Medium alkalization due to carbon metabolism is largely responsible for inhibition of bacterial growth by *Vibrio cholerae* supernatants

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Abstract

Vibrio cholerae is the causative agent of the diarrheal disease cholera. Many *Vibrio* species secrete antimicrobial factors, though the identity of such a factor has not been determined for any *V. cholerae* strain. Such an antimicrobial factor could be relevant to pathogenesis of cholera, which disrupts the intestinal microbiome.

In this study, we investigated the antimicrobial effects of supernatant from 72 hour old cultures of *V. cholerae* C6706 on *Shigella flexneri* CFS100. Inhibition of *S. flexneri* growth was found to be dependent on the alkaline pH of the supernatant. A 1:1 mixture of pH-adjusted supernatant and LB was found to inhibit *S. flexneri* growth at alkaline but not neutral pH, as was pH-adjusted LB alone. In minimal medium, elevation of supernatant pH by *V. cholerae* was dependent on nutritional factors, and this elevation of medium pH also correlated with increased *S. flexneri* growth inhibition. Though medium alkalization in LB is often attributed to amino acid catabolism and the consequent production of ammonia, supplementation of *V. cholerae* cultures in minimal medium with amino acids had a weaker effect on alkalization and inhibition than did supplementation with selected carbon sources. This suggests that some feature of carbon metabolism causes medium alkalization and the resultant antimicrobial activity. Several *V. cholerae* mutants in potentially relevant pathways were screened for alkalization and *S. flexneri* growth inhibition, but none had any effect.

Complicating this picture is the finding that *V. cholerae* grown under microaerobic conditions produce a less alkaline supernatant with stronger *S. flexneri* growth inhibition. The significance of this is unknown.

Introduction

The gram-negative γ-proteobacterium *Vibrio cholerae* causes cholera, a disease characterized by production of a voluminous watery diarrhea. This diarrhea is a dense culture of the bacterium, with approximately 88% of the bacteria in it being *V. cholerae* (Gorbach *et al*., 1970). Analysis of the fecal microbiomes of people recovering from cholera suggests that the disease affects microbiota composition and reduces the overall number of residents (Monira *et al*., 2013; Hsiao *et al*., 2014). This displacement of the native flora could be explained by (1) the rate of fluid movement through the gut and the concomitant alteration of nutrient and oxygen concentrations, (2) the action of antimicrobial compounds, and/or (3) the antimicrobial effect of alkaline pH.

V. cholerae possesses some antimicrobial activity. Differential specificity of this activity against enteric bacteria was used to type *Vibrio* strains in the pre-genetics era, when it was typically attributed to the production of vibriocins (small peptide antibiotics produced by vibrios, in analogy to the generic bacteriocin) (Chakrabarty *et al*., 1970). While vibriocins from other *Vibrio* species have been characterized, no antimicrobials of any kind have been identified in *V. cholerae* (Balakrishnan *et al*., 2014; Jayawardene *et al*., 1970). Members of the family *Vibrionaceae* produce a wide variety of bioactive molecules, including homoserine lactones used for quorum sensing, siderophores, diverse antimicrobials, and tetrodotoxin (Mansson *et al*., 2011). It is thus not implausible that *V. cholerae* C6706, a pathogenic strain of the El Tor biotype, could produce one or more antimicrobial molecules.

V. cholerae elevates the pH of its medium at late stationary phase, and stool from people with cholera is usually alkaline (Almagro-Moreno *et al.,* 2015; Khan *et al*., 1988). This could explain antimicrobial activity in the absence of a specifically antimicrobial natural product, as disruption of pH homeostasis inhibits bacterial growth (Padan *et al.*, 2005). Notably, the small intestine (the

primary site of cholera infection) is acidic at the junction with the stomach and mildly alkaline at and below the hepatopancreatic duct, which secretes a bicarbonate-rich fluid (Evans *et al*., 1988; Millet *et al.*, 2014; Banwell *et al.*, 1970; Steward *et al*., 2005). Bicarbonate and alkaline pH both play a role in regulation of virulence, with (endogenous and exogenous) bicarbonate inducing virulence gene expression and alkaline pH repressing virulence under nutrient starvation (Abuaita & Withey, 2009; Almagro-Moreno *et al.,* 2015). This is a mechanism by which *V. cholerae*-induced medium alkalization could induce the production of an antimicrobial molecule, which would make it difficult to disentangle the antimicrobial effects of pH and such a molecule.

In this study, we show that the antimicrobial activity of supernatant from *V. cholerae* C6706 cultures against *S. flexneri* CFS100, an avirulent strain of *Shigella* and a close relative of *E. coli*, is pH dependent. We demonstrate that medium pH elevation can be modulated by altering the nutrient content of minimal medium. In particular, supplementation with non-nitrogenous carbon sources is sufficient to produce pronounced alkalization and *S. flexneri* growth inhibition. This suggests a primary role for carbon metabolism in alkalization, though it does not rule out the possibility that carbon metabolism facilitates other processes that are ultimately responsible for the effect. Somewhat paradoxically, it is also observed that microaerobic growth produces *V. cholerae* supernatant with both less alkaline pH and stronger inhibition than aerobic growth does.

Materials and Methods

Bacterial strains and media. Bacterial strains used in this study are listed in Table 1. All strains were maintained at -80°C in tryptic soy broth with 20% glycerol. Unless otherwise specified, strains were grown at 37°C in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) or on LB agar (LB, 1.5% agar).

For analysis of nutritional requirements for alkalization and inhibition, strains were grown in T medium (100 mM NaCl, 50 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 20 mM NH_4Cl, 2 mM KH_2PO_4, 1 mM Na_2SO_4, & 100 mM Tris, adjusted to pH 7.3) supplemented 1% with a vitamin stock as prepared in the 100x VA Vitamin Solution of EZ Rich Defined Medium (EZ RDM, https://www.genome.wisc.edu/resources/protocols/ezmedium.htm), a modification of Neidhardt's defined medium ((Simon & Tessman, 1963; Neidhardt *et al*., 1974). Where applicable, amino acids were supplied at 1X concentration jointly as prepared in the 5X Supplement of EZ RDM or singly in the same concentration as in the 5X Supplement. Carbon source was 23.6 or 100 mM pyruvate, 5.8 or 29 mM sucrose, 11.6 mM glucose, 23.4 mM glycerol, or 17.5 mM succinate. Kanamycin was used at 50 μg/mL where applicable.

Cultures were grown microaerobically by the addition of 2 mL light mineral oil to culture tubes, and anaerobically by static incubation in a candle jar with a BD BBL GasPak anaerobic culture system (product reference number 260251).

Thymol blue-based visualization of alkalization. An LB agar plate with 0.0032% thymol blue was inoculated in a single streak with *V. cholerae* C6706 from an overnight culture and incubated statically for 24 hours at 37°C. Images were edited for clarity.

Plate-based visualization of inhibition. An LB agar plate was inoculated in a single streak with *V. cholerae* C6706 from an overnight culture and incubated statically for 48 hours at 37°C. The

resulting lawn was manually removed, and remaining bacteria were killed by exposure to chloroform vapor for 30 minutes. Residual chloroform was allowed to evaporate from the plate for 30 minutes before inoculation with *S. flexneri* CFS100 from an overnight culture in a line perpendicular to the original one. The plate was then incubated statically for 18 hours at 37°C. Images were edited for clarity.

***V. cholerae* supernatant preparation.** 5 mL of medium were inoculated with *V. cholerae* from a plate and incubated at 37°C for 72 hours, shaking at 200 rpm. Cultures were then centrifuged at 6000 x g for 10 minutes at 4°C. Supernatant was filtered through an 0.22 μm PES membrane to sterilize and stored at -20°C. Supernatant pH was measured using a meter accurate to ± 0.005 units or strips accurate to ± 0.25 units (Whatman Indicator Pater pH 4.5 – 10.0, Cat. No. 2614-991). Readings were consistent between the two.

General *S. flexneri* growth inhibition assay. *V. cholerae* supernatant (either diluted with saline or not) or medium at specific pH was mixed 1:1 with a 1:1000 dilution of an *S. flexneri* CFS100 overnight in LB to a total volume of 100 μL in each well of a 96-well plate. Plates were then incubated at 37°C, shaking at 200 rpm. Optical density at 595 nm (OD_{595}) was measured initially and then either at 6 hours or roughly hourly for 16 hours using an Opsys MR platereader. Outer wells were either filled with 100 μL saline or ignored during the analysis, and the layout of supernatant types and dilutions was distributed across the plate to minimize noise due to differential evaporation around edges.

***S. flexneri* pH tolerance determination.** LB was adjusted to various pHs with 10 M NaOH and autoclaved. Supernatant from *V. cholerae* grown in neutral LB was adjusted to various pHs with 1 M NaOH or 1 M HCl, as appropriate, and filter sterilized using an 0.22 μm PES membrane filter. *V. cholerae* supernatant and LB at close pHs were combined 1:1 and their pH re-determined. The

resulting media (either LB or 50% *V. cholerae* supernatant) were then used to perform *S. flexneri* growth inhibition assays as described above, save that media were mixed 20:1 with a 1:100 dilution of an *S. flexneri* CFS100 overnight culture. OD_{595} was measured at the 6 hour timepoint. Four parameter logistic dose-response curves were fit using a nonlinear regression in R.

Lag time-based inhibition assay. Undiluted *V. cholerae* supernatant was used to perform a liquid S. flexneri growth inhibition assay as described above. OD_{595} was measured hourly for roughly 16 hours. A spline-based model was fit for OD_{595} against time using the grofit package in R, from which parameters for lag time length in hours, maximal growth in OD units, maximal growth rate in OD units per hour, and the integral of the curve were extracted (Kahm *et al*., 2010). As lag time length differed most markedly between trials with *V. cholerae* supernatant and saline controls, that parameter was used to compare inhibitory activity of each supernatant.

IC_{50}-based liquid inhibition assay. *V. cholerae* supernatant produced in LB was used to perform *S. flexneri* growth inhibition assays as described above, using supernatant dilutions ranging from 50% to 0% (final concentration). OD_{595} was measured at the 6 hour timepoint. Four parameter logistic dose-response curves were fit using a nonlinear regression in R, and IC_{50} values for each supernatant type were compared.

TABLE 1: Bacterial strains

Strain	Description	Source or reference
S. flexneri CFS100	2457T ΔpVir	C. Fisher
V. cholerae strains		
C6706	Wild type	Lab collection
VC0058	C6706 *VC0058*::kan	Cameron *et al*., 2008
VC0586	C6706 *VC0586*::kan	Cameron *et al*., 2008
VCA0274	C6706 *VCA0274*::kan	Cameron *et al*., 2008
aphA	C6706 *aphA*::kan	Cameron *et al*., 2008
aphB	C6706 *aphB*::kan	Cameron *et al*., 2008
arcA	C6706 *arcA*::kan	Cameron *et al*., 2008
nqrA	C6706 *nqrA*::kan	Cameron *et al*., 2008
speB	C6706 *speB*::kan	Cameron *et al*., 2008
tnaA	C6706 *tnaA*::kan	Cameron *et al*., 2008

Results

Inhibition of *S. flexneri* growth by *V. cholerae* supernatant is pH-dependent.

V. cholerae C6706 produces a factor that inhibits the growth of *S. flexneri* CFS100 (Fig. 1a). Though a plate is shown for demonstration, an assay based on liquid *S. flexneri* and *V. cholerae* cultures is used for quantitative analysis. Supernatant from 72 hour old *V. cholerae* cultures inhibits the growth of *S. flexneri* after 6 hours incubation, but *S. flexneri* supernatant does not (unpublished data, B. Koestler). Initial attempts to isolate an antimicrobial product from V. cholerae supernatant via methanol extraction were unsuccessful, and it was noted that supernatant pH was alkaline (pH 9.0). This alkalization is demonstrated visually in Fig. 1b by the transition of thymol blue from yellow to blue around a streak of *V. cholerae*, which occurs from pH 8.0 to 9.6 (Medalia, 1920). While *S. flexneri* is known to have remarkable tolerance for acidic conditions, with cells able to survive passage through the human stomach, its tolerance for alkaline conditions is less well-described (Lin *et al.*, 1995; Gorden and Small, 1993; Small *et al.*, 1994). To determine

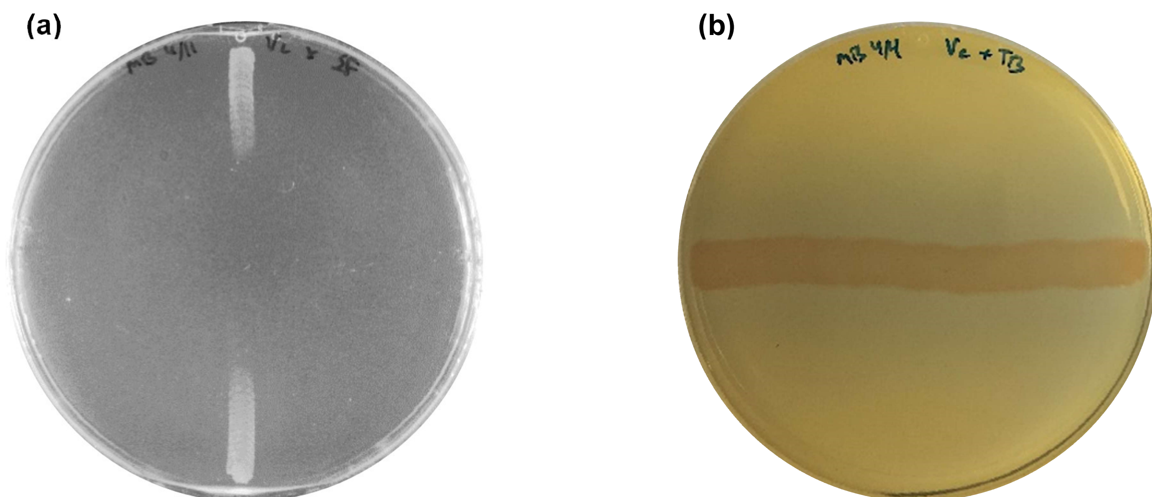


Figure 1. *V. cholerae* secretes a factor that inhibits *S. flexneri* growth and alkalizes its medium. **(a)** *S. flexneri* CFS100 (vertical streak) fails to grow near medium used by *V. cholerae* C6706 (killed, struck horizontally). **(b)** *V. cholerae* C6706 grown 24 hours on LBA with 0.0032% thymol blue alkalizes its medium, shown by transition of the indicator from yellow to blue from pH 8.6 to 9.0.

whether medium alkalization was involved in inhibition of *S. flexneri* growth by *V. cholerae* supernatant, we tested the ability of *S. flexneri* to grow in LB and in 50% *V. cholerae* supernatant adjusted to pHs in the range from 6.7-9.7 (Fig. 2). Both medium types showed pH-dependent inhibition of *S. flexneri* growth. In particular, LB at unaltered pH (6.7) grew *S. flexneri* to the highest observed OD, while 50% *V. cholerae* supernatant at unaltered pH (9.0) completely inhibited *S. flexneri* growth. Conversely, LB at pH 9.0 completely inhibited *S. flexneri* growth, while 50% *V. cholerae* supernatant at pH 6.7 showed the highest *S. flexneri* growth observed in that medium type. This indicates that the alkaline pH of *V. cholerae* supernatants is sufficient to produce at least some of the inhibition observed. However, the supernatant-based medium was generally more inhibitory than LB, with a lower IC_{50} (pH 8.3 for LB, pH 7.8 for supernatant) and less dense growth even at neutral pH.

Failure to reach the same optical density at neutral pH may be due to nutrient depletion of

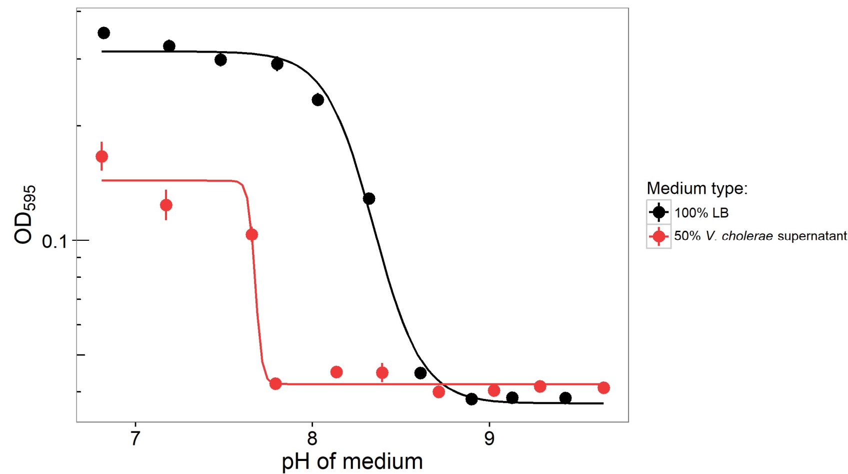


Figure 2. Inhibition of *S. flexneri* growth by *V. cholerae* supernatant is pH-dependent. The ability of *S. flexneri* to grow in either LB (black line) or a 1:1 mixture of LB and *V. cholerae* supernatant (red line) adjusted to pH levels between 6.7 and 9.7 was tested as described in Materials and Methods, and is shown here as the optical density of the *S. flexneri* culture at 6 hours growth. The IC_{50} for growth in LB was 8.34 pH units, and the IC_{50} for growth in 50% *V. cholerae* supernatant was 7.84. Means and standard deviations of at least three *S. flexneri* cultures for each condition are shown.

the supernatant, which does not contain any metals or other nutrients that were held inside cells at time of harvest (72 hours, a timepoint characterized by very little bacterial growth). Nutrient depletion could potentially explain the lower IC_{50} as well, as response to alkaline pH requires energy and resources and is presumably hindered by nutrient deficiency just as every other cellular process is (Padan *et al*., 2005). Due to the difficulty of controlling for differences between undefined media, we cannot say with certainty that the differences in pH-dependent growth inhibition of *S. flexneri* between LB and *V. cholerae* supernatant are due to nutrient depletion or action of an antimicrobial compound.

Choice of carbon source in minimal medium affects alkalization and *S. flexneri* growth inhibition.

Given that *S. flexneri* growth inhibition by *V. cholerae* supernatant can be attributed at least partially to elevated supernatant pH, we may ask how *V. cholerae* elevates the pH of its medium. *V. cholerae* can be grown in T medium, a buffered minimal medium, but supernatants from cultures grown in T medium as originally described with 5.8 mM sucrose as the carbon source are acidic and fail to inhibit *S. flexneri* growth (Figs. 3 & 4b). LB, a rich medium, must contain alkalization-promoting substances that are not present in T. By altering the contents of T medium, we can examine whether alkalization is due to metabolism of any particular nutrient.

To test the effects of carbon source on medium alkalization, *V. cholerae* was grown in T medium with various carbon sources at concentrations normalized to the number of carbon molecules in 5.8 mM sucrose. Growth on sucrose, glucose, and glycerol acidified the medium, while growth on pyruvate and succinate alkalized (Fig. 3). Acidification by the first three is not

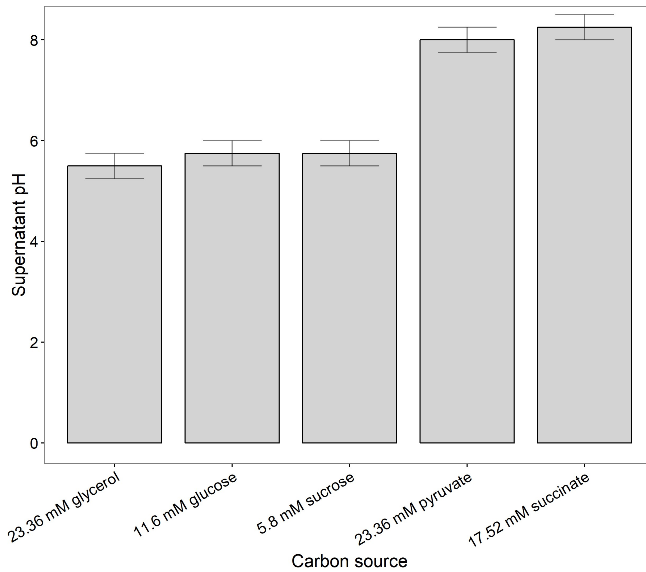


Figure 3. Choice of carbon source affects medium alkalization by *V. cholerae*. The pH of 72-hour old *V. cholerae* cultures grown in T medium supplemented with various carbon sources is reported. Means and measurement error are reported, as measurements for three independent cultures were identical for each. Carbon source concentrations were standardized to the number of carbon atoms per molecule

unexpected, since *V. cholerae* is known known to produce acids when fermenting sugars (Yoon and Mekalanos, 2006). Pyruvate is the final product of glycolysis and feeds into the TCA cycle, and succinate is a TCA cycle intermediate. The TCA cycle does not produce acidic byproducts like fermentation does. This suggests that in the absence of acid production during fermentation, *V. cholerae* elevates the pH of its environment.

To determine whether medium alkalization occurs in response to metabolism of carbon sources or metabolism of sugars masks some other process that causes the alkalization, we tested the effects of carbon source concentration on supernatant alkalization. *V. cholerae* was grown in T medium supplemented with sucrose or pyruvate at 1x or 5x concentration, with 1x standardized to the number of carbon molecules in 5.8 mM sucrose. The higher concentration of sucrose resulted in greater acidification, and the higher concentration of pyruvate resulted in greater alkalization (Fig. 4a). *S. flexneri* growth inhibition of supernatants from the pyruvate-based cultures and the 1x sucrose culture was assayed by the IC_{50}-based method described in Materials & Methods. Supernatant from the 1x sucrose culture had no inhibitory activity at any concentration, while the

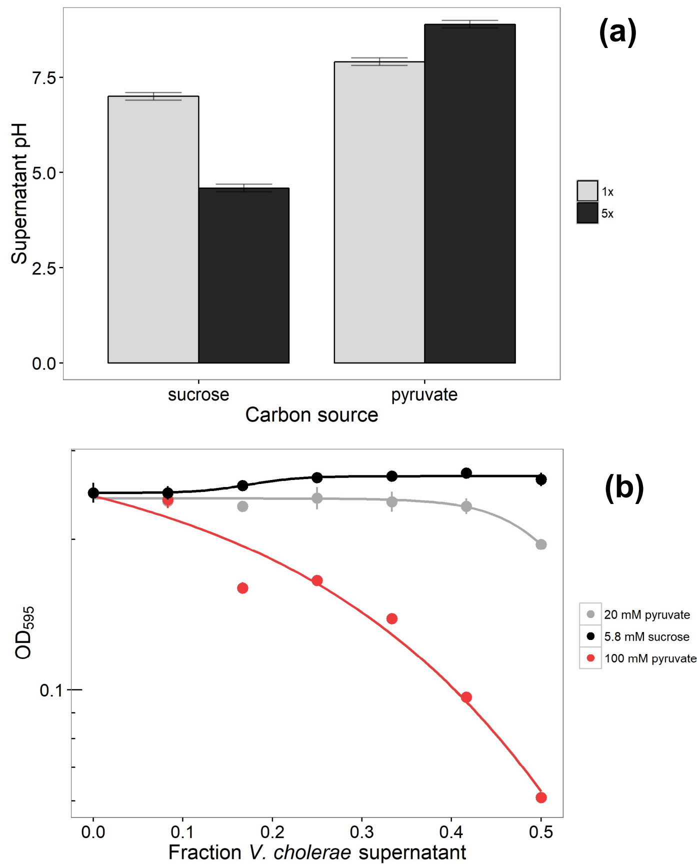


Figure 4. Carbon source concentration and identity affects alkalization and *S. flexneri* growth inhibition. **(a)** Increasing the concentration of selected carbon sources increases change in medium pH. Means and measurement error are reported. **(b)** Supernatant from a 72-hour old *V. cholerae* culture grown with a higher concentration of pyruvate (red line) inhibited *S. flexneri* growth more than that from cultures grown with less pyruvate (black line) or sucrose (grey line). Means and standard deviations of three cultures are shown for each point.

1x pyruvate culture supernatant had very slight inhibition and the 5x pyruvate culture supernatant inhibited *S. flexneri* growth markedly (Fig. 4b).

That increasing the concentration of the carbon source increases the magnitude of the medium alkalization suggests that some feature of metabolism is responsible. This could be a direct effect, in which high concentrations of one or more alkaline metabolites are produced, or it could be an indirect effect in which (for example) increased availability of energy allows the cells to alter buffer conditions or maintain a higher-magnitude transmembrane sodium gradient, which could make influx of positively-charged protons and thus alkalization of the medium more favorable (Häse and Barquera, 2000; Capasso and Supuran, 2014). Interestingly, the addition of carbon, hydrogen, and oxygen alone with no nitrogen is sufficient to produce medium alkalization.

This points to a role for specifically carbon metabolism (or its downstream effects on energy metabolism). However, it could also be that increased energy and carbon availability allows increased nitrogen assimilation from the ammonium in T medium, which could facilitate medium alkalization via amino acid catabolism even in the absence of preformed amino acids in the initial medium.

Amino acid supplementation in minimal medium affects supernatant alkalinity of *V. cholerae* cultures and the resultant *S. flexneri* growth inhibition to a lesser extent than carbon supplementation.

Medium alkalization is typically attributed not to carbon metabolism but to nitrogen metabolism, and specifically to catabolism of amino acids, which produces alkaline byproducts including ammonia and urea (Sezonov *et al.*, 2007; Wolfe, 2005). To determine whether amino acid catabolism could also play a role in alkalization, we tested the effects of amino acid supplementation of *V. cholerae* cultures on supernatant alkalization and *S. flexneri* growth inhibition. Each amino acid was supplemented in proportion to its concentration in the 5x Supplement from EZ RDM. The total amino acid concentration of the 5x Supplement is 23 mM, with most amino acids contributing sub-millimolar amounts. Arginine and serine are the exceptions, at 5.2 mM and 10 mM respectively. Cultures supplemented with 5x Supplement and unsupplemented cultures were used as controls. 20 mM pyruvate was used as the carbon source. Because supernatants from cultures grown in T with 20 mM pyruvate have relatively weak inhibition (Fig. 4b), we were able to use a lag time-based assay to quantify *S. flexneri* growth inhibition. Inhibition by *V. cholerae* supernatant occurs because of an increase in lag time (the amount of time it takes a culture to start growing) of the S. flexneri culture. For supernatants from cultures grown in LB, it may take more than six hours for growth to begin (see Fig. 2, unadjusted

V. cholerae supernatant point (pH 9.0)), but for T-based supernatants with low pyruvate concentration takes only 2.9 – 3.3 hours. Amino acid concentration of the initial supplemented T medium, pH of the V. cholerae supernatant, and optical density of the V. cholerae cultures at time of supernatant harvest were all positively correlated with S. flexneri lag time length (Fig. 4a-c). These correlations, however, are all relatively weak. This suggests that alkalization results from a general cellular process, since cultures that had access to more energy and grew to higher density alkalized their culture more and inhibited *S. flexneri* growth more. That the correlations are weak indicates that amino acids alone are not sufficient to produce inhibition of S. flexneri cultures, at least at the relatively low concentrations tested.

If any particular amino acid were involved in medium alkalization to an unusual degree, that could inform us as to which area(s) of metabolism are responsible. If none were, that might suggest a more general phenomenon (for example, increased energy availability allows more activity of ATP-dependent pumps used to maintain transmembrane ion gradients). Very little inhibition was observed in any amino acid-supplemented T culture. LB-based cultures and cultures grown in T supplemented with high concentrations of pyruvate routinely have no growth at 6 hours in 50% supernatant (i.e., lag time is greater than 6 hours for those conditions). However, within the narrow range of mild inhibitions seen with amino acid supplemented T, some differences were significant. Supernatants from cultures supplemented with methionine and serine had significantly longer lag times than supernatants from unsupplemented cultures, and supernatants from cultures supplemented with leucine, phenylalanine, proline, and tyrosine had significantly shorter lag times than supernatants from cultures grown with 5x Supplement (Fig. 5d). No supernatant type had significantly longer lag times than the 5x Supplement ones, and no supernatant type had significantly shorter lag times than unsupplemented T supernatant.

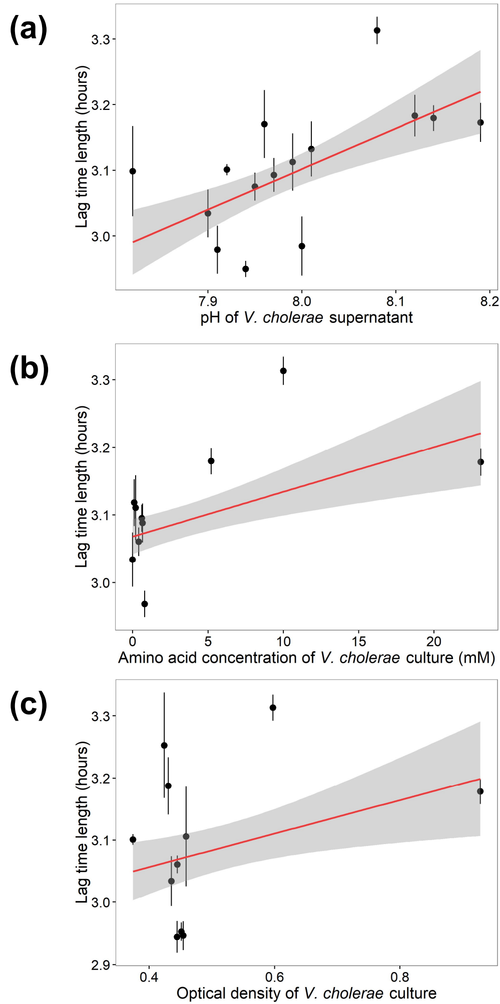
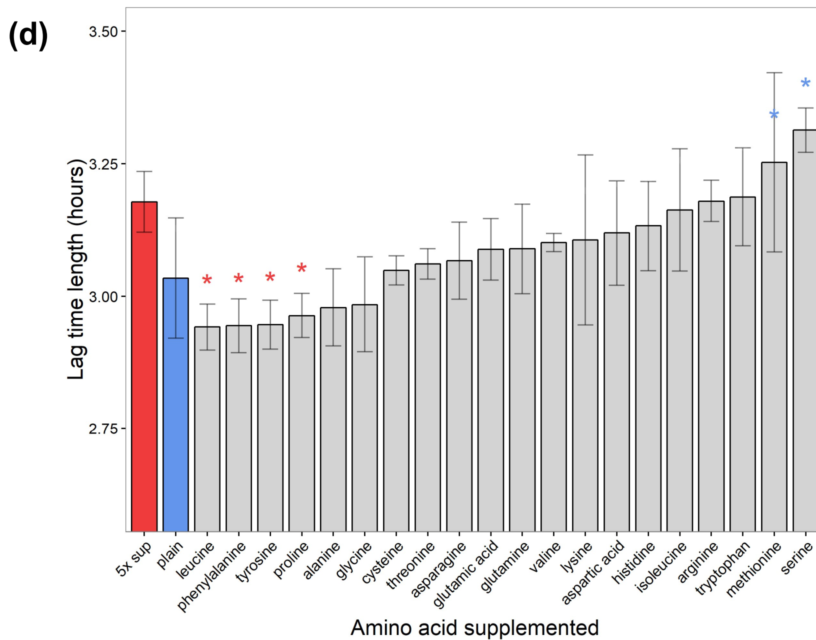


Figure 5. Addition of amino acids to minimal medium affects growth inhibition, supernatant pH, and terminal OD_{650} of *V. cholerae* culture in proportion to amino acid concentration. Means and standard deviations of at least four cultures are shown for each point. **(a-c)** *Shigella* growth inhibition (measured as lag time in 50% supernatant) correlates with supernatant pH (adjusted $R^2 = 0.11$), amino acid concentration of initial *V. cholerae* C6706 culture (adjusted $R^2 = 0.16$), and OD_{650} of *V. cholerae* C6706 cultures at time of supernatant harvest (adjusted $R^2 = 0.10$). These three parameters also all correlate positively with each other ($R^2 = 0.98$ for OD_{650} and amino acid concentration; $R^2 = 0.78$ for OD_{650} and supernatant pH; $R^2 = 0.62$ for amino acid concentration and supernatant pH). Standard error of the regression is shown by the grey outline around the red line. **(d)** *Shigella* growth inhibition by supernatants from cultures grown with specific amino acids. T with leucine, phenylalanine, proline, and tyrosine had significantly shorter lag times than T with 5x Supplement (red bar and asterisks). T with methionine and serine had significantly longer lag times than plain T (blue bar and asterisks). Significance was determined by a pairwise t-test followed by post hoc Bonferroni test with $P < 0.05$.



That supernatant from serine-supplemented cultures had stronger inhibition could be attributed to its relatively high concentration. Methionine, however, was not present at high concentration (0.2 mM). Methionine is used as a source of sulfur, and it and its derivative S-adenosylmethionine are involved in one-carbon metabolism (Rodionov *et al*., 2004). Serine is also involved with one-carbon metabolism. This could indicate a role for that family of pathways in medium alkalization.

All of the amino acids that produced supernatants with inhibition that did not differ from unsupplemented T were present at low concentration (0.8 mM for leucine, 0.4 mM for phenylalanine and proline, 0.2 mM for tyrosine). It may be that these concentrations were too low to have an effect, and supernatants from cultures with the other amino acids present at similar concentrations were insignificantly different from the 5x supplement culture supernatants due to chance and the narrow range of lag times observed. However, methionine was present at similarly low concentrations, and it did significantly increase lag time. This could indicate that amino acid catabolism only contributes to inhibition and alkalization insofar as it contributes to carbon metabolism, like serine and methionine do.

Supernatant from microaerobic *V. cholerae* cultures has stronger *S. flexneri* growth inhibition at a less alkaline pH.

Aerobiosis affects many aspects of physiology in *V. cholerae*. In a related El Tor strain, lack of oxygen caused increased expression of proteins associated with fermentation, glycolysis, anaerobic respiration, virulence, and biofilm, and decreased expression of proteins involved in ferric iron transport and the TCA cycle (Marrero *et al.*, 2008). We tested the effects of aerobiosis on medium alkalization and *S. flexneri* growth inhibition by *V. cholerae*. Anaerobic culture

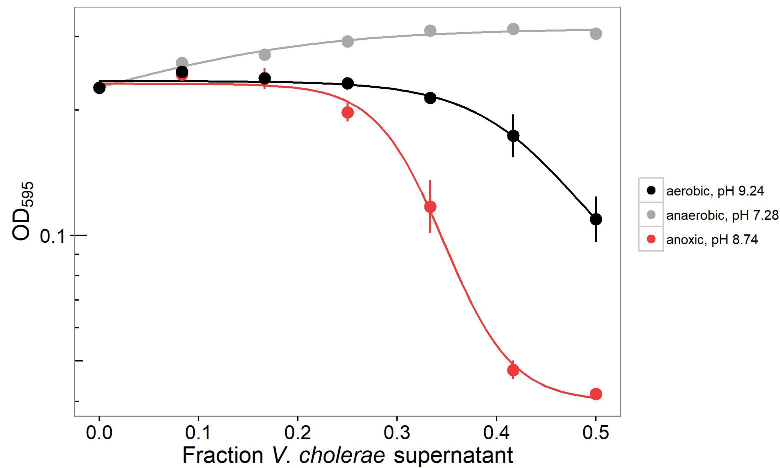


Figure 6. Microaerobic but not anaerobic *V. cholerae* growth enhances *S. flexneri* growth inhibition, even at less alkaline pH. Supernatant from a microaerobic *V. cholerae* culture (red line) decreases *S. flexneri* growth at lower concentrations than supernatant from an aerobic culture (black line), and supernatant from an anaerobic culture (grey line) failed to decrease *S. flexneri* growth at all. pH of each supernatant is given in the legend. Means and standard deviations of three *S. flexneri* cultures are shown for each point.

conditions were produced by static incubation in a candle jar with a BD BBL GasPak anaerobic culture system, and microaerobic culture conditions were produced by shaking incubation with a mineral oil overlay. LB was the medium used. Loss of oxygen led to progressively lower supernatant pH (Fig. 6 legend), which is consistent with the assumption that anoxia causes metabolism to switch to fermentation and glycolysis with resultant accumulation of acidic byproducts (Yoon and Mekalanos, 2006). Despite its lower pH, supernatant from microaerobic cultures inhibited *S. flexneri* growth more strongly than did supernatant from aerobic cultures (Fig. 6). This effect was not seen for supernatant from anaerobic culture, which promoted S. flexneri growth at higher concentrations.

This is not consistent with previous observations of strict dependence of *S. flexneri* growth inhibition on supernatant pH. Because the pH of the supernatant is still alkaline enough to inhibit *S. flexneri* growth (Fig. 2, supernatant pH > 7.8), this might be explained by increased production

of a metabolite that inhibits bacterial growth at high pH but not elevate the pH itself, or by increased production of an alkaline metabolite that inhibits bacterial growth at high pH with its pH-elevating effects masked by production of acidic metabolites during fermentation, or by stronger buffering of anaerobic cultures through some mechanism.

A sampling of *V. cholerae* mutants in key regulatory and metabolic pathways showed no effect on *S. flexneri* growth inhibition.

V. cholerae mutants with insertions in selected genes were screened for alteration of supernatant pH and *S. flexneri* growth inhibition during aerobic growth in LB. All had supernatant pH from 9.0-9.2, which did not differ from the parent strain. None had inhibition that varied significantly from that of the parent strain (Fig. 7). Mutants were sourced from a transposon library made with a Himar1 mariner transposon containing a kanamycin resistance cassette (Cameron *et al*., 2008). Inclusion of kanamycin in cultures used to produce supernatants would maintain the

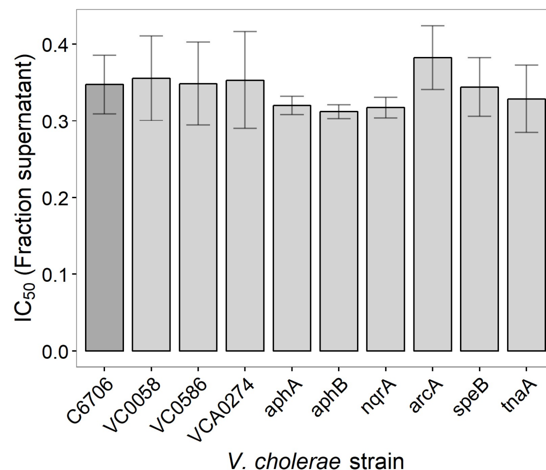


Figure 7. Inhibition of *S. flexneri* growth by supernatant from *V. cholerae* mutants. Supernatant was made using select mutants from a transposon library in *V. cholerae* C6706 (Cameron *et al*., 2008). None differed significantly from the parent strain by a pairwise t-test followed by post hoc Bonferroni test with $P < 0.05$. Means and standard deviations of three independent trials are shown for each point.

insertion in the population, but it would also inhibit *S. flexneri* growth. For the latter reason, kanamycin was omitted from the medium used to culture these mutants for supernatant collection. Additionally, stocks of the mutants were not sequenced to verify position of the insertions. Because of these factors, it is relatively likely that one or more of the results in Fig. 7 is a false negative.

The mutants screened included (1) carbonic anhydrases (*VC0058*, *VC0586*, *VCA0274*), (2) regulators of gene expression (*aphA*, *aphB*, *arcA*), (3) a component of the major electron transport machinery in *V. cholerae* (*nqrA*), and (4) amino acid catabolism enzymes (*speB*, *tnaA*).

1. Carbonic anhydrases catalyze the conversion of carbon dioxide and water to bicarbonate (Smith *et al.*, 1999). That lower pH and lesser inhibition are not observed in these knockouts suggests that either bicarbonate buffering is not involved in pH elevation or that a single knockout is not sufficient to see an effect.
2. *aphA* represses expression of acetoin biosynthesis genes in response to low cell density (Kovacikova *et al.*, 2005). *aphA* cooperates with *aphB* to activate expression of virulence genes (Kovacikova and Skorupski, 1999). *aphB* induces expression of a Na^+/H^+ antiporter and a carbonic anhydrase, among other genes, in response to low pH and anaerobiosis (Kovacikova *et al.*, 2010). *arcA* increases expression of the major virulence regulator *toxT* in response to anaerobiosis, and may have other targets as well (Sengupta *et al.*, 2003). The failure of knockouts of these genes to affect medium alkalization or inhibition suggests that their targets under aerobic conditions are not involved in those phenomena.
3. *nqrA* encodes a subunit of the sodium-translocating NADH quinone reductase, which is the major component of the electron transport chain in *V. cholerae*, knockouts of which are known to acidify their medium in *V. cholerae* O395N1, a distantly-related strain (Minato *et al.*, 2013). That this mutant failed to acidify its medium and did not differ from wild type

inhibition of *S. flexneri* growth may reflect insufficiency of loss of the *nqrA* subunit alone or a difference in metabolism between C6706 and O395N1. If the loss of *nqr* genuinely has no effect on alkalization and inhibition, that suggests that neither respiration nor sodium bioenergetics are involved.

4. *tnaA* encodes a tryptophanase, which cleaves tryptophan into indole, pyruvate, and ammonia, potentially alkalizing the culture (Mueller *et al.*, 2009). Indole is also commonly used as a signaling molecule, so it could conceivably have some effect on *S. flexneri*. *speB* encodes an agmatinase, which converts agmatine to putrescine and urea as a second step in an arginine catabolism pathway (Lee *et al.*, 2009). Putrescine and other polyamines can inhibit bacterial growth in a pH-dependent manner at sufficiently high concentrations (Yohannes *et al.*, 2005). That knockouts of neither of these genes affected medium alkalization or growth inhibition indicates that those pathways not indispensable for the medium alkalization and *S. flexneri* growth inhibition phenotype. Additionally, polyamine-based bacterial growth inhibition is likely not involved.

No genes involved in medium alkalization or *S. flexneri* growth inhibition have yet been identified.

Discussion

The antimicrobial effects of spent media from *V. cholerae* C6706 grown aerobically in LB on an avirulent strain of *S. flexneri* are pH-dependent. Supernatant alkalization and *S. flexneri* growth inhibition can be induced by supplementation of different carbon sources and amino acids in minimal medium, suggesting some feature of metabolism is responsible for alkalization. Despite the frequency with which ammonium production during amino acid catabolism is invoked as a mechanism for medium alkalization, carbon and energy metabolism are the most promising candidates for the responsible area of metabolism (Sezonov *et al*., 2007; Wolfe, 2005). Mutants in several genes affecting bicarbonate buffer balancing, amino acid catabolism, electron transport, and regulation of expression of many other genes were screened for differences in inhibition and alkalization. None differed, but given the limitations of the methodology it is plausible that one or more of these results may be a false negative.

For microaerobic *V. cholerae* cultures, supernatant showed stronger inhibitory activity at a less alkaline pH than supernatant from aerobic cultures (pH 8.6-8.8 vs. pH 9.0-9.2). This finding has not been fully investigated. It is possible that increased inhibition in these supernatants is the result of a separate phenomenon than inhibition in aerobic supernatants. Microaerobiosis could increase production of a primary or secondary metabolite that strongly inhibits bacterial growth, perhaps even at neutral pH. Alternately, it may be based on the same mechanisms as alkalization and inhibition by aerobic supernatants- microaerobiosis could alter regulation of buffer conditions, inhibiting *S. flexneri* growth at lower concentrations by remaining more alkaline, or it could increase the production of a metabolite with pH-dependent antimicrobial activity, with medium alkalization masked by presence of acidic fermentation byproducts. We do not yet have data to identify or distinguish all possible models.

While it seems likely that carbon and/or energy metabolism are the primary causes of alkalization, we have not yet ruled out the possibility that, over the course of the 72-hour incubation, T medium supplemented with pyruvate or succinate comes to contain amino acids which are then metabolized and are the true primary drivers of alkalization. *V. cholerae* must use the carbon sources initially present in the amino acid-free T medium to assimilate nitrogen in order to make proteins and other necessary nitrogenous compounds. As the culture ages, later generations of bacteria might then grow in media that does contain amino acids, depending on how much protein *V. cholerae* secretes during growth or how much lysis occurs during early growth.

The assays to determine nutritional requirements for medium alkalization are somewhat flawed. Because the concentrations of amino acids and carbon sources were not held constant and no controls for osmolarity of the media were implemented, effects of increasing concentrations of amino acids or carbon sources are confounded with increasing osmolarity. Many genes in a related El Tor strain are regulated by the osmolarity-sensitive transcriptional regulator OscR, including genes involved in biofilm formation, virulence, polyamine synthesis, and solute transport (Shikuma and Yildiz, 2009). In particular, increased osmolarity does upregulate expression of Na^+/H^+ antiporters, which extrude sodium and import protons, acidifying the cytoplasm and potentially elevating the pH of the medium. The effects of osmolarity on medium alkalization have not been examined, but they could be relevant.

Additionally, T medium is buffered. Although *V. cholerae* can clearly overcome the buffering and alter the pH of the medium, and internal comparisons between differently supplemented versions of T are valid, comparisons between alkalization in T medium and LB must take into account the buffering.

We cannot rule out the possibility that the antimicrobial effect observed in aerobic cultures is mediated by some molecule with pH-dependent antimicrobial activity. Indeed, given the vast number of primary metabolites and metals that change protonation or charge state at alkaline pH (which alters their ability to cross membranes, propensity to interact with different sites on proteins, and other mechanisms for non-metabolically-mediated bioactivity), it would be quite surprising if this pH-dependent inhibition were mediated purely by increased basicity (Padan *et al.*, 2005; Yohannes *et al.* 2005; Malik *et al.* 2016).

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