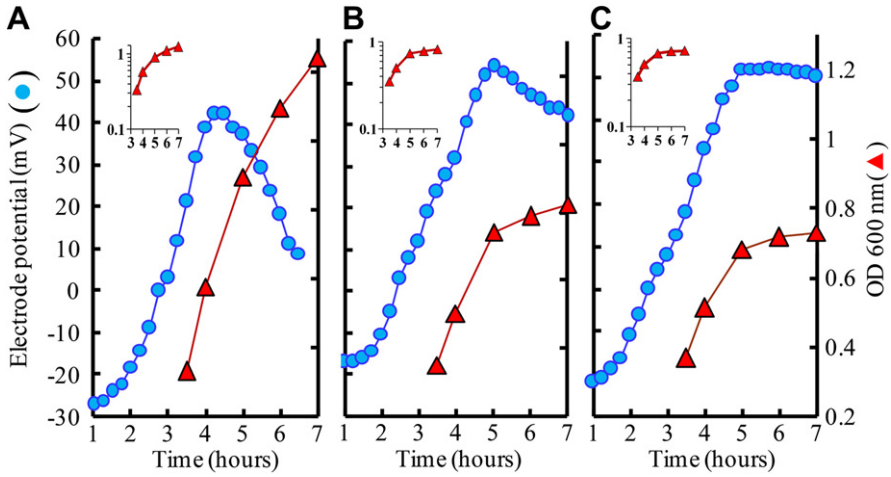

Fig. 2. The kinetics of dissolved CO_2 in EPEC cultures under different SA/V ratios in static flasks. The blue line with circle markers shows CO_2 concentration values as electrode potential readings in mV (*y*-axis on left). Insets show growth curves in semi-log units, with *y*-axis values expressed as $\log_{10}$. The red line with triangle markers shows bacterial density as estimated by optical density at 600 nm (*y*-axis on the right) measured in simultaneous cultures under the same growth conditions. (A) SA/V of 2.5 cm^{-1} (B) SA/V of 1.0 cm^{-1} (C) SA/V of 0.48 cm^{-1}. These cultures were maintained under an atmosphere containing air. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Atmosphere replacement was accomplished in two ways: the cultures were either grown under a CO_2 atmosphere initially or the CO_2 atmosphere was introduced into a culture already in progress. Under the CO_2 atmosphere, growth was decreased in comparison to the control (Fig. 3A). Likewise, the introduction of the CO_2 atmosphere in cultures that had been growing for 4.5 h under air had an inhibitory effect on growth that was detectable 1 h after atmosphere replacement (Fig. 3B). Therefore, the experiments show that exogenous CO_2 was able to inhibit EPEC growth in our culturing system.

2.4. Retention of endogenous CO_2 inhibited EPEC growth

To obtain direct evidence of growth inhibition by endogenous CO_2, we conceived an experiment that involved a sudden shift from a high to a low SA/V in an ongoing culture. If our hypothesis was correct, such a sudden decrease in the SA/V would produce retention of CO_2 and cause inhibition of EPEC growth. As a control for this experiment, the reverse shift was performed, which was expected to promote the escape of CO_2 and to stimulate growth. To perform these experiments, 10 ml cultures were grown in 125 ml Erlenmeyer flasks fitted with a side tube (nephelometric flasks); the SA/V ratio in the tube was 0.1 cm^{-1}, and the SA/V ratio in the flask was 2.5 cm^{-1}. Growth was then initiated either in the flask or in the side tube. After undisturbed growth for 4.5 h, cultures were poured from the flask into the side tube or from the side tube into the flask. In the first case, the SA/V was suddenly decreased from 2.5 cm^{-1} to 0.1 cm^{-1}, whereas in the second case, the SA/V suddenly increased from 0.1 cm^{-1}–2.5 cm^{-1}. Fig. 4 shows that the decrease in SA/V caused the retention of CO_2 and the inhibition of growth (Fig. 4B).

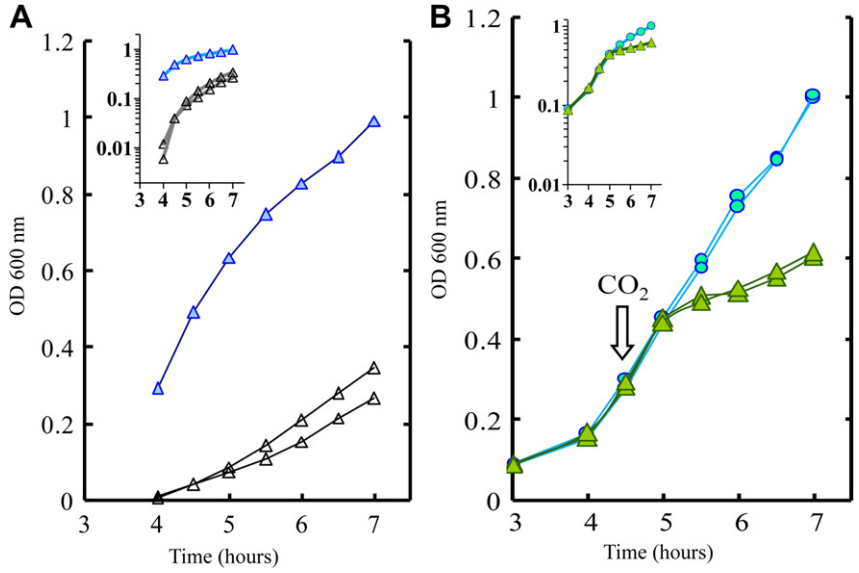

Fig. 3. The inhibition of EPEC growth by exogenous CO_2. In both panels, the *y*-axis shows OD_{600}, and the *x*-axis shows time in hours. (A) Growth of EPEC under a CO_2 atmosphere. The lines with empty triangle markers represent two independent cultures grown under CO_2, while the line with blue triangle markers represents the control culture under a regular air atmosphere. (B) Growth of EPEC under an aerial atmosphere, followed by replacement of air with a CO_2 atmosphere (green triangles) or without atmosphere substitution (blue-green circles). The vertical arrow indicates the time of atmosphere replacement (4.5 h). In both (A) and (B), the insets show growth curves in semi-log style, with the *y*-axis values expressed in logarithmic form. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

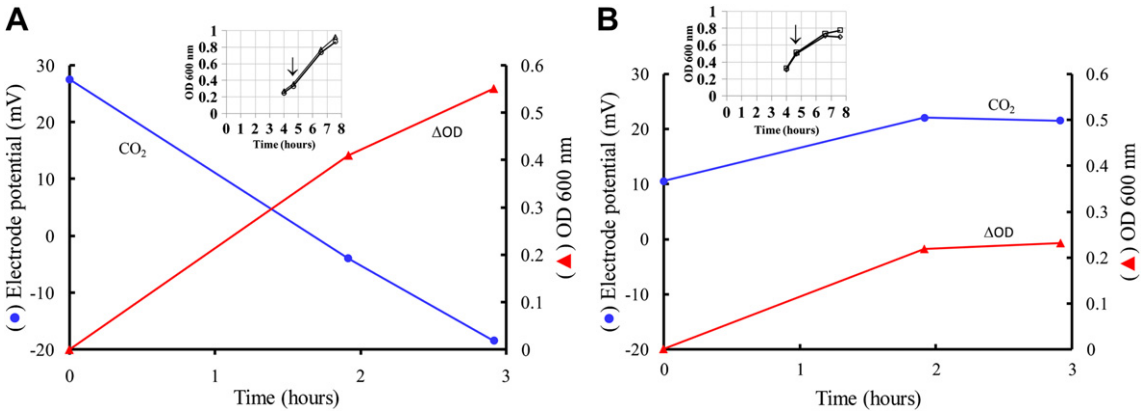

Fig. 4. The effect of shifts in the SA/V on EPEC growth and on endogenous CO_2 levels. (A) The effect of a shift from a low SA/V (0.1 cm^{-1}) to a high SA/V (2.5 cm^{-1}), which is reflected in the continuation of growth and a descent in CO_2. Growth change is shown as ΔOD according to the right *y*-axis (OD 600 nm), while absolute CO_2 levels are shown as the electrode potential in mV (left *y*-axis). (B) The effect of the shift from a high SA/V (2.5 cm^{-1}) to a low SA/V (0.1 cm^{-1}). In this instance, growth was dampened (ΔOD), and CO_2 levels did not decrease. The results are the average of two independent determinations in each case. For appreciation of the original OD_{600} values, corresponding insets are included in each panel with the point at which the SA/V was shifted indicated with an arrow.

The increase in SA/V promoted the escape of CO_2 and stimulated growth (Fig. 4A). These results were consistent with the proposal that endogenous CO_2 inhibits EPEC growth. The SA/V shifts shown in Fig. 4 were performed in air, but according to the results shown in Fig. 1, substitution of the aerial atmosphere should not change the general outcome of the experiment. However, for additional confirmation, the experiments shown in Fig. 4 were repeated under a helium atmosphere without performing CO_2 measurements to avoid atmosphere disruption. Under helium, the shift from low to high SA/V led to continued growth with an increase in optical density at 600 nm (OD_{600}) from 0.362 to 0.694, while the opposite shift from a high to a low SA/V led to a reduction in the growth rate with a change in OD_{600} from 0.446 to 0.550. These results were similar to those shown in Fig. 4, except that the final OD_{600} was approximately 35% lower under helium than in air.

To examine the proposal that CO_2 could escape the medium and that this escape is proportional to the SA/V of cultures, uninoculated sterile medium was pre-loaded with CO_2 by bubbling to allow for changes in CO_2 levels to be monitored over time under different SA/V ratios at 37 °C. Experiments showed the spontaneous escape of CO_2 and a direct correlation between the CO_2 escape rate and the SA/V

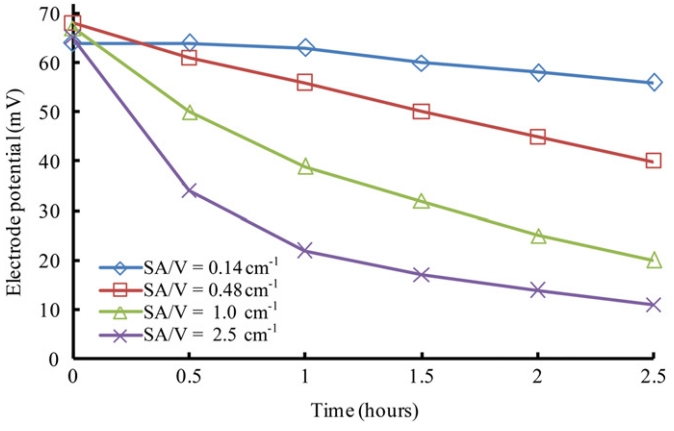

Fig. 5. Spontaneous escape of CO_2 from the culture medium directly correlates with the SA/V. Uninoculated culture medium was bubbled with CO_2 in flasks at SA/V ratios of 0.14 cm^{-1}, 0.48 cm^{-1}, 1.0 cm^{-1} and 2.5 cm^{-1}, as indicated in the graph. Then, spontaneous changes in CO_2 levels were measured at various time points at 37 °C. As seen, CO_2 escape directly correlated with the SA/V, with maximal loss at a SA/V of 2.5 cm^{-1} and highest retention of CO_2 at a SA/V of 0.14 cm^{-1}.

(Fig. 5). These results demonstrated that dissolved CO_2 had the capacity to leave the medium and that this escape was directly affected by the SA/V. Thus, the decrease in CO_2 after reaching a maximum concentration (Fig. 2A) could be explained by its spontaneous escape. However, part of the endogenous CO_2 could be re-incorporated by the bacterial cell for anabolism via its conversion into bicarbonate. To obtain evidence of possible intracellular conversion of CO_2 into bicarbonate, the effect of a carbonic anhydrase inhibitor on CO_2 levels was tested. We found that an EPEC culture, under the conditions described for Fig. 2A but in the presence of 10 mM acetazolamide, reached an OD_{600} of 0.74 and a CO_2 reading of +24 mV after 8 h of growth, while the control culture reached an OD_{600} of 1.08 and a CO_2 reading of −7 mV. The higher CO_2 level in the presence of acetazolamide indicated reduced conversion into bicarbonate, while the lower bacterial density could be attributed to inhibition by CO_2. However, it may be necessary to discard a latent effect of decreased availability of bicarbonate in the cell. Regardless, these results indicate that endogenous CO_2 is partly re-incorporated by the bacterial cell via its conversion into bicarbonate.

2.5. Accumulation of CO_2 may induce the secretion of virulence-related proteins

We considered the possibility that the accumulation of CO_2 might influence EPEC virulence, perhaps by affecting the secretion of virulence-related proteins from the T3SS, which has been shown to occur when exogenous CO_2 is present [20,21]. Accordingly, we analyzed TCA-precipitated proteins from culture supernatants at various culture time points. The results show that EPEC protein secretion was responsive to the accumulation of CO_2. Explicitly, we observed that secretion of the EspB and EspC proteins could be detected at around hour 5 (Fig. 6), when the concentration of CO_2 was near its maximum and prior to the post-exponential growth stage (Fig. 2). This finding suggested that endogenous CO_2 could be a signal to trigger the secretion of virulence-related proteins.

3. Discussion

3.1. Potential role of CO_2 as a growth inhibitor

Taken together, our results support the view that the direct relationship between (post-exponential) bacterial growth and the SA/V is due to the self-inhibition of growth. The effect of the SA/V

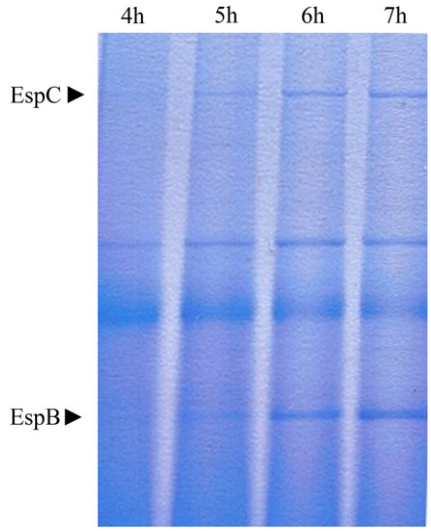

Fig. 6. The kinetics of secreted EPEC proteins. EPEC-secreted proteins as observed in SDS-PAGE after staining with Coomassie blue. Arrowheads indicate the position of proteins EspB and EspC, which were identified by LC–MS. Culturing time is indicated above the lanes. Note the increase in EspB and EspC concentrations near 5 h of growth; this time is similar to the time at which maximum CO_2 concentrations were achieved in the cultures (as seen in Fig. 2).

can be explained by either retention of endogenous CO_2 (under decreasing SA/V), which inhibits growth, or by the escape of CO_2 (under increasing SA/V), which promotes growth. This straightforward mechanism is supported by the following findings: a) the correspondence between the kinetics of CO_2 and the inhibition of bacterial growth (Fig. 2); b) the inhibition of growth by exogenous and endogenous CO_2 (Figs. 3 and 4) and c) the demonstration of SA/V-dependent escape of CO_2 from the medium (Fig. 5).

Theoretically, metabolites other than CO_2 might also inhibit growth. However, as previously mentioned, our initial analysis by GC–MS revealed no significant signals for other volatile metabolites. Nonetheless, it is possible that the accumulation of other excreted metabolites, such as fermentation acids and/or ethanol [22–24], may play a role in the self-inhibition of EPEC growth. Experiments are underway to determine the potential involvement of other metabolites, either in conjunction with CO_2 or independent of it.

The experiments reported here were performed in a rich growth medium. However, we found that the growth of EPEC in M9 minimal medium with 0.4% glucose gave an analogous response to changes in the SA/V (data not shown). This result indicates that the culture medium is not critical for the self-inhibition of growth or, by extension, for the proposed inhibitory role of endogenous CO_2. Likewise, the employed *E. coli* organism was not essential, as analogous behavior was found in *E. coli* JM83, *E. coli* HB101, *Salmonella enterica* and *Vibrio cholerae* (unpublished data). We consequently suggest that CO_2 accumulates in bacterial cultures depending on the SA/V and that the common laboratory practice of shaking cultures improves growth largely because it reduces the self-inhibition of growth by endogenous CO_2. Lastly, the release of CO_2 would be expected to be more efficient when bacteria are growing on a solid surface. Thus, we hypothesize that the free escape of CO_2 may be a reason why EPEC and other enteric bacteria are able to reach high cell densities on agar plates.

Concerning the potential inhibition mechanism, externally supplied CO_2 has previously been shown to be capable of inhibiting the growth of many microbial organisms, including *E. coli* [7]. However, studies on the effect of CO_2 on *E. coli* are infrequent and often difficult to compare, as cultures are frequently carried out under conditions designed to investigate how to avoid microbial growth in food or how to enhance yields in fermentations. Nonetheless, in a study performed under typical growth conditions and aimed at investigating transport across membranes, 40% inhibition of *E. coli* growth was produced with an external 5% CO_2 atmosphere [6]. How exogenous or endogenous CO_2 may inhibit growth is not immediately obvious, but it is likely that its effect is indirect. For example, the effect of CO_2 likely does not entail physical disturbance of the bacterial membrane [6], even though the deletion of *msbB*, a gene that encodes an enzyme that myristoylates lipid A, confers acute sensitivity to CO_2 in *Salmonella* [25]. The effect of CO_2 may instead involve lowering the intracellular pH, which could negatively affect cell metabolism in multiple ways [5]. CO_2 may also cause reductions in the concentration of the essential carbonic anhydrase [26], or it may exacerbate reactive oxygen species toxicity by increasing oxidative cellular lesions [27]. An effect associated with pH would be in agreement with the SA/V-linked differences in the external pH after 5 h of EPEC growth, as we registered a pH of 6.01 for a culture with a SA/V of 2.5 cm^{-1} and a pH of 5.83 for a culture with a SA/V of 0.48 cm^{-1}. Experiments are in progress to determine whether this reduction in the extracellular pH is linked to the proposed growth inhibition by CO_2. The characterization of an inhibition mechanism in EPEC and other bacteria could lead to a better understanding of analogous processes in other unicellular organisms, given that endogenous CO_2 has also been proposed to inhibit growth in the protozoan *Tetrahymena pyriformis* [28].

3.2. Potential role of CO_2 as inducer of virulence in EPEC

It has been suggested that a 5% CO_2 atmosphere may affect the regulation of the T3SS [20,21], although others have reported no effect of CO_2 on the T3SS [29]. Under our culturing conditions, we investigated whether, in addition to its effect on EPEC growth, the endogenous CO_2 affected the expression of virulence-related proteins. The analysis of secreted protein profiles (Fig. 6) showed increases in EspB and EspC in the culture medium when the level of CO_2 in cultures was near its maximum. We hypothesize that this relationship indicates an induction of virulence expression by endogenous CO_2, as has been postulated for exogenous CO_2 [20]. The explanation for this phenomenon is not immediately clear, but it is possible that the diffusion of CO_2 back into the cell followed by its conversion into bicarbonate might increase intracellular bicarbonate, which may induce the expression and/or secretion of EspB and EspC. The conversion of endogenous CO_2 into bicarbonate under our growth conditions is supported by our experiments with acetazolamide (Section 2.3). A role for endogenous bicarbonate is suggested because exogenously added bicarbonate has been shown to induce virulence expression in other T3SS-holding organisms. For example, in enterohemorrhagic *E. coli* (EHEC), exogenously added bicarbonate induces the T3SS [30], although similar induction was not found in EPEC [29]. Analogously, in *Citrobacter rodentium*, exogenous bicarbonate activates the RegA protein [31], RegA activates *ler* expression, and the Ler protein induces T3SS expression [32]. The T3SS and the presence of the Ler regulator in *C. rodentium*, EHEC and EPEC are all similar. In EPEC, Ler stimulates expression of the genes encoding EspB and EspC. However, no RegA homologue has been identified in EPEC. Therefore, if the hypothesized stimulation by endogenously generated bicarbonate occurs in EPEC, it involves a different protein.

4. Materials and methods

4.1. Strain, growth conditions and CO_2 measurements

The EPEC E2348/69 serotype O127:H6 [33], kindly provided by Dr. José Luis Puente at Instituto de Biotecnología, UNAM, México

was used in this study. For culturing, we used a glucose-free medium containing 1.5% (w/v) Bacto-Peptone (DIFCO), 0.4% yeast extract (DIFCO) and 0.5% NaCl [34,35]. The SA/V was defined as the ratio of the exposed surface area to the culture volume, with units of cm^{-1}. The liquid surface area was calculated using direct measurements (i.e., internal diameter), in reference flasks of the various capacities. Exposed surface areas in cultures with the same amount of medium were assumed to be similar when flasks were of the same capacity. Such an assumption agreed with an approximate maximum variability in liquid height of ±5%. Bacterial cultures were started with a bacterial suspension in saline solution as previously described [35]. The EPEC strain was grown either in spectrophotometer cuvettes of 3.5 ml capacity, in Erlenmeyer flasks under different SA/V at 37 °C or, for protein secretion, in 6-well tissue culture plates containing 2 ml of medium with a calculated SA/V of 5 cm^{-1}. Growth under a helium atmosphere in spectrophotometer cuvettes and flasks was achieved by replacing the air in the headspace with helium grade 4.5 (99.995%, Praxair, Mexico). Growth under pure CO_2 (USP grade, UN1013, Infra, Mexico) was carried out in flasks. When the atmosphere was replaced, spectrophotometer cuvettes and flasks were fitted with hermetic seals. When the atmosphere was not replaced, the flasks were fitted with a cotton plug to allow for gas exchange with the surrounding environment. In some experiments, Erlenmeyer flasks fitted with a side tube (nephelometric flasks) were used. When growth was carried out in spectrophotometer cuvettes, the cuvettes were kept stationary, but the culture medium was gently mixed by a rotating magnet. The flasks were either kept static or shaken orbitally at 150 rpm. To facilitate appreciation of post-exponential growth, we plotted the data using linear *x*- and *y*-axes. However, semi-log plots have been included in graphs to give an approximate idea of the time at which exponential growth ended. Changes in dissolved CO_2 in air-exposed cultures in static flasks were measured with a gas-sensitive submersible electrode (Instrulab, S.A., Mexico). The electrode response to the reference bicarbonate-CO_2 solution in the range of 0.1 mM–10 mM indicated that an electrode potential of +50 mV was equivalent to 2.5 mM dissolved CO_2, while an electrode potential of +1 mV corresponded to 0.25 mM dissolved CO_2.

4.2. Protein secretion

EPEC protein secretion was monitored by SDS-PAGE followed by Coomassie blue staining. Terminal cultures were withdrawn from incubation every hour from 4 to 7 h. Bacteria were pelleted by centrifugation, and supernatants were subjected to precipitation with 10% trichloroacetic acid (TCA). TCA precipitates were resuspended in 1 M Tris pH 8. The resuspension volumes were adjusted according to the optical density (600 nm) of the cultures to compensate for differences in bacterial density. For protein identification, stained protein bands were excised from gels and sent for analysis by Liquid Chromatography–Mass Spectrometry (LC–MS) to the Proteomics Unit at the Instituto de Biotecnología, UNAM Campus Morelos, México.

Acknowledgments

The first author (HM) gratefully acknowledges a current CONACYT scholarship for PhD studies. This work was supported by CONACYT projects CB-2007/79924 and CB-2010-01/156257.

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