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The mechanism of carbonate killing of *Escherichia coli*

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Aims: To define the mechanism of carbonate killing in *Escherichia coli*.
Methods and Results: Sodium carbonate (150 mM) and ethylenediaminetetraacetic acid (EDTA, 60 mM) both killed *E. coli* K-12 when the pH was 8·5, but ammonium chloride (150 mM) was ineffective. EDTA was a 5-fold more potent agent than carbonate, but some of this difference could be explained by ionization. At pH 8·5, only 1·6% of the carbonate is CO^{-2}, but nearly 100% of the EDTA is $EDTA^{-2}$.
Conclusions: As carbonate and EDTA had similar effects on viability, cellular morphology, protein release and enzymatic activities, the antibacterial activity of carbonate seems to be mediated by divalent metal binding.
Significance and Impact of the Study: Cattle manure is often used as a fertilizer, and *E. coli* from manure can migrate through the soil into water supplies. Previous methods of eradicating *E. coli* were either expensive or environmentally unsound. However, cattle manure can be treated with carbonate to eliminate *E. coli*, and the cost of this treatment is less than $0·03 per cow per day.

INTRODUCTION

Escherichia coli inhabits the intestinal tract of warm blooded mammals, and it can survive for long periods of time in manure (Himathongkham *et al.* 1999). Most *E. coli* are harmless, but some strains (e.g. *E. coli* O157:H7) are highly pathogenic (Armstrong *et al.* 1996). Recent work indicated that *E. coli* counts decreased rapidly if sodium carbonate and alkali were added to dairy cattle manure, but the mechanism of this antimicrobial activity was not defined (Diez-Gonzalez *et al.* 2000). Carbonate anion is a reactive molecule that forms insoluble divalent metal ion complexes at alkaline pH (Merck 1996). A variety of bacterial enzymes (e.g. kinases) are activated by magnesium and other divalent cations, and magnesium cross-bridges are needed to stabilize the outer membrane of Gram-negative bacteria like *E. coli* (White 1995). The following experiments sought to define more precisely the mechanism of carbonate killing of *E. coli* by comparing the effects of carbonate with EDTA, a known chelator of magnesium and other divalent cations.

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MATERIALS AND METHODS

Bacterial growth. *Escherichia coli* K-12 (ATCC 12435, J. Lederberg W1485), provided by Dr Valley Stewart, Ithaca, NY, was grown aerobically in Luria broth (10 mg Trypticase, 5 mg yeast extract and 5 mg NaCl per ml, pH 7·5) in tubes (4·5 ml, 13 × 100 mm) or flasks (50 or 125 ml) at 37°C. Stationary phase cultures (100 μl, approximately 10^7 cells ml^{-1}) were mixed in equal parts with sodium carbonate (150 mM), ammonium chloride (150 mM) or disodium EDTA (60 mM) that had been adjusted to pH 8·5 with either HCl or NaOH. After 0–24 h of incubation (37°C), the treated cultures were 10-fold serially diluted using lauryl sulphate broth (35·6 mg lauryl tryptose per ml) in sterile 96-well microtitre plates, which were incubated at 37°C for 24 h to determine the number of surviving cells.

Spectrophotometry and microscopy. Optical density of LB-grown *E. coli* K12 cultures incubated in LB with EDTA (60 mM, pH 8·5), NH_4Cl (150 mM, pH 8·5) or Na_2CO_3 (150 mM, pH 8·5) was monitored (model 260, Gilford Instruments, Oberlin, OH) using a 1-cm light path cuvette at 600 nm. Phase contrast microscopy of the same cultures was undertaken using a Zeiss light microscope (Carl Zeiss

Ltd, Oberkochen, Germany) and a 100-X objective oil immersion lens. An LB-grown *E. coli K12* culture (pH 8·5) was included for comparative purposes for light microscopy.

SDS-PAGE gels. Polyacrylamide gel electrophoresis was performed as previously described (Laemmli 1970). Protein samples were combined with 5X SDS loading buffer [SDS, 2·0 g; Tris (1 M, pH 6·8), 8·0 ml; glycerol, 10 ml; bromophenol blue, 20 mg; dH_20–100 ml] to achieve a 1X solution. Samples were boiled for 5 min in the presence of dithiothreitol (50 mM) and loaded onto 15% SDS-polyacrylamide gels using an approximately equal amount of protein (5 μg) in each lane. The electrophoresis was performed at 4°C, 130 V for 10 h. Protein bands were detected using a silver stain kit according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

Enzyme assays. Stationary phase cultures of *E. coli* K-12 (50 ml) were exposed to EDTA (60 mM, pH 8·5), Na_2CO_3 (150 mM, pH 8·5) or NH_4Cl (150 mM, pH 8·5) for 12 h at 37°C. Cells were harvested by centrifugation (10 000 *g*, 10 min) and resuspended in phosphate buffer (100 mM, pH 7·5). Suspensions were centrifuged as before and resuspended in 5 ml of phosphate buffer and sonicated (model 450 Branson Sonifier, microtip, output 6 for 15 min at 0°C). Sonicates were centrifuged (10, 000 × *g*, 10 min) to remove cell debris and cell free extracts were stored at 4°C.

Lactate dehydrogenase (EC 1·1.1·27) and aldolase (EC 4·1.2·13) activities were followed by measuring the disappearance of NADH at 340 nm using the methods of Freier and Gottschalk (1987), and Bruns and Bergmeyer (1963), respectively. Glucokinase (EC 2·7.1·2) (Leder 1963) and isocitrate dehydrogenase (EC 1·1.1·42) (Siebert 1963) activities were followed by the reduction of NADP at 340 nm. Alkaline phosphatase (EC 3·1.3·1) was assayed colourimetrically using p-nitrophenol phosphate at 400 nm (Garen and Levinthal 1960). Catalase (EC 1·11·1.6) was qualitatively assayed using the absence or presence of oxygen bubble production when an 11% H_2O_2 solution (50 μl) was brought into contact with 50 μl of *E. coli* broth cultures on a glass microscope slide.

RESULTS

When *E. coli* K-12 was treated with increasing amounts of ammonium chloride (24 h, 22°C, pH 8·5), there was little decrease in optical density (Fig. 1a), and the viable cell count remained more or less constant (Fig. 1b). The optical density of carbonate-treated *E. coli* K-12 cultures (24 h, 22°C, pH 8·5) declined by as much as 50% (Fig. 1a). Even low concentrations of carbonate caused a reduction in viability, and viable cells could not be detected if the carbonate concentration was greater than 120 mM (Fig. 1b).

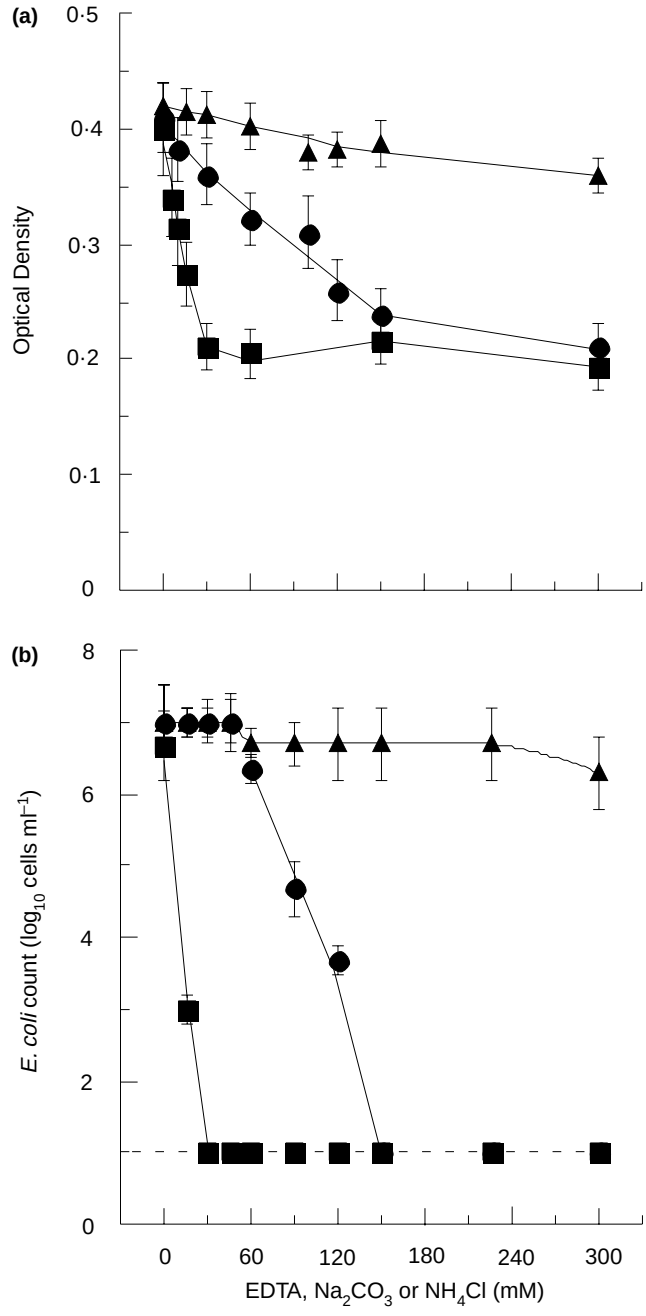

Fig. 1 The effect of EDTA (■), sodium carbonate (●) or ammonium chloride (▲) on the optical density of stationary phase *E. coli* K-12 cultures after 24 h of exposure at 22°C (a). Viable counts are shown in part (b)

E. coli K-12 was even more sensitive to EDTA (24 h, 22°C, pH 8·5), and as little as 30 mM EDTA caused a 50% decrease in optical density (Fig. 1a), and a complete loss of viability (Fig. 1b). The optical density and viability of untreated cells did not decrease (data not shown).

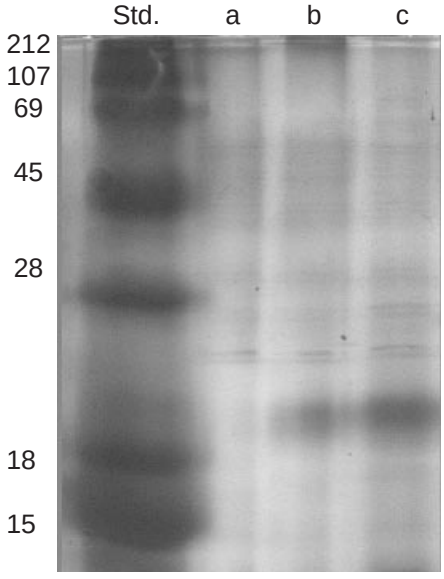

Fig. 2 An SDS polyacrylamide gel showing proteins that were released from *E. coli* K-12 cultures that had been treated with (A) ammonium chloride (150 mM, pH 8·5) (B) sodium carbonate (150 mM, pH 8·5) or (C) EDTA (60 mM, pH 8·5)

Phase contrast microscopy indicated that untreated cells and ammonium chloride-treated cells were morphologically similar, but the EDTA- and sodium carbonate-treated cells were elongated and less dense. Ammonium chloride-treated cells lost some protein into the cell-free supernatant, and this protein had a relatively high molecular weight (Fig. 2a). Cells that had been treated with sodium carbonate (Fig. 2b) or EDTA (Fig. 2c) lost more protein in the cell-free supernatant, and much of this cell-free protein had a low molecular weight (approximately 22 kDa).

Sonicated cell extracts of untreated and ammonium chloride-treated cells had similar alkaline phosphatase, glucokinase, aldolase, lactate dehydrogenase and isocitrate dehydrogenase activities (Table 1). The carbonate- and EDTA-treated cells had little alkaline phosphatase, glucokinase, aldolase or isocitrate dehydrogenase activity, and the lactate dehydrogenase activity was lower than untreated or ammonium chloride-treated cells.

DISCUSSION

Previous work indicated that carbonate could kill *E. coli* and other bacteria, and tests with manure taken from commercial dairy farms indicated that sodium carbonate was highly effective so long as some sodium hydroxide was added to ensure that the pH was 8·5 or greater (Diez-Gonzalez *et al.* 2000). The sodium carbonate and alkali treatment was rapid, and viable *E. coli* cells in treated manure could not be detected after 5 d (Diez *et al.* 2000). Based on a chemical cost of \$0·15 and \$0·07 per kg for sodium carbonate and sodium hydroxide, respectively, and a manure output of 14 000 kg of manure per year per cow, the carbonate and alkali treatment had an estimated cost of less than \$0·03 per cow per d or as little as \$10 per cow per year. Because a 3-fold water dilution did not diminish effectiveness, it appears that treatment can be used in modern dairy farms that store their manure outdoors or add other water sources (e.g. milking parlour wash).

Sodium accumulation in soil can have a negative impact on plant productivity (Black 1968), However, in the north-east and north central United States, rainfall is moderate, and sodium accumulation in the soil is generally not a problem. Because previous work indicated that potassium carbonate was as effective as sodium carbonate (Diez-Gonzalez *et al.* 2000), potassium salts could be used if sodium posed an environmental concern. Potassium salts are approximately twice the price of sodium salts, but their benefit as a fertilizer would offset some of this increased expense.

CO_3^{-2} is a divalent anion that forms complexes with a variety of divalent cations (Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}), and metal salts of carbonate are insoluble at alkaline pH (Merck 1996). Divalent cations can play a variety of important roles in bacteria. Many enzymes utilize metal ions as cofactors, and these ions act as general acids under physiological conditons by virtue of their positive charge. Metals ions

Table 1 The enzymatic activities of untreated cells and cells treated with ammonium chloride, EDTA, and sodium carbonate

Enzyme	Enzyme location	Treatment			
		Untreated	NH_4Cl	EDTA	Na_2CO_3
Catalase	membrane	+ + +	+ + +	$+^w$	$+^w$
Alkaline phosphatase[a]	periplasm	1478 ± 141	1292 ± 100	< 10	< 10
Glucokinase	cytoplasm	539 ± 57	390 ± 10	< 10	< 10
Aldolase	cytoplasm	411 ± 44	393 ± 14	< 5	< 5
Lactate dehydrogenase	cytoplasm	557 ± 19	570 ± 55	29 ± 5	103 ± 19
Isocitrate dehydrogenase	cytoplasm	6450 ± 1289	4610 ± 786	< 5	< 5

[a]Activities are expressed as nmol min^{-1} mg $protein^{-1}$. + + + strong reaction, $+^w$ weak reaction.

have a larger size than protons, but this difference is partially compensated by the larger number of positive charges on the metal. In these cases, the metal ion (e.g. Mg^{2+}, Mn^{2+} or Zn^{2+}) acts as a Lewis acid that accepts an electron pair and promotes acid catalysis.

Many metal ion–protein interactions are not particularly strong, and traditional methods of protein purification can cause a loss of the metal. However, in some cases the metal ion is tightly bound to the protein as a prosthetic group (e.g. Fe^{2+} or Co^{2+} porphyrins). Metal prosthetic groups participate in the transport of electrons, and some are needed to remove toxic hydrogen peroxide (Lowe and Ingraham 1974).

Most bacteria have a high internal osmotic pressure that would rupture the cell membrane, but this membrane is surrounded by a rigid peptidoglycan. The outer membrane of gram-negative bacteria lies outside the peptidoglycan, but the lipopolysaccharides (LPS) are stabilized by divalent cations that form salt bridges and neutral repulsive forces on neighbouring LPS molecules and or proteins (Marvin *et al.* 1989). If these bridges are de-stabilized, the LPS losses its structural integrity, and periplasmic proteins are released from the cells.

When *E. coli* K12 was treated with EDTA or carbonate, the optical density decreased and the cells became less dense, but less than 50% of the protein was lost. Alkaline phosphatase is a magnesium requiring enzyme that has often been used as a marker for periplasmic proteins (White 1995). EDTA- and carbonate-treated cells had essentially no alkaline phosphatase activity, and this activity was not restored if magnesium was added to the assay buffer. Based on these results, it appeared that carbonate was causing the inactivation and release of periplasmic proteins.

When *E. coli* cultures are grown under adverse conditions, the cells produce a variety of stress proteins, and many of these proteins have a low molecular weight (Phillips *et al.* 1987). Ammonium chloride-treated cells released little protein into the cell-free supernatant, and most of these proteins had molecular weights that ranged from 45 to 200 kDa. EDTA- and carbonate-treated cells released more protein, and much of this protein was low molecular weight (approximately 22 kDa).

Catalase is an iron containing cell membrane-associated protein (Poole and Ingledew 1987) and preliminary experiments indicated that EDTA- and carbonate-treated cells were no longer catalase positive. Glucokinase and aldolase also require magnesium as a cofactor, and EDTA- and carbonate-treated cells had virtually no activities even if magnesium was added to the assay buffer. Because lactate and citrate dehydrogenase activities of EDTA- and carbonate-treated cells were also markedly lower than untreated controls or ammonium chloride-treated cells, the effect of carbonate and EDTA on the cell extracts could not be explained solely by cofactor availability.

Previous workers indicated that sodium bicarbonate had antibacterial properties, but this activity was pH-dependent (Corral *et al.* 1988). When pH was more alkaline, the amount of bicarbonate could be reduced, and the pH of our manure treatment was also critical (Diez-Gonzalez *et al.* 2000). If fermentation acid production of bacteria in manure decreased the pH, the treatment was no longer effective. When the pH is alkaline, the bicarbonate buffer system shifts towards carbonate anion, and this pH dependency is consistent with the idea that carbonate rather than bicarbonate is toxic to bacteria.

Titration experiments (Fig. 1) indicate that approximately 5-fold more sodium carbonate was needed to kill *E. coli* than EDTA (150 *vs.* 30 mM), but this difference could be at least partially explained by ionization. At pH 8·5, only 1·6% of the carbonate is CO^{-2}, but nearly 100% of the EDTA is $EDTA^{-2}$. Carbonate and EDTA had similar effects on viability, cellular morphology, protein release and enzymatic activities, but further work is needed to see if the antibacterial activity of carbonate is mediated by divalent metal binding.

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