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Metabolism

Effect of chlorate, molybdate, and shikimic acid on *Salmonella enterica* serovar Typhimurium in aerobic and anaerobic cultures

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ABSTRACT

Experiments were conducted to determine factors that affect sensitivity of *Salmonella enterica* serovar Typhimurium to sodium chlorate (5 mM). In our first experiment, cultures grown without chlorate grew more rapidly than those with chlorate. An extended lag before logarithmic growth was observed in anaerobic but not aerobic cultures containing chlorate. Chlorate inhibition of growth during aerobic culture began later than that observed in anaerobic cultures but persisted once inhibition was apparent. Conversely, anaerobic cultures appeared to adapt to chlorate after approximately 10 h of incubation, exhibiting rapid compensatory growth. In anaerobic chlorate-containing cultures, 20% of total viable counts were resistant to chlorate by 6 h and had propagated to 100% resistance ($>10^9$ CFU mL^{-1}) by 24 h. In the aerobic chlorate-containing cultures, 12.9% of colonies had detectable resistance to chlorate by 6 h, but only 1% retained detectable resistance at 24 h, likely because these cultures had opportunity to respire on oxygen and were thus not enriched via the selective pressure of chlorate. In another study, treatment with shikimic acid (0.34 mM), molybdate (1 mM) or their combination had little effect on aerobic or anaerobic growth of *Salmonella* in the absence of added chlorate. As observed in our earlier study, chlorate resistance was not detected in any cultures without added chlorate. Chlorate resistant *Salmonella* were recovered at equivalent numbers regardless of treatment after 8 h of aerobic or anaerobic culture with added chlorate; however, by 24 h incubation chlorate sensitivity was completely restored to aerobic but not anaerobic cultures treated with shikimic acid or molybdate but not their combination. Results indicate that anaerobic adaptation of *S.* Typhimurium to sodium chlorate during pure culture is likely due to the selective propagation of low numbers of cells exhibiting spontaneous resistance to chlorate and this resistance is not reversible by molybdenum supplementation.

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1. Introduction

Salmonella is the second most common bacterial cause of foodborne illness in the U.S., causing a third of reported bacterial foodborne illness [1]. An estimated 1.4 million cases of *Salmonella* food-poisoning occur each year in the U.S., with approximately 15,000 hospitalizations and 400 deaths, and an estimated annual cost of 0.5–2.3 billion dollars [1,2]. Antibiotic resistant *Salmonella* strains are a cause of growing concern worldwide. Multidrug resistant (MDR) *Salmonella enterica* serovar Typhimurium have increased from 15% of all tested isolates in 1992 to 42% in 2001 according to a survey of 29 mostly industrialized countries of Europe, the Americas, Africa, Asia, and Oceania [3]. Of concern is the recent development of resistance by *S.* Typhimurium to fluoroquinolones, since these are the drugs of choice for treatment of severe *Salmonella* infection in humans [3]. Because of concerns about resistance to antibiotics, alternative means of controlling *Salmonella* in food animals need to be explored.

A recently developed experimental chlorate-containing product has shown promise in the pre-slaughter reduction of the human pathogens *Salmonella* and *Escherichia coli* O157:H7 from the gastrointestinal tracts of food animals. Chlorate, a valence state analog of nitrate, is thought to be metabolized to cytotoxic chlorite

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by the molybdoenzyme component of respiratory nitrate reductase [4–6]. Consequently, it was hypothesized and subsequently demonstrated that chlorate selectively targets bacteria possessing membrane bound respiratory nitrate reductase activity but not beneficial anaerobes lacking the enzyme thereby conserving the competitive exclusion potential of the host's normal flora [7]. The selective bactericidal activity of chlorate has been demonstrated in several laboratory and field studies with both ruminants and monogastrics, achieving significant reductions in *Salmonella* and *E. coli* thus supporting its conceptual use as a feed additive to reduce gut concentrations of foodborne pathogens [4]. The effect of chlorate treatment on numbers of *Salmonella* and *E. coli* O157:H7 varied during these and other studies [8,9], with reductions ranging from approximately 100 to 100,000 cells suggesting that the possibility that the bactericidal activity of chlorate may be limited depending on cultural and feeding conditions. Additionally, in some batch culture studies the consumption of chlorate or its bactericidal effect appeared to be transient, with considerable regrowth of the pathogens occurring by 24 h of incubation, thereby suggesting the possible acquisition of resistance or other adaptive mechanisms [10,11]. The development of chlorate resistance within the gut of animals could potentially reduce the efficacy of chlorate products administered as feed additives. Consequently, we conducted several studies to quantify the effects of chlorate on growth and acquisition of chlorate resistance by *S.* Typhimurium and to investigate potential factors such as oxygen, molybdate and shikimic acid that may potentially mitigate the acquisition of chlorate resistance.

2. Materials and methods

2.1. Media and culture conditions

Brain heart infusion (BHI) broth and agar (Difco; Becton, Dickinson and Company; Sparks, MD) were reconstituted according to manufacturer's instructions. Glucose limited minimal medium contained (mg L^{-1}): K_2HPO_4, 225.0; KH_2PO_4, 225.0; $(NH_4)_2SO_4$, 450.0; NaCl, 450.0; $MgSO_4 \cdot 7H_2O$, 45.0; $CaCl_2$, 22.5; resazurin, 1.0; cysteine-HCl, 500.0; Na_2CO_3, 4000.0; Bacto peptone, 3700.0. Cysteine-HCl and Na_2CO_3 were added after the medium containing the other ingredients was adjusted to pH 7.0. Anaerobic broth was prepared by boiling and then cooling the medium on ice while flushing with oxygen-free 100% N_2 [12]. The medium was anaerobically distributed (9 mL) to 18 × 150 mm glass walled crimp top culture tubes which were immediately closed with rubber stoppers, crimped with aluminum seals and autoclaved. Aerobic broth was prepared similarly except without N_2 and was distributed to 18 × 150 mm straight walled glass tubes and closed with stainless steel closures. Respective additions of sodium chlorate, sodium nitrate, sodium molybdate, shikimic acid or glucose were made to sterilize media via aseptic addition (0.1–0.2 mL) of concentrated filter sterilized stock solutions. Final volumes of all tubes were equalized via addition of appropriate volumes of sterile reverse osmosis water. All incubations were carried out at 39 °C.

2.2. Effect of chlorate on growth and resistance during anaerobic and aerobic growth

S. enteric serovar Typhimurium was incubated aerobically or anaerobically in triplicate BHI broth tubes supplemented with 5 mM nitrate and with or without 5 mM chlorate (Experiment 1). Cultures were inoculated with 0.1 mL of an overnight-grown culture of *S.* Typhimurium. Growth was determined by measuring absorbance at 600 nm (A600) on a spectrophotometer (Spectronic 20D+, Spectronic Instruments, Inc., Rochester, NY) during a 24 h incubation period. Lag times varied for different treatments; therefore, maximum specific growth rates were calculated during the steepest part of logarithmic growth [13].

To enumerate proportions of chlorate susceptible and chlorate resistant *S.* Typhimurium, samples (1 mL) were collected from each culture tube at 0, 3, 6 and 24 h and serially diluted (10-fold) in phosphate buffer. One-tenth mL of each 10^{-2}–10^{-6} dilution and 0.5 mL of each 10^{-1} dilution were spread onto BHI agar (prepared according to manufacturer's instructions) and colonies were enumerated after 24 h aerobic incubation at 39 °C. Our limit of detection was 600 cells (2.8 $\log_{10}$) mL^{-1} (based on a plate spread with 0.5 mL of 10^{-1} dilution yielding a minimum of 30 CFU). Up to three successively diluted plates were selected from each sample at each sampling time for determination of nitrate reductase activity using the agar overlay method of Glaser and DeMoss [14], in which colonies capable of reducing nitrate to nitrite turn purple, while nitrate reductase deficient (chlorate resistant) colonies remain the original ivory color. Chlorate susceptible and resistant colonies were counted.

2.3. Stability of a chlorate resistant phenotype

To determine the stability of a chlorate resistant phenotype, a colony originating from a 24 h anaerobic culture grown in BHI broth supplemented with 5 mM nitrate and 5 mM chlorate was picked from a colony propagated after 24 h incubation in a Bactron Anaerobic Chamber (Sheldon Labs Manufacturing Inc., Cornelius, OR) on BHI agar supplemented likewise. Chlorate resistance was defined as the ability of the *S.* Typhimurium to grow and survive in broth containing 5 mM chlorate. The chlorate resistant isolate was then conditioned to growth with or without 5 mM chlorate via anaerobic subculture for two successive 24 h passages in anaerobic glucose limited minimal broth supplemented accordingly. Thereafter, the conditioned cultures were each used to inoculate (in duplicate) tubes containing glucose limited minimal broth supplemented with or without 5 mM chlorate (Experiment 2) or with and without 5 mM nitrate (Experiment 3). Growth and mean specific growth rates in the presence or absence of 5 mM added chlorate were determined as described above. Growth and mean specific growth rates of a chlorate susceptible *S.* Typhimurium cultured similarly were measured for comparison.

To determine if reduced expression of nitrate reductase activity by the chlorate resistant phenotype persisted following culture in the absence of chlorate, cells of the chlorate resistant phenotype conditioned to growth with or without 5 mM chlorate after 2 consecutive passages (24 h each) in 30 mL anaerobic BHI broth supplemented with 5 mM nitrate were harvested by centrifugation (20 min at 10,000 g). Chlorate and nonchlorate-conditioned cells were each resuspended separately in 5 mL of anaerobic dilution solution [15] containing 5 mM nitrate and distributed (1.4 mL) to 18 × 150 mm crimp top glassed wall tubes (in triplicate). Tubes were immediately closed with rubber stoppers and a H_2:CO_2 (1:1) gas phase was used with H_2 serving as the reducing substrate for reduction of nitrate. Cells of a chlorate susceptible *S.* Typhimurium grown in nitrate supplemented BHI broth without added chlorate were harvested and distributed likewise. The tubes were incubated at 39 °C and samples (0.2 mL) collected at 0, 3, 6 and 24 h were analyzed colorimetrically for determination of nitrate concentration [16]. Protein content of the cell suspensions was determined using a commercial modified Lowry Protein Assay Kit (Pierce, Rockford, IL, USA). Specific nitrate reductase activity was calculated as the linear rate of nitrate disappearance divided by protein concentration within the reaction fluids.

2.4. Effect of shikimic acid, molybdate or their combination on growth and resistance of S. Typhimurium during aerobic and anaerobic growths with or without added chlorate

To determine the effect of additions of molybdate, shikimic acid or their combination on the bactericidal activity of chlorate against *S.* Typhimurium, cultures were incubated in triplicate aerobically and anaerobically (Experiment 4) in BHI broth supplemented with 5 mM nitrate and with or without 5 mM chlorate, 0.34 mM shikimic acid, 1 mM molybdate or their combinations. Growth and viable cell counts were determined as previously described.

2.5. Determination of rates of chlorate metabolism

3 mL samples of the 10^{-1} dilution from each culture were frozen until subsequent analysis of chlorate by HPLC. Samples were thawed at room temperature, and prepared and chromatographed according to the method of Beier et al. [10]. Rates of chlorate disappearance were calculated using linear regression.

2.6. Statistical analysis

Data from each experiment were analyzed separately to avoid confounding effects between experiments using Statistix 9 Analytical Software (Tallahassee, FL). Maximum specific growth rates, maximum optical densities and specific nitrate reductase activities were analyzed to determine effects of treatments using a general analysis of variance. Treatment means were compared to means from controls (cultures incubated without chlorate and treatment) using a Dunnett's procedure and were considered significant at $P < 0.05$. Log_{10} transformations of viable cell counts (CFU mL^{-1}) were likewise analyzed using general analysis of variance at each respective sampling time during incubation but due to the greater variability associated with viable cell counts a more conservative $P < 0.01$ was considered significant for these mean comparisons. Rates of chlorate metabolism determined in chlorate-supplemented cultures were also analyzed by analysis of variance but because rates were nonexistent from controls lacking chlorate, least squares' means of all chlorate-supplemented cultures were compared using a Tukey's multiple comparison procedure. Regression analysis was used to compare slopes of estimated and observed growth curves.

3. Results and discussion

3.1. Effect of chlorate on growth and resistance during anaerobic growth

In our first experiment, chlorate inhibition was apparent early during anaerobic culture of *S.* Typhimurium with added chlorate, as evidenced by an extended 9–10 h lag in growth as well as by a decrease ($P < 0.05$) in viable numbers of *S.* Typhimurium compared to nonchlorate-supplemented controls (Fig. 1A, B). The maximum specific growth rate during logarithmic growth for the anaerobic cultures incubated with chlorate was > 50% slower ($P < 0.05$) than that of aerobic controls or that of anaerobic cultures incubated without chlorate (Table 1).

The ability of *Salmonella* and *E. coli* to grow in the presence of chlorate has long been recognized and attributed to genetic defects in *chl* alleles which more recently have been renamed to more accurately describe deficiencies in molybdenum uptake and metabolism (*moa, mob, nar, mod, moe, mog*) [17]. These genetic defects often are expressed as a deficiency in membrane bound respiratory nitrate reductase activity, but inactivation of other molybdenum containing enzymes such as dimethyl sulfoxide reductase, trimethylamine N-oxide reductase and thiosulfate

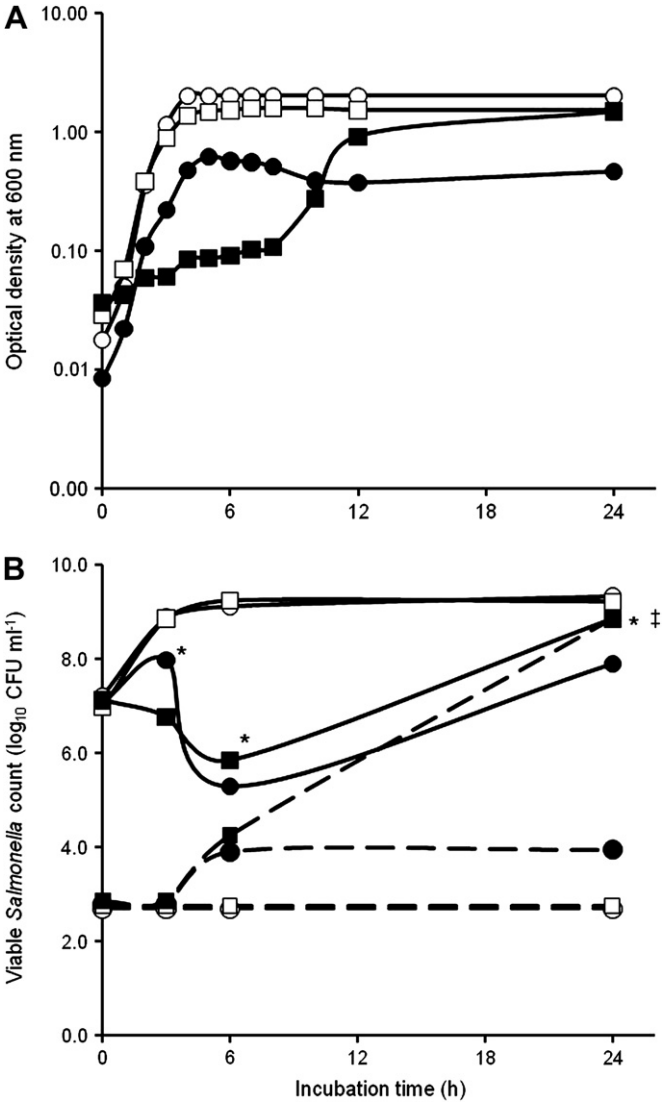

Fig. 1. Growth curves (A) and viable cell counts (B) of *Salmonella* Typhimurium grown aerobically (circles) or anaerobically (squares) in nitrate supplemented (5 mM) tryptic soy broth without (open symbols) or with (closed symbols) 5 mM added chlorate (Experiment 1). Total viable counts (solid lines) with values ≤ * denotation and nitrate reductase deficient counts (dashed lines) with values ≥ ‡ denotation differ from aerobic controls incubated without added chlorate; SEM were ≤ 0.83 and 0.24 for total viable counts and for nitrate reductase deficient counts, respectively.

Table 1
Comparative mean specific growth rates and maximum optical density during aerobic and anaerobic culture of *Salmonella* Typhimurium without or with chlorate[a]

	Comparative response during aerobic and anaerobic growths (Experiment 1)			
	Mean specific growth rate (ΔA_{600} h^{-1})		Maximum optical density (A_{600})	
Supplement	Aerobic	Anaerobic	Aerobic	Anaerobic
None	1.59	1.28	1.99	1.58[b]
Chlorate	1.16[b]	0.60[b]	0.61[b]	1.46[b]

[a] Cultures in triplicate were grown aerobically or anaerobically (100% N_2) in nitrate supplemented (5 mM) tryptic soy broth without or with 5 mM added chlorate. SEM = 0.09 and 0.01 from a general analysis of variance of mean specific growth rates and maximum optical densities, respectively.

[b] Least squares means differ ($P < 0.05$) from aerobic controls incubated without added chlorate.

reductase may also occur [18]. Results from the present study are consistent with the most widely accepted view that chlorate toxicity affects molybdoenzymes such as membrane bound nitrate reductase A (NarA) which catalyze the reduction of chlorate to chlorite, the later which is toxic to the bacteria, probably by causing oxidative damage [5–7,18–20]. The NarA enzyme, which is expressed under anaerobic conditions, is purported to be the primary contributor to this reduction, although the constitutively expressed NarZ which can account for approximately 10% the nitrate reductase activity may also contribute to chlorate toxicity [11].

Tests for nitrate reductase proficiency (which was our measure of chlorate sensitivity) in the current study revealed that 20 and 100% of the total viable non-$\log_{10}$ transformed *S.* Typhimurium counts after 6 and 24 h anaerobic incubation, respectively, with added chlorate were deficient in nitrate reductase activity (Fig. 1B). The appearance of chlorate resistant *S.* Typhimurium observed in our study is likely due to the selective propagation of low numbers of cells expressing spontaneous resistance to chlorate within the population rather than to a mutagenic effect as chlorate has been reported to be nonmutagenic when tested via the Ames test [21]. For instance, in cells grown without selective pressure of nitrate or chlorate the frequency of recovering chlorate resistant *S.* Typhimurium was estimated to occur at 1.56×10^{-6} [22]. Based on this frequency, we calculate that 21 ± 5.9 ($1.32 \log_{10}$) mL^{-1} resistant cells would have been present at the start of our incubations. These low numbers of resistant cells would have been unobserved as our limit of detection was 600 ($2.8 \log_{10}$) mL^{-1} cells. A comparison of regression lines obtained from theoretical logarithmic growth curves estimated from the propagation of these low numbers of chlorate resistant cells to the line plotted from counts observed during anaerobic culture with added chlorate revealed that the lines did not significantly ($P > 0.05$) differ (not shown). In our cultures incubated without added chlorate, nitrate reductase deficient *S.* Typhimurium were not observed at any time (Fig. 1B), but this is not unexpected as these cultures contained nitrate and thus would have selected for nitrate proficient phenotypes.

Results from our first experiment do not allow us to discount the possibility that the long lag phase that occurred during anaerobic incubation with chlorate may alternatively reflect an adaptive response manifested by a chlorate-stimulated down-regulation of Nar gene expression. However, when a genetically uncharacterized chlorate resistant *S.* Typhimurium isolate obtained from our study was harvested from subcultures grown 2 successive passages without or with exposure to chlorate they similarly expressed low nitrate reductase activity (0.024 ± 0.04 and 0.019 ± 0.03 μmol mg prot.$^{-1}$ h^{-1}, respectively). This finding indicates that the resistant phenotype persisted regardless of chlorate exposure. By comparison, nitrate reductase activity expressed by cells of a chlorate sensitive isolate grown without chlorate exposure (0.83 ± 0.06 μmol mg prot.$^{-1}$ h^{-1}) was much higher ($P < 0.05$). Moreover, in Experiment 2 we observed that propagation of the chlorate resistant isolate for two consecutive 24 h transfers in a glucose minimal medium lacking added chlorate did not restore the cells to a sensitive or non-adaptive state. For instance, growth curves (Fig. 2A) and maximum specific growth rates (Table 2) were nearly equivalent whether subsequently cultured with or without chlorate. The chlorate resistant cultures did grow more slowly during the first 3 h of culture than the parental chlorate susceptible *S.* Typhimurium grown without added chlorate which suggests that some adaptation or differential regulation may occur during early growth of the chlorate resistant *S.* Typhimurium but this lag was observed independent of whether or not the resistant cultures were grown with or without chlorate. By 3 h of incubation the chlorate resistant cultures began to grow more rapidly than the non-chlorate-supplemented susceptible cultures and achieved an equivalent maximum optical density after 22 h incubation indicating that the energetic cost associated with chlorate resistance was minimal during fermentative growth in this energy restricted medium (Fig. 2).

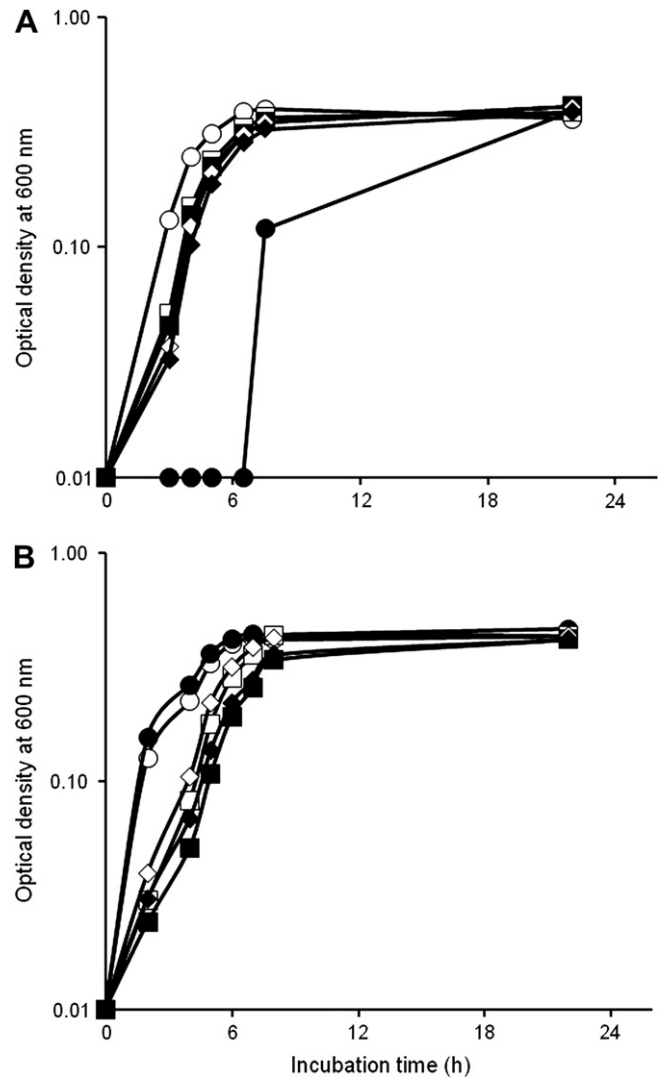

Fig. 2. Growth curves of a chlorate susceptible (circles) and an uncharacterized nitrate reductase deficient (chlorate resistant) *S.* Typhimurium grown anaerobically in glucose minimal broth without (open symbols) or with (closed symbols) 5 mM added chlorate (A, Experiment 2) or nitrate (B, Experiment 3). The chlorate resistant isolate had been conditioned immediately prior via two 24 h cultures in medium lacking (diamonds) and containing added chlorate (squares).

When the chlorate resistant *S.* Typhimurium was grown in a glucose minimal medium with or without added nitrate (Experiment 3) it also grew more slowly during the first 3 h of culture but subsequently grew more rapidly than the parental chlorate susceptible *S.* Typhimurium grown without added nitrate (Fig. 2B). The resistant isolate also achieved higher maximum specific growth rates than the parental chlorate susceptible *S.* Typhimurium grown without added nitrate, although the rate was not significantly higher for cultures previously conditioned in the absence of chlorate and then grown with added nitrate (Table 2). In this case, however, chlorate resistant cultures grown with added nitrate achieved lower ($P < 0.05$) maximum optical density than resistant cultures grown without nitrate and the susceptible cultures grown without added nitrate thus indicating an energetic cost of chlorate

Table 2
Comparative mean specific growth rates and maximum optical densities during anaerobic culture of a chlorate susceptible and a preconditioned chlorate resistant *S.* Typhimurium during subsequent growth with or without chlorate or nitrate [a]

	Mean specific growth rate (ΔA_{600} h^{-1})			Maximum optical density (A_{600})		
Supplement	Susceptible isolate	Resistant isolate		Susceptible isolate	Resistant isolate	
		Grown prior without chlorate	Grown prior with chlorate		Grown prior without chlorate	Grown prior with chlorate
Comparative response to growth with or without chlorate (Experiment 2)[b]						
None	0.47	0.76[d]	0.77[d]	0.39	0.38	0.40
Chlorate	0.26	0.78[d]	0.75[d]	0.39	0.41	0.38
Comparative response to growth with or without nitrate (Experiment 3)[c]						
None	0.44	0.60[d]	0.64[d]	0.42	0.42	0.42
Nitrate	0.43	0.50	0.53[d]	0.44	0.38[d]	0.39[d]

[a] Cultures in duplicate were incubated anaerobically (100% N_2) in glucose minimal medium supplemented without or with 5 mM sodium chlorate (Experiment 2) or sodium nitrate (Experiment 3). Chlorate resistant cells used as inocula had been conditioned via two prior 24 h cultures in similar medium supplemented with or without chlorate; chlorate susceptible cells used as inoculum had been grown similarly without chlorate.
[b] SEM = 0.02 and 0.01 from a general analysis of variance of mean specific growth rates and maximum optical densities, respectively.
[c] SEM = 0.04 and 0.01 from analysis of mean specific growth rates and maximum optical densities, respectively.
[d] Least squares means within experiment differ ($P < 0.05$) from chlorate susceptible control incubated without added chlorate or nitrate.

resistance when nitrate was present, possibly due to induction of incomplete respiratory function. Chlorate susceptible cultures grown with added nitrate achieved the highest maximum optical density measurement which is as expected because nitrate respiration is considered to be more efficient than fermentation [18]. As observed in our first experiment, chlorate susceptible *S.* Typhimurium cultured with added chlorate did not exhibit logarithmic growth until after 7 h of incubation, achieving a slower ($P < 0.05$) maximum specific growth rate (0.26 h^{-1}) but an equivalent maximum optical density than chlorate susceptible controls incubated without chlorate (Fig. 2B). This observation is consistent with our conclusion that cells from both cultures were conserving energy via fermentative metabolism rather than respiration.

3.2. Effect of chlorate on growth and resistance during aerobic growth

In contrast to that observed during anaerobic culture with added chlorate, the maximum specific growth rate, which was achieved within the first 4 or 5 h of incubation, by aerobic cultures incubated with added chlorate did not differ significantly from that of control cultures incubated aerobically without chlorate (Table 1). However, by 6 h of incubation a persistent inhibition of growth was evident during aerobic culture with added chlorate as the maximum optical density (Table 1) and viable cell counts achieved at this time or thereafter were significantly lower than aerobic controls incubated without chlorate (Fig. 1B). As was observed during anaerobic culture without added chlorate, viable nitrate reductase deficient *S.* Typhimurium were not recovered at any time from cultures incubated without the selective pressure of added chlorate (Fig. 1B). Nitrate reductase deficient *S.* Typhimurium were recovered after 6 h aerobic culture with added chlorate at numbers (12.9% of the total viable non-$\log_{10}$ transformed population) nearly equivalent to those recovered during anaerobic culture, but numbers recovered after 24 h culture with chlorate accounted for only 1% of the total viable *S.* Typhimurium population (Fig. 1B). Substantial propagation of nitrate reductase deficient *S.* Typhimurium would not be expected with oxygen-respiring cultures, however, as NarA nitrate reductase activity is suppressed in favor of more energetically preferred respiration on oxygen, which has a higher redox couple potential than nitrate (pϵ of +12.7 and +13.8, respectively) [5,23–25]. In our study as well, aerobic respiration was more efficient than anaerobic respiration on nitrate in cultures lacking added chlorate as evidenced by higher optical density values (Fig. 1A). Under aerobic conditions, an intermediate product produced from nitrate reductase reduction of chlorate has been proposed to exert a toxic effect on *E. coli* by inhibiting an early step in the biosynthesis of aromatic amino acids or in ubiquinone-8 biosynthesis via the shikimic acid pathway [26–29]. Giordano et al. [30] also suggested that the mechanism of chlorate toxicity during aerobic growth may involve the oxidation of quinols as *E. coli* were also able to reduce nitrate under aerobic conditions. Inhibition of

Table 3
Comparative mean specific growth rates and maximum optical density during aerobic and anaerobic culture of *S.* Typhimurium without or with chlorate and treatments with or without molybdate, shikimic acid or their combination[a]

	Comparative response during growth with potential mitigating treatments (Experiment 4)					
	Mean specific growth rate (ΔA_{600} h^{-1})		Maximum optical density (A_{600})		Rate of chlorate disappearance (μmol mL^{-1} h^{-1})	
Supplement	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
Without chlorate						
None	0.97	0.79	1.19	1.08	–	–
Molybdate	1.09	0.76	1.19	1.09	–	–
Shikimic acid	0.99	0.74	1.18	1.08	–	–
Combination	1.03	0.75	1.19	1.09	–	–
With chlorate						
None	0.57[b]	0.52[b]	0.52[b]	0.39[b]	4.39	5.51
Molybdate	0.64[b]	0.57[b]	0.48[b]	0.36[b]	6.61	4.21
Shikimic acid	0.65[b]	0.54[b]	0.50[b]	0.34[b]	4.18	4.79
Combination	0.61[b]	0.61	0.47[b]	0.37[b]	7.38	4.50

[a] Cultures in triplicate were grown aerobically in nitrate supplemented (5 mM) tryptic soy broth without or with 5 mM added chlorate and with or without treatments of 1 mM sodium molybdate, 0.34 mM shikimic acid or their combination. SEM = 0.04, 0.01 and 1.84 from a general analysis of variance of mean specific growth rates, maximum optical densities and rates of chlorate disappearance, respectively.
[b] Least squares means within columns differ ($P < 0.05$) from controls incubated without added chlorate.

the electron carrier function of various quinols may explain the decreased growth efficiency observed during respiratory growth in the absence of added chlorate but with nitrate as an electron acceptor (Fig. 2B). It is possible, however, that chlorate may have been reduced in our aerobic cultures by aerobically expressed nitrate reductase NarZ, which is also purported to reduce chlorate [31], or possibly via expression of NarA in oxygen deficient areas of the media as the tubes were not continually agitated.

3.3. Effects of molybdate, shikimate or their combination on growth and resistance during anaerobic and aerobic growth

To further investigate the toxicity of chlorate, *S.* Typhimurium was cultured similar to that in our first experiment except with or without additions of shikimic acid, molybdate or a combination of these compounds. In this study (Experiment 4), all cultures with added chlorate, regardless of whether treated or not with shikimic acid, molybdate or their combination, had achieved equivalent lower maximum specific growth rates (Table 3), lower maximum optical densities and lower total viable *S.* Typhimurium than controls cultured without chlorate (Fig. 3). Consistent with observations from our first experiment, nitrate reductase deficient *S.* Typhimurium were not detected in any of the nonchlorate-supplemented cultures or from any of the chlorate-supplemented cultures at the 0 h sampling time (Fig. 3). In aerobic cultures, nitrate reductase deficient *S.* Typhimurium were not detected after 4 h of incubation and were recovered at nearly equivalent numbers from all the cultures with added chlorate at 8 h of incubation (Fig. 3A). After 24 h of aerobic incubation, nitrate reductase deficient *S.* Typhimurium were recovered only from chlorate-supplemented cultures treated with neither or with both shikimic acid and molybdenum (Fig. 3A). However, only in the cultures treated with both shikimic acid and molybdate were numbers of nitrate reductase deficient *S.* Typhimurium significantly higher than the limit of detection value determined in controls grown without added chlorate (Fig. 3A). In cultures incubated anaerobically, nitrate reductase deficient *S.* Typhimurium were recovered in significantly higher numbers than from controls incubated without added chlorate after 8 h and 24 h incubation but were similar between treatments (Fig. 3B). As done in Experiment 1, a comparison of the regression line obtained from a theoretical logarithmic growth curve, estimated via the propagation of 12 ± 6.8 ($1.1 \log_{10}$) mL^{-1} resistant cells initially present, to that from counts observed during anaerobic culture with added chlorate revealed that the lines did not differ ($P > 0.05$).

Our observation of decreased recovery of nitrate reductase deficient *S.* Typhimurium from chlorate-supplemented aerobic cultures treated with molybdate alone (Fig. 3B) is consistent with earlier findings that when provided in excess, as done here, molybdate restored nitrate reductase activity in *E. coli* mutants deficient in molybdenum uptake *modABCD* (*chlD*) thereby overcoming chlorate resistance [14].

The transient appearance of nitrate reductase deficient *S.* Typhimurium at 8 h but not at 24 h may be reflective of greater proportional amounts of functional enzyme produced during later stages of growth as NarZ expression is increased as cells approach or are in stationary phase [32]. Molybdate did not restore chlorate sensitivity during anaerobic culture with chlorate but the reason for this is not clear. We speculate that the more abundant expression of NarA during anaerobic growth may have rapidly consumed available molybdenum or overwhelmed the capacity of the culture to incorporate molybdenum. Alternatively, it is possible that differences exist in the uptake and incorporation of molybdenum during the different growth conditions or that anaerobiosis may differentially select for chlorate resistance not caused by defects in molybdenum uptake. Our finding that initial rates of chlorate

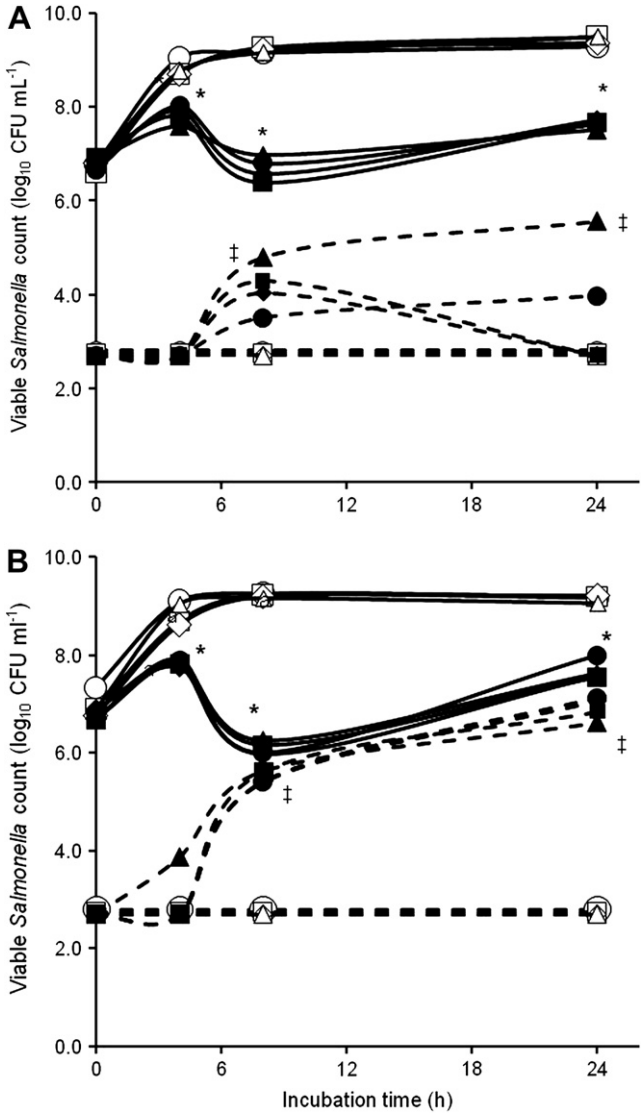

Fig. 3. Viable cells' counts of *S.* Typhimurium grown aerobically (A) or anaerobically (B) in nitrate supplemented (5 mM) BHI broth without (open symbols) or with (closed symbols) 5 mM added chlorate (Experiment 4). Cultures were also treated without (circles) or with 0.34 mM shikimic acid (squares), 1 mM molybdate (diamonds) or their combination (triangles). Total viable counts (solid lines) with values $\leq$ * denotation and nitrate reductase deficient counts (dashed lines) with values $\geq$ ‡ denotation differ from controls incubated without added chlorate; SEM were ≤ 0.14 and 0.24 for total viable counts and ≤ 0.43 and 0.38 for nitrate reductase deficient counts during aerobic and anaerobic growths; respectively.

disappearance (Table 3) and amounts of chlorate consumed (20% of initial 457.5 ± 59.3 ppm) did not differ between aerobic or anaerobically grown cultures argues against that former hypothesis. However, quantitative aspects of chlorate metabolism within *Salmonella* and *E. coli* have not been thoroughly investigated and it is possible that our measurements of chlorate disappearance reflect intracellular uptake of chlorate rather than its reduction to chlorite. Unlike nitrate, uptake of chlorate is not influenced by oxygen [33].

Newman et al. [26] also showed that molybdate additions were able to restore nitrate reductase activity and hence chlorate susceptibility in aerobically grown *E. coli*. They further showed that additions of shikimic acid alleviated chlorate toxicity presumably by restoring aromatic amino acid biosynthesis otherwise inhibited by nitrate reductase-mediated reduction of chlorate [26]. In our study, however, shikimic acid did not lessen chlorate toxicity either aerobically or anaerobically and appeared to counteract molybdate restoration of

nitrate reductase activity during aerobic growth as evidenced by higher recovery of nitrate reductase deficient *S.* Typhimurium from cultures co-treated with both shikimic acid and molybdate (Fig. 3B). A possible explanation for the antagonism observed with co-addition of shikimic acid and molybdate may be that biosynthesis of the functional quinone electron carriers may also be enhanced as shikimic acid is an intermediate for the production of chorismate. Chorismate is a common intermediate for production of phenylalanine, tryptophan, tyrosine and for ubiquinone and menaquinone [29,34]. Alternatively, as a central intermediate in the biosynthetic shikimic acid pathway, which includes branches for the synthesis of the aromatic amino acids, quinols, folate and other compounds [35], it is conceivable that different metabolic demands could dictate how shikimic acid is used. It is also possible that molybdate supplementation could shift the direction of shikimic acid use from one biosynthetic branch to another that contains a key molybdoenzyme. In addition, a major difference between our study and that of Newman et al. [26] is the species of bacteria investigated: we tested *S.* Typhimurium while the Newman group examined *E. coli*.

In summary, chlorate differentially inhibited growth of *S.* Typhimurium during anaerobic and aerobic culture, with inhibition occurring more rapidly in anaerobically grown cultures. Chlorate resistant *S.* Typhimurium, as defined by a deficiency in nitrate reductase activity, were recovered from all chlorate-supplemented cultures but propagated to a much higher level during anaerobic culture, most likely due to the selective propagation of low numbers of *S.* Typhimurium exhibiting spontaneous resistance to chlorate. The propagation of chlorate resistant populations within the gut of animals administered chlorate would naturally be problematic as far as maintaining efficacious control of targeted pathogens. From a practical perspective, however, this occurrence may be unlikely in mixed populations as Callaway et al. [36] reported that a chlorate resistant *E. coli* O157:H7 strain did not persist when experimentally inoculated into continuous flow cultures of mixed ruminal microbes. In the presence of chlorate, an uncharacterized nitrate reductase deficient *S.* Typhimurium appeared to be as fit as nitrate proficient *S.* Typhimurium grown in glucose limited medium lacking chlorate as long as energy was conserved via fermentative metabolism. However, Callaway et al. [36] observed that when pure cultures of *E. coli* O157:H7 were desensitized to chlorate during broth culture in an energy rich medium containing added chlorate, the population was rapidly resensitized upon subsequent transfer to broth lacking chlorate, presumably via enrichment of chlorate sensitive populations within the culture. Thus, it is reasonable to suspect that chlorate resistance may impede an organism's competitive fitness during growth in a less restrictive medium or in the presence of competing organisms. From a theoretical perspective, this seems likely as genetic defects in molybdenum uptake or molybdoenzyme synthesis responsible for chlorate resistance can also affect the function of other molybdenum containing enzymes such as formate dehydrogenase [6,18]. Selective propagation of chlorate resistant *S.* Typhimurium was not observed to the same extent during aerobic culture presumably because these bacteria had opportunity to respire on oxygen. Molybdate or shikimic acid added individually, but not in combination, was able to restore chlorate sensitivity in aerobic cultures, but not in anaerobic cultures. The latter result is consistent with earlier observations reported by Callaway et al. [36].

4. Conflict of interest

Mention of a trade name, proprietary product, or specific equipment do not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

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