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ARTICLE

Chlorate Metabolism in Pure Cultures of *Escherichia coli* O157:H7 Pretreated with either Nitrate or Chlorate

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Previous research has suggested that nitrate-respiring pathogens such as *Escherichia coli* O157:H7 and *Salmonella* spp. are susceptible to chlorate salts due to the conversion of chlorate to chlorite by respiratory nitrate reductase. This study was conducted to determine the effect of chlorate on *E. coli* O157:H7 growth and chlorate biotransformation and to determine whether chlorite is produced in anaerobic culture of *E. coli* O157:H7. Final concentrations of *E. coli* O157:H7 were generally decreased by about 2 log units in incubations containing ≥5 mM chlorate, except when bacteria were pretreated with 10 mM chlorate. [^{36}Cl]Chlorate metabolism by pure cultures of *E. coli* O157:H7 was not measurable above chlorate concentrations of 5 mM, but measurable chlorate reduction occurred in cultures containing 0.5, 1, or 5 mM [^{36}Cl]chlorate. Pretreatment of *E. coli* O157:H7 with 5 mM nitrate did not increase the rate of chlorate conversion to chloride, suggesting that nitrate did not induce nitrate reductase isoforms capable of metabolizing chlorate in *E. coli* O157:H7. Pure cultures of *E. coli* O157:H7 preconditioned with 10 mM chlorate had an attenuated ability to transform [^{36}Cl]chlorate to [^{36}Cl]chloride with measurable chlorate reduction only occurring in 0.5 mM chlorate treatments. The hypothesis that *E. coli* O157:H7 is sensitive to chlorate by virtue of the reduction of chlorate to chlorite ion (ClO_2^-) was supported, but not proven, by the direct measurement of low concentrations of [^{36}Cl]ClO_2^- in incubation media containing 0.5 mM [^{36}Cl]ClO_3^-. Collectively these results indicate that growth of *E. coli* O157:H7 in pure culture will be reduced in the presence of 5 mM or greater concentrations of sodium chlorate and that *E. coli* O157:H7 is capable of producing chlorite ions during the metabolism of chlorate.

KEYWORDS: Chlorate; nitrate; chlorite; *E. coli*; food safety

INTRODUCTION

Throughout the history of sodium chlorate's ($NaClO_3^-$; **Table 1**) use as a herbicide, chlorite (ClO_2^-) or other chlorate reduction products such as chlorine dioxide (ClO_2) or hypochlorite ion (ClO^-) have been suspected as the toxic factor(s) associated with chlorate's activity (*1*). Similarly, chlorate's bactericidal activity in nitrate-respiring bacteria (*2*−*4*), including the economically important strains of *Escherichia coli* and *Salmonella*, has been hypothesized to be related to the enzymatic formation of chlorate reduction products. Pathogenic strains of these facultative anaerobes are the causative agents of an estimated 1.5 million cases of foodborne enteric diseases each year in the United States (*5*), and their continued presence in and on food animal products represents a risk to consumers worldwide. Tamási and Lantos (*6*) first demonstrated in 1983 that chlorate salts have the potential to reduce the concentrations of *E. coli* and *Salmonella* in mixed cultures of cattle wastes. Subsequently, the concept of using chlorate to reduce the burdens of enteropathogens in live food-animal species has been proposed (*7*), and this notion has been advanced for poultry (*8*−*10*), swine (*11*, *12*), sheep (*13*, *14*), and cattle (*15*−*17*). The mechanism through which chlorate salts reduce or eliminate the enteropathogens in food animals is believed to be through the intracellular, enzymatic reduction of chlorate to chlorite (*18*), although antimicrobial activity through chlorate reduction to either chlorine dioxide or hypochlorite cannot be ruled out.

In vertebrate food animals, chlorite has not been identified as a stable metabolite of chlorate. For example, chlorite residues have not been measured in tissues or excreta of farm animals treated with [^{36}Cl]chlorate (*19*−*22*) even though chlorite was reported by Abdel-Rahman et al. (*23*, *24*) to be a metabolite excreted into the

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Table 1. Oxidation States of Chlorine [Adapted from Gordon (*39*)]

oxidation state	species	formula
+7	perchlorate ion	ClO_4^-
+5	clorate ion	ClO_3^-
+4	chlorine dioxide	ClO_2
+3	chlorite ion	ClO_2^-
	chlorous acid	$HClO_2$
+1	hypochlorite ion	OCl^-
	hypochlorous acid	HOCl
0	chlorine	Cl_2
−1	chloride ion	Cl^-

urine of [^{36}Cl]chlorate-dosed rats. Subsequent studies of chlorate metabolism in rats (*25*) were not able to replicate the findings of Abdel-Rhaman (*23*, *24*), but did demonstrate that chlorite is fairly unstable in rat urine. Further studies with [^{36}Cl]chlorite have demonstrated that its half-life was only 4.5 min in bovine ruminal fluid (*26*). In bacterial systems, studies supporting the bacterial reduction of chlorate to chlorite have relied on relatively insensitive (paper chromatography) and (or) nonspecific, indirect analyses (titration) for measurement of chlorite (*2*, *4*, *27*). These studies, on which the basis of the chlorate-to-chlorite hypothesis rests, have reported the formation of millimolar concentrations of chlorite in test media.

Given the difficulty of directly demonstrating the formation of chlorite in biological systems and the role that nitrate reductases presumably play in bacterial chlorate reduction (*18*), it was of interest to revisit the hypothesis that chlorite is produced by nitrate-respiring bacteria in sufficient quantities for measurement. Therefore, the objectives of this study were (1) to determine the effect of nitrate or chlorate pretreatment on the viability of pure cultures of *E. coli* O157:H7 in various levels of chlorate, (2) to determine the effect of nitrate and chlorate pretreatment on the metabolism of chlorate in pure culture, and (3) to provide data either supporting or refuting the hypothesis that chlorate is metabolized by *E. coli* O157:H7 to the toxic intermediate chlorite.

MATERIALS AND METHODS

Radioactive sodium chlorate (0.575 mCi/mmol; Ricerca Biosciences, Columbus, OH; radiochemical purity, 94.6% [^{36}Cl]chlorate, remainder [^{36}Cl]chloride) was diluted with unlabeled sodium chlorate (EKA Chemical Co., Marietta, GA) to a specific activity of 150 dpm/μg for 1, 5, 15, and 30 mM preparations of sodium [^{36}Cl]chlorate in tryptic soy broth (TSB) and to a specific activity of 5000 dpm/μg for use in 0.5 mM sodium [^{36}Cl]chlorate treatments. Corrections were made for the radiochemical purity of the source sodium [^{36}Cl]chlorate when stock TSB solutions were prepared. Sodium [^{36}Cl]chlorite having a specific activity of 13920 dpm/μg and a radiochemical purity of >99% was synthesized in-house from sodium [^{36}Cl]chlorate as described by Hakk et al. (*25*).

Novobiocin and nalidixic acid resistant *E. coli* O157:H7 (*7*) were propagated overnight in 40 mL of TSB (controls), TSB containing 5 mM sodium nitrate (to induce respiratory nitrate reductase), or TSB containing 10 mM sodium chlorate (to select for chlorate-insensitive bacteria (*28*)). Novobiocin (an aminocoumarin) and nalidixic acid (a quinolone) resistance is conferred to bacteria through mutations of DNA gyrase, and resistance is not linked to nitrate reductase activities (*29*, *30*). The resistant strain was used because resources to isolate and characterize wild-type *E. coli* O157:H7 were not readily available. Aliquots (100 μL) of bacterial cultures were diluted into 9.9 mL of TSB, each dilution was mixed, and each was used to inoculate respective batch vials (n = 126 for each enrichment) containing 10 mL of TSB fortified with 0, 0.5, 1, 5, 15, or 30 mM sodium [^{36}Cl]chlorate (21 vials per chlorate level). Triplicate samples were harvested after 0, 2, 4, 8, 12, 16, and 24 h of incubation at 37 °C, and *E. coli* O157:H7 was quantified after dilution in phosphate-buffered saline (10^{-1}−10^{-8}) and plating onto MacConkey agar (limit of quantitation = 10 cfu/mL). Sampling occurred during and after logarithmic growth. Sample aliquots that were not used for quantifying *E. coli* O157:H7 were diluted 1:1 with isopropyl alcohol, flash frozen in a dry ice acetone bath, and subsequently analyzed for [^{36}Cl]chlorate content. Such treatment effectively inactivated the bacterial cells as direct plating of thawed samples on MacConkey agar did not yield colonies.

Parent [^{36}Cl]chlorate and [^{36}Cl]chloride were quantified after precipitation of [^{36}Cl]chloride with silver nitrate as described by Oliver et al. (*26*); reported chlorate concentrations are corrected for the starting radiochemical purity (i.e., T0 incubations contained 5.6% [^{36}Cl]chloride). Studies counting radioactivity in incubation supernatant before and after pelleting bacterial cells by centrifugation revealed no difference in supernatant activity. Thus, radioactivity was not sequestered in bacterial cells. [^{36}Cl]Chlorite was measured at sample harvest in 0.5 or 15 mM [^{36}Cl]chlorate-treated cells by immediately removing 1 mL aliquots of incubation supernatant, filtering through a 0.45 μm PTFE filter, and direct injection of 100 μL of filtrate onto Dionex 11HC guard and analytical anion exchange columns. Chlorite, chloride, and chlorate were eluted (1 mL/min) using isocratic 5 mM NaOH (0−12 min) followed by a gradient to 20 mM NaOH from 12 to 15 min, with a final hold of 20 mM NaOH for 10 min. Radioactive analytes were detected with a Packard Flow Scintillation analyzer 500 TR using Ultima Flow AP LSC fluid at a flow rate of 2 mL/min. Peaks were integrated using Packard software. Limit of detection (LOD) for the ion chromatographic determination of [^{36}Cl]chlorite was approximately 60 ng/mL (about 30 dpm above background) with limits of quantification set at 10× the LOD, or 600 ng/mL (~1% of injected radiochlorine; about 300 dpm above background). Paper chromatographic resolution of [^{36}Cl]chlorite, [^{36}Cl]chloride, and [^{36}Cl]chlorate was performed as described in the source literature (*2*, *4*) except that Whatman no. 1 paper was substituted for the Munktell 20/150 paper used by Goksøyr (*2*). Approximately 10000−25000 dpm (10−25 μL) of each ion was spotted prior to developing plates with the appropriate solvent system. Detection of radiocarbon on paper chromatographic strips was achieved using a Bioscan System 200 (Bioscan, Washington, DC); each lane was counted for 1 h. Data were collected, downloaded as ASCI-II files, and plotted using GraphPad Prism 5.02 (San Diego, CA).

Bacterial counts were analyzed within pretreatment (i.e., TSB only; nitrate, or chlorate) by time and dose by two-way analysis of variance. When appropriate, differences in means of control (no chlorate) values and chlorate-treated cultures were determined by the Bonferonni method. Within the TSB-only, nitrate, and chlorate pretreatments, data across chlorate dose (excluding the 0.5 mM chlorate dose) were pooled and means at each time were compared to determine if, within pretreatment category, overall effects of chlorate were evident. The 0.5 mM chlorate dose was excluded from pooling because bacterial numbers were clearly intermediate between the zero-chlorate level and cultures containing ≥1 mM chlorate (**Figure 1**). Such pooling was appropriate because significant ($P > 0.05$) interactions of time and dose were not detected in the original data set.

Percentages of chlorate remaining in incubation cultures were analyzed within pretreatment (TSB only, nitrate- and chlorate-adapted) by two-way analysis of variance. Within each time period, the percentages chlorate remaining in the 0.5, 1, 5, and 15 mM chlorate incubations were compared to the percentage chlorate levels remaining in the 30 mM chlorate incubation. Visual inspection of the data (**Figure 2**) clearly shows that insignificant percentages of the 30 mM chlorate incubations were degraded, regardless of the treatment. Statistical values were calculated using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA).

To prove that the expression of chlorite dismutase could not contribute to chlorate resistance in *E. coli* O157:H7, its expression was probed using polyclonal antibodies against chlorite

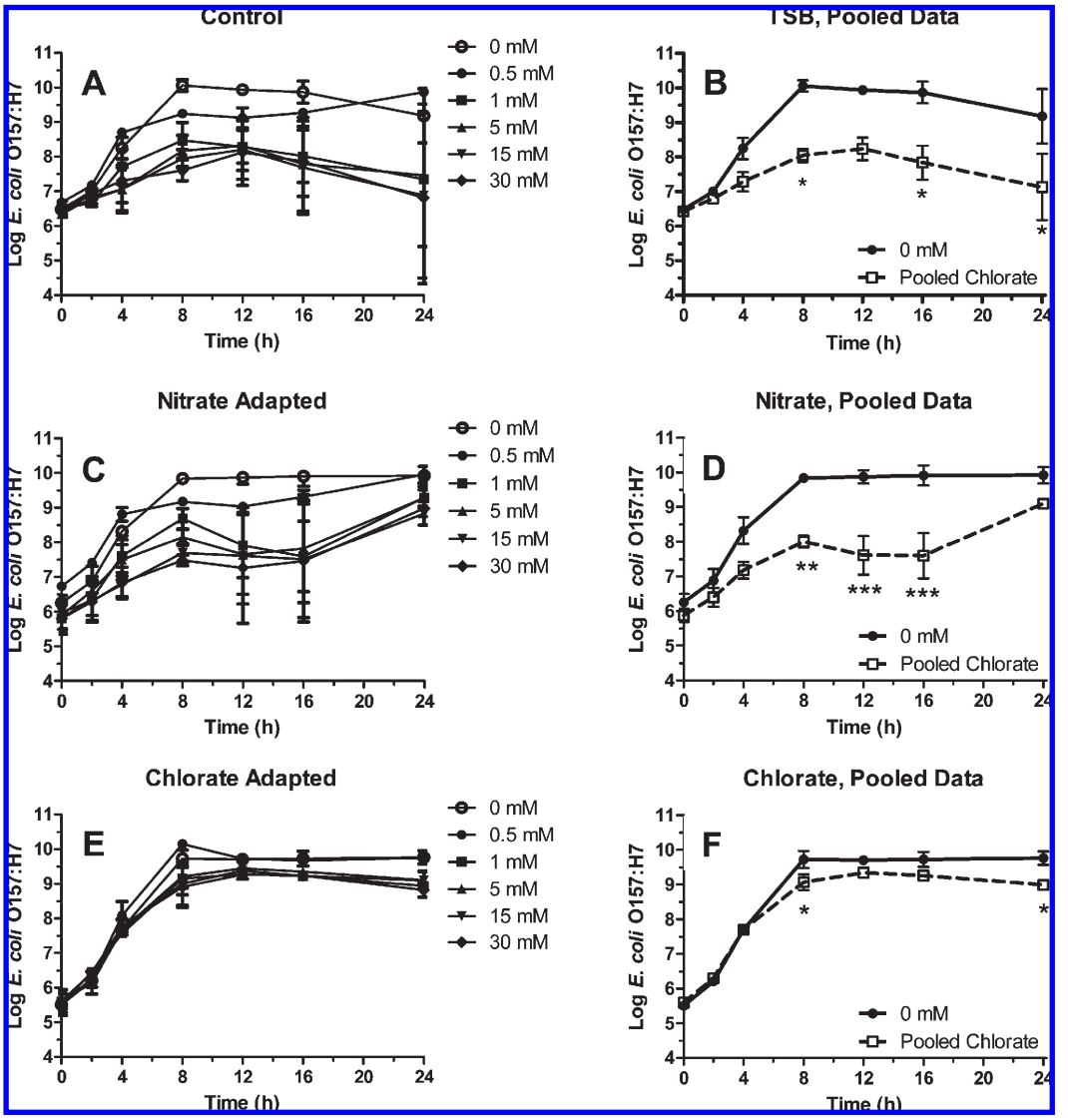

Figure 1. Growth of *E. coli* O157:H7 preconditioned in tryptic soy broth (controls; **A**, **B**), tryptic soy broth containing 5 mM sodium nitrate (**C**, **D**), and tryptic soy broth containing 10 mM sodium chlorate (**D**, **E**). Panels **B**, **D**, and **F** show the overall (pooled) effects of 1, 5, 15, and 30 mM chlorate on bacterial numbers. Data are plotted as means ± SEM (∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$).

dismutase as described by O'Connor and Coates (*31*). Briefly, *E. coli* O157:H7 was propagated in TSB, TSB containing 5 mM nitrate, and TSB containing 10 mM sodium chlorate. A positive control organism, *Dechloromonas agitata* (ATCC 700666), known to express chlorite dismutase (*31*), was propagated in ATCC medium 2106. Cells were collected by centrifugation (15000*g*), rinsed in phosphate-buffered saline, and lysed at 20000 psi with a French press. Cell lysates were centrifuged, and the supernatant protein concentration was quantified using the Bradford method (*32*) for the immunological analysis of chlorite dismutase by Western blot. Proteins from cell lysates were separated by SDS-PAGE (12% polyacrylamide) using the mini-Protein II system (Bio-Rad, Hercules, CA). Proteins were separated at 35 mA for approximately 1 h in Tris/glycine/SDS buffer (0.025 M Tris base, 0.192 M glycine, 0.1% SDS, pH 8.3) and subsequently transblotted onto Immobilon P transfer membrane (Millipore Corp., Bedford MA) using mini-transblot electrophoretic transfer cells (Bio-Rad) in Towbin buffer (0.025 M Tris base, 0.192 M glycine, pH 8.3, 20% methanol; 350 mA for 1 h). The nonspecific binding sites of the transferred membrane were blocked by incubating the membrane for 30 min at room temperature with 1 mg/mL bovine serum albumin mixed into phosphate-buffered saline and 0.1% Tween 20 (PBST). The membrane was then incubated with chlorite dismutase antibody (J. Coates, University of California at Berkeley) at a 1:500 dilution for 60 min at room temperature. The membrane was washed five times with PBST and then incubated with anti-rabbit IgG−horseradish peroxidase (Sigma-Aldrich Corp., St. Louis, MO) at a 1:500 dilution for 60 min at room temperature. The membrane was again washed five times with PBST, metal-enhanced 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate (Pierce Biotechnology, Inc., Rockford, IL) was added, and the mixture was incubated at room temperature for 10 min before the reaction was stopped with water.

RESULTS

Effects of Chlorate on *E. coli* O157:H7 in Pure Culture. Figure 1 shows the effects of nitrate or chlorate adaptation and chlorate concentration on the growth of pure cultures of *E. coli* O157:H7 in TSB. When bacteria were propagated in TSB and then inoculated into unfortified TSB or TSB containing 0.5, 1, 5, 15, or 30 mM chlorate, bacterial numbers increased through 8 h regardless of chlorate concentration. Although bacteria grown in TSB without chlorate (**Figure 1A**) were roughly 2 log units more concentrated than bacteria grown in TSB containing 1, 5, 15, and 30 mM chlorate at 8−16 h, statistical differences did not occur

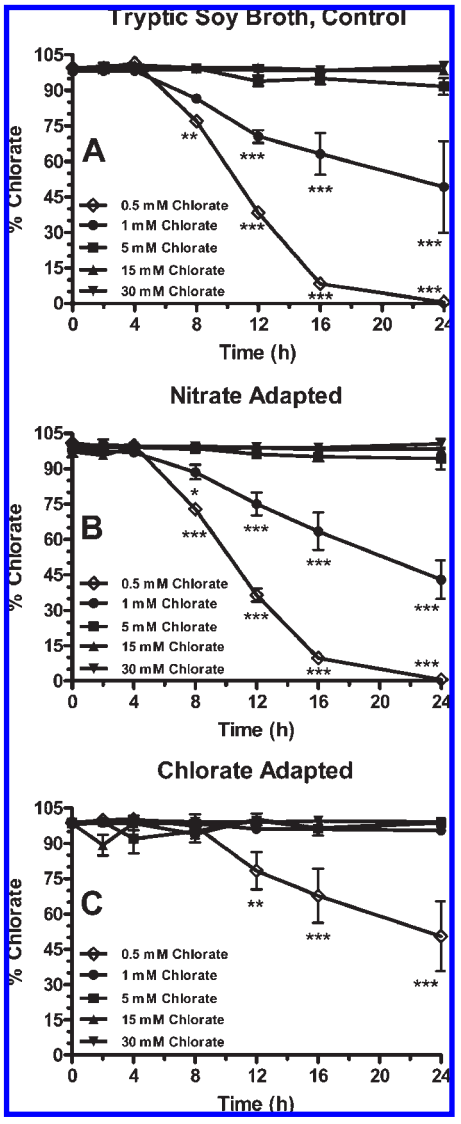

Figure 2. Metabolism of 0.5, 1, 5, 15, and 30 mM [^{36}Cl]chlorate in control (**A**) cultures of *E. coli* O157:H7 and in cultures adapted to 5 mM nitrate (**B**) or 10 mM chlorate (**C**). Data are presented as means ± SEM (n = 3; ∗ P < 0.05; ∗∗, P < 0.01; ∗∗∗, P < 0.001).

($P > 0.05$) at any time point. Numbers of bacteria grown in 0.5 mM chlorate were intermediate relative to those grown in control TSB and in TSB containing 1 mM chlorate or greater. Because no interactions were present ($P > 0.05$), concentrations of bacteria in the 1, 5, 15, and 30 mM chlorate treatments were pooled within time (**Figure 1B**) and compared to controls. When the pooled data were compared to controls, concentrations of bacteria grown in chlorate were less ($P < 0.05$) than those grown in control TSB at 8, 16, and 24 h.

In cells preconditioned with 5 mM nitrate (**Figure 1C**), *E. coli* O157:H7 concentrations grown in 1 mM chlorate were less than controls ($P < 0.05$) at 16 h and were less than controls at 12 h when grown in 5 mM chlorate. Likewise, bacterial numbers were less than controls at 12 and 16 h when grown in 15 mM chlorate and less than controls at 8, 12, and 16 h in the presence of 30 mM chlorate. By 24 h, there was no effect ($P > 0.05$) of chlorate on *E. coli* O157:H7 concentrations of nitrate-propagated bacteria. Again, because no time−chlorate concentration interactions occurred ($P > 0.05$), the 1, 5, 15, and 30 mM chlorate treatments were pooled and compared to control incubations (**Figure 1D**). Overall, chlorate decreased ($P < 0.01$) *E. coli* O157:H7 growth

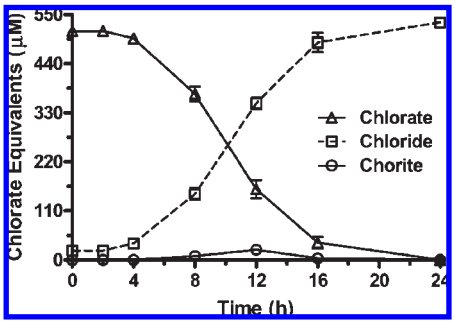

Figure 3. Metabolism of 0.5 mM [^{36}Cl]chlorate of control and nitrate-adapted (data combined for clarity) *E. coli* O157:H7. Data are presented as means ± SD (n = 3) of micromolar concentrations of chlorite, chloride, and chlorate (chlorate equivalents). Only one of the three observations at 16 h had a quantifiable chlorite level.

rates so that at 8, 12, and 16 h chlorate-treated microbes were less concentrated than controls; by 24 h no differences ($P > 0.05$) between chlorate-treated and untreated cells occurred. In cells propagated in 10 mM chlorate (**Figure 1E**), subsequent incubation in 1 mM chlorate reduced ($P < 0.05$) *E. coli* O157:H7 concentrations relative to controls at 24 h, and 30 mM chlorate reduced ($P < 0.05$) *E. coli* O157:H7 concentrations at 8 and 24 h relative to incubations containing no chlorate. When 1, 5, 15, and 30 mM chlorate treatments were pooled (**Figure 1F**), chlorate decreased ($P < 0.05$) *E. coli* 0157:H7 at 8 and 24 h relative to controls. Mean concentrations of *E. coli* O157:H7 in control and nitrate-treated incubations were similar ($P > 0.05$) at 0, 2, 4, 8, 12, and 16 h (bacterial numbers across chlorate level were pooled). At 24 h, however, *E. coli* concentrations were greater ($P < 0.05$) in the nitrate treatment than the control treatment.

Chlorate Metabolism by Nitrate or Chlorate Preconditioned Cells. Figure 2 shows the reduction of 0.5, 1, 5, 15, and 30 mM [^{36}Cl]chlorate as a function of time by *E. coli* O157:H7 adapted in control TSB, 5 mM nitrate in TSB, and 10 mM chlorate in TSB. Chlorate reduction to chloride was complete in incubations containing 0.5 mM chlorate (**Figure 2A**,**B**) for the control and nitrate-adapted bacteria. In chlorate-adapted bacteria (**Figure 2C**), the only indication of significant ($P < 0.01$) chlorate reduction occurred in the 0.5 mM treatment, but reduction was not significant until 12 h, whereas chlorate reduction in 0.5 mM treatments of control and nitrate-adapted bacteria was significant ($P < 0.05$) at 8 h. Significant ($P < 0.05$) reduction of 1 mM chlorate occurred in control and nitrate-adapted cells by 8−12 h, but was not different ($P > 0.05$) from controls in chlorate-pretreated cells. Visual inspection of the data from the control and nitrate-adapted treatments suggests that chlorate tended to be reduced at the 5 mM concentrations, but the degree of reduction was not significant ($P > 0.05$).

Chlorite Production by *E. coli* O157:H7. Figure 3 shows the relationship between chlorate, chlorite, and chloride in 0.5 mM chlorate incubations from control and nitrate-enriched bacteria. [^{36}Cl]Chlorite was quantifiable by ion chromatography (**Figure 4**) with radiochemical detection in replicates (n = 3) at 8 and 12 h; at 16 h, chlorite was quantifiable in only one of the three replicates. Maximal chlorite concentrations, which occurred at 12 h, were 23 ± 8.1 μM. Chlorite was not detectable at any time point in any of the incubations containing 15 mM chlorate (data not shown). Chlorite measurements were not performed in 0.5 mM incubation of [^{36}Cl]chlorate in chlorate-pretreated bacteria because preliminary experiments provided no evidence of chlorate metabolism in chlorate pretreated cells.

Chromatographic Resolution of [^{36}Cl]Chlorite, [^{36}Cl]Chloride, and [^{36}Cl]Chlorate. Figure 4 shows radiochromatograms of [^{36}Cl]-chlorite, [^{36}Cl]chloride, and [^{36}Cl]chlorate standards (upper left

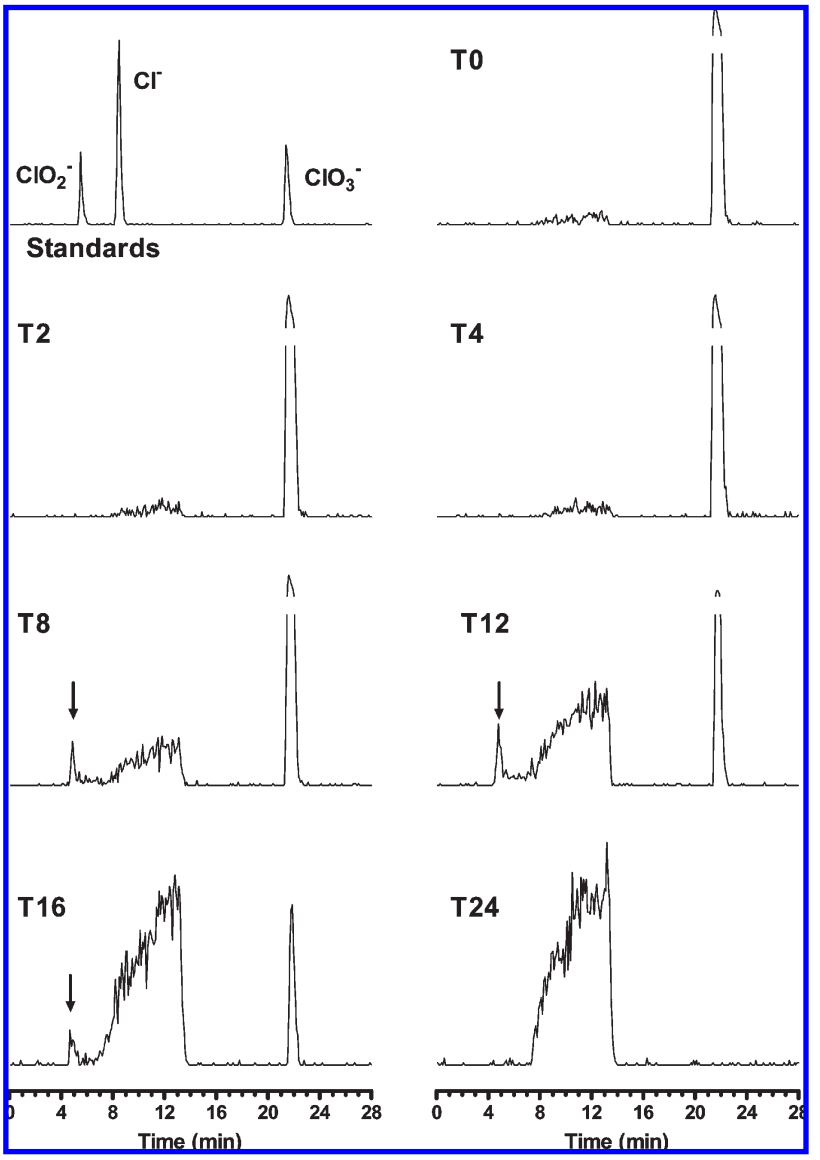

Figure 4. Radiochromatograms of [^{36}Cl]chlorite, [^{36}Cl]chloride, and [^{36}Cl]chlorate standards (top left) and of 0.5 mM [^{36}Cl]chlorate incubated with *E. coli* O157:H7 in TSB for 0, 2, 4, 8, 12, 16, or 24 h. In the metabolic incubations [^{36}Cl]chloride elutes as a very broadband from about 7 to 14 min because the sodium chloride content of TSB is approximately 86 mM and [^{36}Cl]chloride is diluted by unlabeled chloride. The arrows in T8, T12, and T16 show the elution of [^{36}Cl]chlorite ion. The response scales of chromatograms for T0, T2, T4, T8, and T12 are split so that the lower 80% of each scale is normalized to the response scales of T16 and T24.

chromatogram) separated by ion chromatography. Standards were well resolved with excellent separation between chlorite and chloride ions. Subsequent chromatograms present in **Figure 4** show the progressive metabolism of 0.5 mM [^{36}Cl]chlorate to [^{36}Cl]chloride over time (T0, T2, T4, T8, T12, T16, and T24) by cultures of *E. coli* O157:H7. Noteworthy in the chromatographic progression is the decrease in chlorate content with the concomitant increase in the broad chloride peak (eluting from about 7 to 14 min). The chloride peak is broad because the chloride content of TSB is approximately 86 mM and the [^{36}Cl]chloride formed from [^{36}Cl]chlorate during the progression of the incubation period is diluted by the unlabeled chloride. Nevertheless, the progressive increase of [^{36}Cl]chloride over the course of the experiment is clearly observed, consistent with data from the silver nitrate assay (chloride curve) presented in **Figure 3**. Although small, the presence of [^{36}Cl]chlorite is clearly indicated at 4−5 min in the chromatograms of T8, T12, and T16 h incubations.

In contrast, paper chromatographic systems used previously to identify chlorite production in bacterial cultures (*2*, *4*) were unable to clearly resolve radiochemically pure (> 95%) standards of [^{36}Cl]chlorate, [^{36}Cl]chlorite, or [^{36}Cl]chloride, (**Figure 5**). The chromatographic system of Sánches-Crispín (*4*) seemed to have the greatest resolving power of the three systems tried, but the chromatographies of chlorate and chloride were ambiguous as chlorate and chloride each separated into mutually exclusive peaks. Separation of each ion into separate peaks was likely due to an ion-pairing or salt-forming phenomenon.

Chlorite Dismutase Expression by *E. coli* O157:H7 and *D. agitata*. It could be argued that the chlorate resistance observed by Callaway et al. (*28*) and replicated in this study, which occurs in pure cultures of *E. coli* O157:H7 after exposure to chlorate, might occur by the induction of chlorite dismutase. Chlorite dismutase induction would mask the production of chlorite and would also render bacteria resistant to the effects of chlorate. Western blot analyses demonstrated that chlorite dismutase was not present in control *E. coli* O157:H7 or in *E. coli* O157:H7 preconditioned with either nitrate (5 mM) or sodium chlorate (10 mM). Whereas Western blots of cell lysates of *D. agitata*

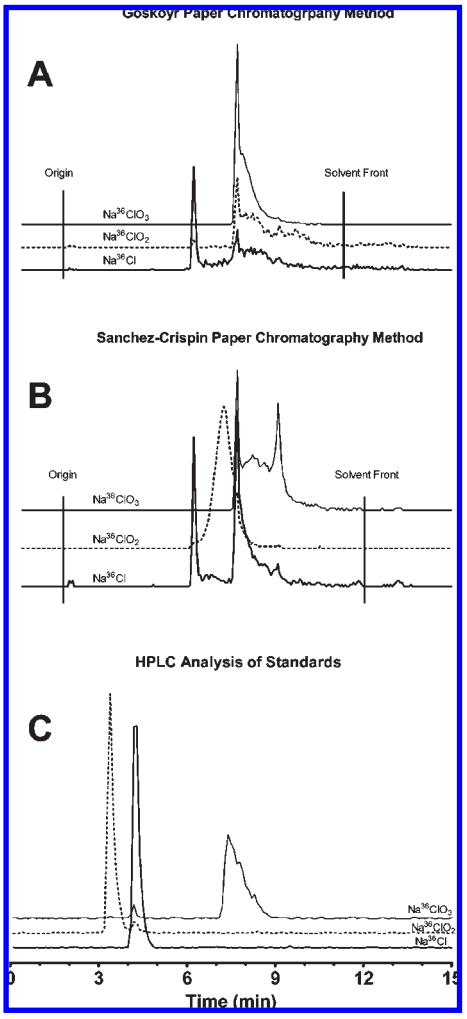

Figure 5. Paper chromatographic resolution of [^{36}Cl]chlorate (top line of each graph), [^{36}Cl]chlorite (middle line of each graph), and [^{36}Cl]chloride according to the methods of Goksøyr (*2*) (**A**) and Sánches-Crispín et al. (*4*) (**B**), and contemporaneously measured radiochemical purity (**C**) of each standard by ion chromatography with radiochemical detection.

grown in growth medium containing chlorate were strongly positive for the presence of chlorite dismutase (data not shown), similar Western blots of *E. coli* O157:H7 cell lysates stained only very lightly. Because the faint staining was not in the molecular weight range of chlorite dismutase (32000), this staining was interpreted as nonspecific binding.

DISCUSSION

Extensive evidence indicates that pathogenic strains of *E. coli* are sensitive to the effects of chlorate under a variety of conditions. For example, Callaway et al. (*28*) found that treatment of pure cultures of *E. coli* O157:H7 with >5 mM sodium chlorate decreased *E. coli* O157:H7 populations by 100-fold. These findings were verified in the current study (**Figure 1**). In vitro studies using mixed culture have consistently shown that *E. coli* O157:H7 populations are sensitive to chlorate concentrations greater than about 1 mM in buffered ruminal fluid (*7*), cattle feces (*15*), and swine feces (*12*). In live cattle, *E. coli* O157:H7 in intestines was decreased 100-fold by orally dosed chlorate (*15*), and chlorate was effective against *E. coli* O157:H7 in both ruminal fluid and fecal matter of cattle orally administered chlorate (*15*−*17*). In other food animals, orally dosed chlorate has decreased *E. coli* O157:H7 in both lambs (*13*, *14*) and pigs (*11*, *12*). Collectively, data over numerous studies indicate that chlorate salts tend to reduce concentrations of enteric *E. coli* O157:H7 by 2−3 log units within 12−24 h of dosing. As a result, chlorate salts are of interest as a potential preharvest food safety tool for use in live animals.

Co-administered nitrate enhances the bactericidal effects of chlorate on *Salmonella* in mixed cultures (*8*, *12*, *33*), and nitrate sometimes (*33*), but not always (*12*, *16*), enhances the bactericidal effects of chlorate on *E. coli* O157:H7. In both species, the presumed action of chlorate is through the induction of bacterial respiratory nitrate reductase. Respiratory nitrate reductase is believed to metabolize chlorate to chlorite, which is cytotoxic. In this study, pretreatment of *E. coli* O157:H7 with nitrate did not influence the susceptibility to chlorate relative to controls. It could be argued that nitrate induction might quickly be lost after the transfer of cells out of nitrate containing TSB and the subsequent rapid growth rates in fresh media. Nevertheless, initial responses to chlorate in nitrate-treated *E. coli* O157:H7 were not different from responses of *E. coli* O157:H7 grown in control TSB.

Oliver et al. (*26*) found that in vitro ruminal fluid cultures were capable of reducing moderate concentrations of chlorate (0.8 and 2.8 mM) during 24 h incubations. The degree of chlorate reduction (42% of total) for the 0.8 mM treatment of Oliver et al. (*26*) was comparable to the degree of chlorate reduction (51%) for the 1 mM treatment of this study (**Figure 2A**,**B**). In anaerobic artificial cattle lagoons, 0.94 mM (100 ppm) of sodium chlorate was completely reduced to chloride ion by 7 days (*34*), and in mixed cattle wastes (urine, feces, soil) 0.16, 0.31, and 0.63 mM sodium chlorate was metabolized to chloride ion with half-lives ranging from 0.1 to 29 days, depending upon temperature and whether aerobic or anaerobic conditions were present (*35*). Typically, chlorate half-lives were <1 day for cultures at 30 °C, <5 days for cultures at 20 °C, and 3−29 days for cultures at 5 °C. In the present study using pure cultures of bacteria, there was little evidence that sodium chlorate was metabolized to a significant degree when concentrations of sodium chlorate were ≥ 5 mM (532 ppm; **Figure 2**).

The fact that chlorite was a metabolic product of chlorate is consistent with earlier studies stating as much (*2*−*4*, *27*). As discussed by Oliver et al. (*35*), investigators that have previously measured chlorite formation in cultures of target organisms (*2*−*4*, *27*) have typically utilized indirect detection methods or have used methods that are inherently insensitive. For example, Quastel (*27*), Goksøyr (*2*), and Pichinoty (*3*) measured chlorite either qualitatively (*27*) or quantitatively (*2*, *3*) by using potassium iodide in acidic media with a starch indicator. According to Goksøyr (*2*) the chlorite measurements were "very insecure" due to variability and interferences. Neverthless, Goksøyr (*2*) measured chlorite concentrations of >5000 μM. Pichinoty et al. (*3*) also used potassium iodide titration to measure chlorite, but cautioned that too much acid could bias the assay and that the presence of bacterial cells would interfere with the assay. Pichinoty et al. (*3*) also indicated that they relied on the results of Goksøyr (*2*) as a validation of the specificity of the potassium iodide titration toward chlorite.

With regard to method specificity, Goksøyr (*2*) and Sanchez-Crispin (*4*) both relied on paper chromatography to identify chlorite produced in bacterial cultures. Goksøyr (*2*) stated that concentrations of chlorite required for detection were "abnormally high", with the result that the paper chromatography method "could not be used generally in this work". Five millimolar concentrations of chlorite were typically required for paper chromatographic detection. Sánchez-Crispín et al. (*4*), using potassium [^{36}Cl]chlorate, paper chromatography, and autoradiography, had nanogram-level sensitivity, but measured chlorite production only in membrane vesicles and a mutant strain of

E. coli. Notably, they did not detect chlorite in whole cell incubations of wild-type *E. coli* strains. In our hands, neither of the paper chromatography methods offered by Goksøyr (*2*) or Sánchez-Crispín et al. (*4*) provided satisfactory resolution of chloride, chlorite, and chlorate (**Figure 5**). Thus, the veracity of the data supporting the long-held hypothesis that chlorite is a measurable product of chlorate metabolism in nitrate-respiring bacteria remained questionable.

Method sensitivity is an issue for the detection of chlorite in bacterial culture. In this study, where chlorite was directly detected, only low quantities ($23 \pm 8\ \mu$M) of chlorite were present in pure culture at 12 h (**Figure 3B**), the time when chlorate reduction rates were maximal. Previously, we determined that the half-life of modest quantities (28 μM) of [^{36}Cl]chlorite under anaerobic culture conditions (live ruminal fluid) was about 4.5 min (*26*). In preliminary work for this study, we measured the stability of low concentrations (0.24 μM) of chlorite in TSB to ensure that, if formed, chlorite could be detected in our matrix; after 1 h in TSB, 86% of the starting chlorite remained (data not shown), indicating that the TSB itself would not quench the presence of chlorite. Because chlorite's reduction in live ruminal fluid is rapid and because chlorite stability in biological fluids is limited (*25*), we anticipated that the time from sampling anaerobic cultures to analysis would necessarily be short. For this reason, "raw" bacterial cultures were only prefiltered prior to analysis by ion chromatography with radiochemical detection. With the use of a high-purity (> 98%) [^{36}Cl]chlorite standard (*25*) and the direct detection of radioactivity, we were able to unambiguously determine the presence of chlorite in pure cultures of *E. coli* O157:H7 (**Figure 4**) and provide direct evidence for the formation of chlorite from chlorate by *E. coli* O157:H7. Obviously, the qualitative conclusion is not different from that of previous researchers (*2*, *27*) but, in accordance with the data of Sánchez-Crispín et al. (*4*), we believe that the magnitude of chlorite production in susceptible bacteria is less than previously reported (i.e., mM concentrations of chlorite were not present in incubation media) and, once formed, chlorite will be reduced to chloride in anaerobic systems. Goksøyr (*2*), who reported the formation of millimolar quantities of chlorite by "*B. coli*", seemed surprised that so much chlorite would be formed stating, "It is remarkable that the reduction of chlorate stops at the chlorite level, as this is very difficultly obtained by ordinary reduction of chlorate; the reaction always proceeds to chloride...". When the present work is compared with previous work, one caveat should be added: that is, Quastel (*27*), Goksøyr (*2*), and Pichinoty et al. (*3*) used washed, "resting" cells that had been suspended in minimal media. Cells growing in this study were not washed and were allowed to grow in TSB; however, the length of study period (24 h) encompassed both growth and resting phases, as is clearly indicated by the growth curves of **Figure 1**. In addition, the possibility remains that in addition to chlorite, the formation of hypochlorite could also explain the bactericidal effects of chlorate.

Despite the formation of at least some chlorite, populations of *E. coli* O157:H7 survived and grew in the culture media regardless of whether high or low concentrations of chlorate were present (**Figure 1**). During the first 4 h of incubation, *E. coli* cells were rapidly growing, yet there was little evidence of chlorate transformation, even in treatments containing the lowest concentration (0.5 mM) of chlorate. By 8 h, chlorate conversion to chloride ion was underway and concentrations of cells in the incubation media were generally reaching maxima. The most rapid rates of chlorate biotransformation occurred between 8 and 16 h, a time when *E. coli* O157:H7 numbers were generally static, suggesting that chlorate may be most rapidly metabolized by "resting" bacteria.

Regardless of the mechanism, chlorate did result in a 2−3 log reduction in overall *E. coli* numbers by the end of the 24 h incubation period in control and nitrate-pretreated cells, consistent with many previous results. Thus, there was an inhibitory effect of chlorate on *E. coli* O157:H7 growth, even in pure culture. In cells pretreated with 10 mM chlorate, however, there was little evidence for an overall chlorate effect and little evidence of chlorate metabolism (**Figure 2**), except for the 0.5 mM treatment in which about 50% of the chlorate was transformed to chloride. The consistent effectiveness of chlorate against *E. coli* and *Salmonella* pathogens in mixed culture may be a function of both the weakly toxic effects of chlorate and the rather acute selection pressure exerted by the multitudes of commensal bacteria competing for nutritional resources and exerting their own defense/survival mechanisms. The metabolism of chlorate to modest quantities of chlorite apparently provides just enough selection pressure to negatively affect overall populations of sensitive bacteria. The direct bactericidal effect of chlorite on *E. coli* is realized only when chlorite is administered in millimolar concentrations (*3*).

Resistance to chlorate was not due to the expression of chlorite dismutase, an enzyme in chlorate- and perchlorate-respiring bacteria that rapidly converts chlorite to chloride ion and oxygen (*36*, *37*). Such a finding is consistent with the data of Bender et al. (*38*), who demonstrated that genes for chlorite dismutase were not expressed in *E. coli*. Our results confirm that pathogenic *E. coli* O157:H7 do not produce chlorite dismutase and eliminate the possibility that this enzyme could in any manner be responsible for the development of chlorate resistance. The collective evidence indicates that chlorate sensitivity is related to chlorate metabolism or through interference with nitrate assimilation, presumably through nitrate reductases in nitrate-respiring species. The development of chlorate insensitivity by susceptible species seems to be related to the complete elimination of chlorate metabolism. For example, preconditioning cells with 10 mM chlorate caused a chlorate insensitivity (**Figure 1**) similar to that observed by Callaway et al. (*28*). The metabolism of chlorate in these cells was completely eliminated in the 1, 5, 15, and 30 mM treatments. Reduction of 0.5 mM chlorate occurred, but was delayed by about 4 h relative to the metabolism of 0.5 mM chlorate in control and nitrate-adapted cells (**Figure 2**). It is possible that at 0.5 mM chlorate, two populations of *E. coli* O157:H7 were present: a sensitive and an insensitive population. Callaway et al. (*28*) reported that pure cultures of *E. coli* O157:H7 resistant to chlorate could be sensitized to chlorate after several passages in media free of chlorate. It is unknown whether desensitization of *E. coli* O157:H7 requires threshold levels of chlorate, but it is possible that the chlorate reduction observed in 0.5 mM treatment of chlorate insensitive cells may have been due to a growing population of sensitive cells. Although resistance to chlorate is readily developed in pure cultures of *E. coli* O157:H7, chlorate resistance in mixed cultures was not observed (*28*).

In summary, these studies of chlorate metabolism in pure culture demonstrated that (a) nitrate does not sensitize *E. coli* O157:H7 to chlorate in pure culture, (b) development of *E. coli* O157:H7 insensitivity to chlorate when incubated in pure culture was not due to the expression of chlorite dismutase, (c) development of chlorate insensitivity is not because of the rapid decomposition of chlorate through metabolism, and (d) the toxic intermediate, chlorite, is produced by *E. coli* O157:H7, albeit in modest quantities.

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