AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

A Bacterial Microcompartment Is Used for Choline Fermentation by *Escherichia coli* 536

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ABSTRACT Bacterial choline degradation in the human gut has been associated with cancer and heart disease. In addition, recent studies found that a bacterial microcompartment is involved in choline utilization by *Proteus* and *Desulfovibrio* species. However, many aspects of this process have not been fully defined. Here, we investigate choline degradation by the uropathogen *Escherichia coli* 536. Growth studies indicated *E. coli* 536 degrades choline primarily by fermentation. Electron microscopy indicated that a bacterial microcompartment was used for this process. Bioinformatic analyses suggested that the choline utilization (*cut*) gene cluster of *E. coli* 536 includes two operons, one containing three genes and a main operon of 13 genes. Regulatory studies indicate that the *cutX* gene encodes a positive transcriptional regulator required for induction of the main *cut* operon in response to choline supplementation. Each of the 16 genes in the *cut* cluster was individually deleted, and phenotypes were examined. The *cutX*, *cutY*, *cutF*, *cutO*, *cutC*, *cutD*, *cutU*, and *cutV* genes were required for choline degradation, but the remaining genes of the *cut* cluster were not essential under the conditions used. The reasons for these varied phenotypes are discussed.

IMPORTANCE Here, we investigate choline degradation in *E. coli* 536. These studies provide a basis for understanding a new type of bacterial microcompartment and may provide deeper insight into the link between choline degradation in the human gut and cancer and heart disease. These are also the first studies of choline degradation in *E. coli* 536, an organism for which sophisticated genetic analysis methods are available. In addition, the *cut* gene cluster of *E. coli* 536 is located in pathogenicity island II ($PAI\text{-}II_{536}$) and hence might contribute to pathogenesis.

KEYWORDS microcompartment, carboxysome, choline, *E. coli* 536

Hundreds of bacterial species produce complex proteinaceous organelles known as bacterial microcompartments (MCPs) (1–9). MCPs are among the largest known multiprotein complexes. They are typically 100 to 150 nm in diameter and are built from thousands of protein subunits of 10 to 20 different types. The function of MCPs is to optimize metabolic pathways by sequestering toxic or poorly retained metabolic intermediates and by increasing enzymatic reaction rates (7–9). Overall, MCPs consist of metabolic pathways encapsulated within a selectively permeable protein shell. This architecture allows the selective confinement of toxic pathway intermediates that would otherwise damage DNA and cytoplasmic components (10–14). It also prevents the loss of intermediates that would normally diffuse out of the cell resulting in the loss of valuable carbon (8, 11, 15). In addition, MCPs concentrate enzymes together with their substrates, increasing reaction rates, with the most notable example being the carboxysome MCP, which is used to enhance CO_2 fixation by RubisCO via a carbon dioxide-concentrating mechanism (8).

Bacterial MCPs are present in many ecologically diverse and important bacteria and

Received 20 December 2017 **Accepted** 23 February 2018

Accepted manuscript posted online 5 March 2018

Citation Herring TI, Harris TN, Chowdhury C, Mohanty SK, Bobik TA. 2018. A bacterial microcompartment is used for choline fermentation by *Escherichia coli* 536. J Bacteriol 200:e00764-17. https://doi.org/10.1128/JB.00764-17.

Editor William W. Metcalf, University of Illinois at Urbana Champaign

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have a number of potential biotechnology applications. Bioinformatics analyses indicate that MCPs are produced by about 20% of bacteria distributed across 11 kingdom-level taxa, and physiological studies have shown that MCPs are involved in 10 or more metabolic processes ranging from carbon dioxide fixation to the catabolism of 1,2-propanediol, ethanolamine, choline, glycerol, rhamnose, fucose, and fucoidan (15–22). In addition, MCPs have been linked to pathogenesis in *Salmonella* and *Listeria* spp. (23–26), as well as to heart disease and cancer in humans due to their metabolic roles in the gut microbiome (27–30). Moreover, several labs have begun to develop MCPs as a platform for protein-based containers for use in renewable chemical production, drug delivery, and the expression of toxic proteins (31–42). However, as of yet, only three MCP types have been studied in any detail: the 1,2-propanediol utilization (Pdu) MCP, the ethanolamine utilization (Eut) MCP, and the carboxysome. Hence, a great deal still remains to be learned about the physiology and ecology of diverse MCPs, as well as their associations with human health and their potential biotechnology applications.

The genes for MCPs are typically found in large operons that encode both the shell proteins and the enzymes for the encapsulated metabolic pathway (1–9). The pathway enzymes vary according to the substrate metabolized which defines the MCP type. The shells of MCPs are primarily built from bacterial microcompartment (BMC) domain proteins and a bacterial microcompartment vertex (BMV) protein (43–47). MCP operons typically encode a single BMV protein that is thought to help impart curvature to the shell and multiple BMC domain proteins which fulfill various functional roles (44, 47). Hexameric BMC domain proteins have central pores that mediate the selective transport of small molecules across the MCP shell (43, 48, 49). The trimeric class of BMC domain proteins includes members proposed to have allosterically gated pores for the transport of larger molecules, as well as members with FeS clusters thought to mediate electron transfer across the MCP shell (50–57). Moreover, BMC domains are found fused to a variety of protein domains of unknown function, and MCP operons show extensive variation in the number and types of BMC domain proteins they encode. Presumably, this diversity is needed for the optimal function of varied MCP types operating in diverse host organisms. However, the specific functions of many BMC domain proteins are unknown.

Recently, a choline utilization (*cut*) gene cluster was identified in *Desulfovibrio desulfuricans* (58). This cluster included a gene for a glycyl-radical choline TMA-lyase (CutC), as well as multiple genes for bacterial MCP shell proteins. Physiological and genetic studies demonstrated that choline TMA lyase and the *cut* gene cluster were required for choline utilization by *Desulfovibrio alaskensis* and *Proteus mirabilis* (58–61). Bioinformatic analyses showed that *cut* gene clusters are widely but unevenly distributed among bacteria (58, 61). Studies also indicated that the *cutC* gene is diagnostic for choline-degrading bacteria and provides a useful marker for metagenomic analyses (61). In addition, recent electron microscopy of *P. mirabilis* and fitness studies in *D. alaskensis* G20 substantiated that a bacterial MCP was involved in choline degradation (59, 60). However, many aspects of choline degradation, including its physiological role, regulation, and mechanisms have not been investigated fully.

In this report, we characterize choline degradation by *Escherichia coli* 536. *E. coli* 536 is a uropathogen, and in this organism, the choline gene cluster is found in a pathogenicity island (PAI-II$_{536}$) (62).

RESULTS

Choline utilization by *E. coli* 536. Prior bioinformatic analyses identified a choline utilization (*cut*) gene cluster in PAI-II$_{536}$ (58, 61, 62). This *cut* cluster encodes a putative choline TMA lyase (CutC) which is diagnostic for choline utilization, as well as multiple homologs of MCP shell proteins, suggesting that a bacterial MCP is involved in choline degradation in *E. coli* 536. Growth studies were conducted to determine whether *E. coli* 536 is indeed capable of choline degradation. Choline did not serve as a sole carbon and energy source under aerobic or anaerobic conditions. However, under anaerobic conditions, choline simulated the growth of *E. coli* 536 on minimal medium that also

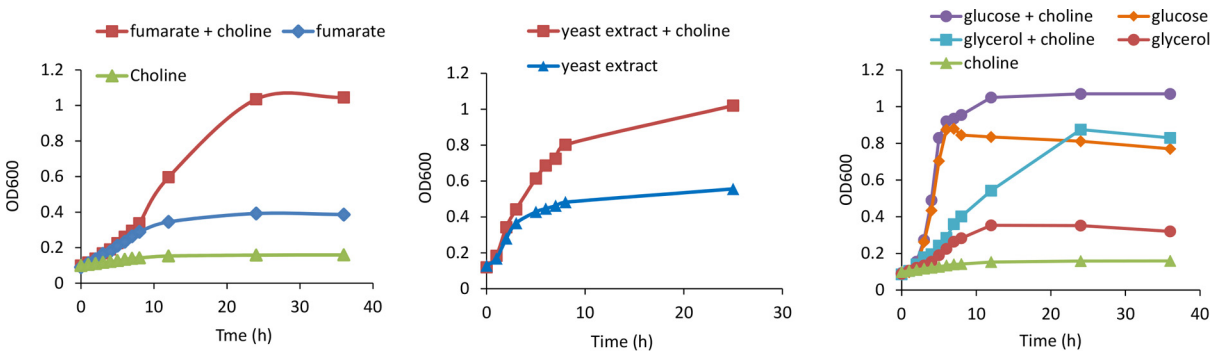

FIG 1 Effect of choline on anaerobic growth of *E. coli* 536. Cells were grown on NCE minimal medium supplemented with 1 mM $MgSO_4$, 50 μM ferric citrate, and the following: choline (1%), fumarate (50 mM), yeast extract (0.2%), glucose (10 mM), and/or glycerol (10 mM). Cultures were incubated anaerobically in sealed tubes at 37°C with shaking. All growth curves were repeated three or more times with nearly identical results.

contained fumarate or small amounts of yeast extract (0.2%) (Fig. 1). Choline also stimulated anaerobic growth of *E. coli* 536 on medium containing glucose and glycerol (Fig. 1). In further tests, *E. coli* 536 was unable to use choline as a carbon source for anaerobic respiration with nitrate, trimethylamine-*N*-oxide (TMAO), or dimethyl sulfoxide (DMSO) as terminal electron acceptors. As a positive control, *E. coli* 536 was grown on glycerol in combination with nitrate, TMAO, or DMSO as terminal electron acceptors. Growth was positive under these conditions. We also tested *E. coli* 536 for anaerobic respiration of choline or glycerol with tetrathionate as a terminal electron acceptor but with negative results. For these studies, *Salmonella enterica* serovar Typhimurium LT2 was used as a positive control and was found to grow well by anaerobic respiration of glycerol with tetrathionate as terminal electron acceptor (63).

Choline utilization among the ECOR collection. The *E. coli* Reference (ECOR) collection is a set of 72 natural isolates of *E. coli* intended to represent the genetic diversity of this species (64). To test the ECOR collection for the ability to use choline, MacConkey-choline indicator medium was used. On this medium, positive strains are red due to acid production from choline. Among 72 ECOR strains tested, 5 (6.9%) strains were positive for choline utilization in this test. They were ECOR 38-41 and 64 (see Fig. S1 in the supplemental material).

The five choline-positive ECOR strains were tested for the presence of the choline TMA lyase gene by PCR, as described previously (61). PCR amplification followed by DNA sequencing indicated that each positive ECOR strain had a *cutC* TMA lyase gene. Thus, it is likely that five ECOR strains use the choline TMA lyase pathway of choline degradation. We also note that MacConkey-choline agar provides a simple test for bacterial choline degradation that should prove helpful in various studies of choline metabolism. Even though choline metabolism occurs only in the absence of oxygen, the MacConkey-choline plates were incubated under aerobic conditions. This works because as colonies expand, their centers become anaerobic.

***E. coli* 536 produces bacterial microcompartments during growth on choline.** The presence of MCP structural genes in the *cut* operon of *E. coli* 536 suggested that a bacterial MCP might be involved in choline degradation in this organism. To test this possibility, *E. coli* 536 was grown anaerobically on LB medium with and without choline, and thin sections were prepared and examined by transmission electron microscopy. Protein complexes similar in size and appearance to bacterial MCPs were observed in cells grown in the presence of choline but not in its absence (Fig. 2).

Overall structure of the *E. coli* 536 *cut* operon. *E. coli* 536 has a type II choline utilization gene cluster similar to that of *P. mirabilis* (59) (Fig. 3). This cluster is composed of 16 genes all transcribed in the same direction. At the ends of the operon, flanking the *cutW* and *cutV* genes, are intergenic regions (the distance to the nearest ORF) of 302 and 295 bp, respectively. Each gene in the *cut* cluster is separated by ≤53 bp, with the exception of the *cutY* and *cmcA* genes, where there is an intergenic region

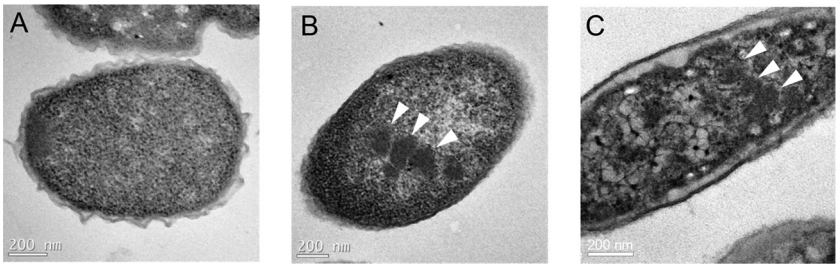

FIG 2 Electron microscopy images of *E. coli* 536 grown in the presence of choline. (A and B) *E. coli* 536 grown anaerobically on yeast extract (A) or yeast extract supplemented with choline (B). (C) *S. enterica* grown under conditions where Pdu MCPs form. Arrowheads point to the microcompartments.

of 519 bp (IGR1). The IGR1 region contains no predicted genes transcribed in the same direction as the main *cut* operon and only one small putative gene encoding a protein of 86 amino acids transcribed in the reverse direction of the main *cut* operon, based on GeneMark.hmm analyses (65). We also note that the start site of the *cutD* gene was likely misidentified during annotation of the genome available through the PATRIC website. Analyses with both BLASTP and the GeneMark.hmm software indicate that the start of the *cutD* gene is a GTG 159 bp upstream of the proposed ATG start site (65, 66). Analysis with RegRNA 2.0 (67) predicts two intrinsic terminators: one that starts 147 bp downstream of the *cutY* stop codon (TGAGCAAAAGCGCATTTTTT) and a second that begins 80 bp downstream of the *cutV* gene (GCGGCTTCCACAACAGGGAGCCGTTTTCTT). Overall, analyses of the *cut* gene cluster suggests the existence of two operons: one consisting of three genes, *cutW-cutX-cutY*, and a second main operon consisting of 13 genes, *cmcA-cmcB-cmcC-cutF-cmcD-cutO-cmcC-cutD-cmcE-cutH-cutT-cutU-cutV*.

For this report, we named previously unnamed choline utilization genes. We used both *cut* and *cmc* acronyms. This was done to prevent conflicts with the gene names previously used for type I choline gene clusters (61). The acronym *cmc* is for choline microcompartment. Each *cmc* gene has homology to MCP shell proteins.

Genes in the *cut* operon are required for choline degradation by *E. coli* 536. Each gene of the *cut* cluster and IGR1 was deleted individually by linear recombination of PCR products (68). In each case, the entire gene (except for the ribosome binding region of the upstream gene [~20 bp]) was deleted (68). The target gene was initially replaced with a chloramphenicol resistance marker, which was removed with the FLP recombinase, leaving behind only an FLP recombination target (FRT) site (68). This approach is designed to produce nonpolar deletions (68). In some cases, we attempted to replace the target gene with a kanamycin resistance cassette. This worked poorly in our study due to a large number of false positives.

For each mutant, anaerobic growth in liquid medium (0.2% yeast extract, 50 μM ferric citrate, and 1% choline) and on MacConkey-choline plates was used to test for the

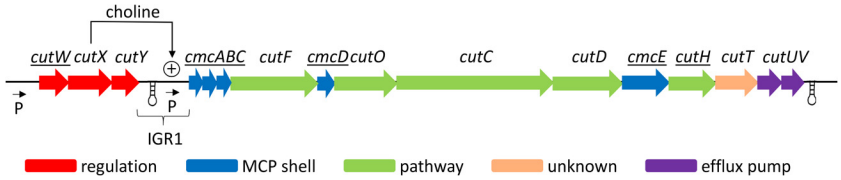

FIG 3 The *cut* gene cluster of *E. coli* 536. Bioinformatic analyses indicated that the choline utilization (*cut*) gene cluster of *E. coli* 536 is composed of two operons: a three-gene operon consisting of *cutW-cutX-cutY* and a main choline operon consisting of *cmcA-cmcB-cmcC-cutF-cmcD-cutO-cmcC-cutD-cmcE-cutH-cutT-cutU-cutV*. General gene functions are indicated by color. Underlined gene names indicate gene deletions that did not impair growth stimulation by choline (Table 1). The genes (proposed function) shown are *cutW* (transcriptional regulator), *cutX* (transcriptional regulator), *cutY* (regulation), *cmcA*, *cmcB*, *cmcC*, *cmcD*, and *cmcE* (MCP shell proteins), *cutF* (aldehyde dehydrogenase), *cutO* (alcohol dehydrogenase), *cutC* (choline TMA lyase), *cutD* (choline TMA lyase-activating enzyme), *cutH* (phosphotransacylase), *cutT* (unknown), *cutU* (efflux pump), and *cutV* (efflux pump).

TABLE 1 Phenotypes of *cut* and *cmc* mutants

Genotype[a]	Function of closest homolog of deleted gene	MacConkey-choline plate[b]	Choline utilization[c]	Protein product (accession no.)
Wild type		+	+	
Δ*cutW*::frt	Transcriptional regulator	+	+	WP_000926369.1
Δ*cutX*::frt	Transcriptional regulator	−	−	WP_001270145.1
Δ*cutY*::frt	Unknown	−	−	WP_001217008.1
ΔIGR1::frt	Intergenic region (519 bp)	+/−	−	
Δ*cmcA*::frt	BMC protein	+	+	WP_000502008.1
Δ*cmcB*::frt	BMC protein	+	+	WP_000502010.1
Δ*cmcC*::frt	BMC protein	+	+	WP_001206281.1
Δ*cutF*::frt	Aldehyde dehydrogenase	−	−	WP_000570989.1
Δ*cmcD*::frt	BMV protein	+	+	WP_000599365.1
Δ*cutO*::frt	Alcohol dehydrogenase	+/−	−	WP_001288714.1
Δ*cutC*::frt	Choline TMA lyase	−	−	WP_000035052.1
Δ*cutD*::frt	Choline TMA lyase-activating enzyme	−	−	WP_001275637.1
Δ*cmcE*::frt	BMC protein	+	+	WP_001086628.1
Δ*cutH*::frt	Phosphotransacetylase	+	+	WP_000564322.1
Δ*cutT*::frt	Unknown	+	+	WP_000139438.1
Δ*cutU*::frt	Efflux pump	−	−	WP_000095890.1
Δ*cutV*::frt	Efflux pump	−	−	WP_000481835.1

[a]The wild-type strain was *E. coli* 536.
[b]MacConkey-choline is MacConkey agar base supplemented with 0.8% choline chloride (pH 7.0).
[c]Choline utilization was measured as growth stimulation by choline in liquid medium containing 0.2% yeast extract, 50 μM Fe citrate, and 1% choline chloride (Fig. S2).

ability to utilize choline. The results of these tests are summarized in Table 1. Growth curves and MacConkey tests are shown in Fig. S2 and S3. Deletions of *cutX*, *cutY*, IGR1, *cutF*, *cutO*, *cutC*, *cutD*, *cutU*, and *cutV* eliminated choline utilization in anaerobic liquid cultures. All of these deletions also prevented the production of a red color on MacConkey-choline indicator plates, except *cutO*::*frt* and IGR1::frt, which reduced but did not eliminate color formation. Individual deletions of seven choline utilization genes (*cutW*, *cutT*, *cmcA*, *cmcB*, *cmcC*, *cmcD*, and *cmcE*) did not noticeably impair choline degradation in liquid medium or on MacConkey-choline indicator plates. The reasons for these various phenotypes are addressed in the Discussion.

The *cut* operon of *E. coli* 536 is induced by choline supplementation. To examine the regulation of the choline operon of *E. coli* 536, we used the *cutF* gene as a reporter for induction of the main *cut* operon (Fig. 3). The *cutF* gene encodes a close homolog of aldehyde dehydrogenases (ALD) whose production can be measured by a relatively easy enzyme assay (69). To test for induction of the *cutF* gene by choline, wild-type *E. coli* 536 was grown anaerobically without or with 1% choline, and ALD assays were performed (Table 2). Extracts from cells grown anaerobically without or with choline had activities of 100 ± 19 and 810 ± 121 nm · min^{-1} · mg^{-1}, respectively. In contrast, controls showed that the ALD activities in a Δ*cutF* mutant were 70 ± 7 and 100 ± 20 nm · min^{-1} · mg^{-1} without and with choline, respectively. Together, these

TABLE 2 Effect of selected mutations on induction of the main *cut* operon using the CutF aldehyde dehydrogenase as a reporter

	Aldehyde dehydrogenase (CutF) activity (nm · min^{-1} · mg^{-1})[a]			
	Anaerobic		Aerobic	
Strain	None	Choline	None	Choline
Wild type	100 ± 19	810 ± 121	10 ± 10	50 ± 21
Δ*cutF* mutant	70 ± 7	100 ± 20	10 ± 10	10 ± 5
Δ*cutW* mutant	70 ± 3	730 ± 118	10 ± 6	20 ± 5
Δ*cutX* mutant	70 ± 19	100 ± 10	10 ± 1	20 ± 15
Δ*cutY* mutant	60 ± 7	60 ± 2	4 ± 3	6 ± 4

[a]Each strain was grown anaerobically or aerobically on LB, 1 mM $MgSO_4$, and 50 μM ferric citrate without or with choline. Cell extracts were prepared, and ALD activity was measured as described in Materials and Methods.

results indicated that choline induced expression of the *cutF* gene and that CutF activity would be a useful reporter for induction of the main *cut* operon.

CutX is a positive regulator of the main *cut* operon. We also looked at expression of the CutF ALD in strains carrying a Δ*cutW*, Δ*cutX*, or *cutY* mutation (Table 2). The CutX protein has sequence similarity to transcriptional regulators. A *cutX* deletion mutant was grown anaerobically on medium without or with choline. Cells extracts were prepared, and the ALD activities were measured to be 70 ± 19 and 100 ± 10 nm · min^{-1} · mg^{-1}, respectively. Hence, in contrast to the wild-type strain, choline did not significantly induce expression of the CutF ALD in a *cutX* deletion strain (Table 2). These results (in conjunction with the finding the CutX has a predicted DNA-binding domain) indicate that *cutX* encodes a transcriptional activator that induces the main *cut* operon in response to choline supplementation.

We also looked at the effects of a *cutW* deletion on expression of the *cut* operon. Like CutX, the CutW protein has sequence similarity to transcriptional regulatory proteins. However, in contrast to the results obtained with the *cutX* deletion, a *cutW* deletion did not significantly affect induction of the *cut* operon by choline supplementation. Extracts prepared from Δ*cutW* cells grown without and with choline produced 70 ± 3 and 730 ± 118 nm · min^{-1} · mg^{-1} of ALD activity compared to the wild type, which produced 100 ± 19 and 810 ± 121 nm · min^{-1} · mg^{-1} of ALD activity, respectively.

CutY is also needed for induction of the main *cut* operon. Bioinformatic analyses indicate that *cutW*, *cutX*, and *cutY* are in the same operon (Fig. 3). Unlike CutW and CutX, the CutY protein lacks sequence similarity to known transcription factors. Hence, it was somewhat unexpected that a *cutY* deletion prevented induction of the *cut* operon by choline under anaerobic conditions (Table 2). Cell extracts prepared from Δ*cutY* cells grown without or with choline produced 60 ± 7 and 60 ± 2 nm · min^{-1} · mg^{-1} of ALD activity compared to the wild type, which produced 100 ± 19 and 810 ± 121 nm · min^{-1} · mg^{-1} of ALD activity. Presumably, *cutY* has a role in the regulation of the *cut* operon, and some possibilities are considered in Discussion.

The *cut* operon is not induced under aerobic conditions. The effects of oxygen on induction of the *cut* operon were investigated (Table 2). Strains carrying Δ*cutW*, Δ*cutX*, *cutY*, and Δ*cutF* mutations as well as the wild type were grown aerobically without or with choline, cell extracts were prepared, and CutF ALD activities were measured. The activities, without and with choline, respectively, were as follows: wild type, 10 ± 10 and 50 ± 21 nm · min^{-1} · mg^{-1}; Δ*cutF* mutant, 10 ± 10 and 10 ± 5 nm · min^{-1} · mg^{-1}; Δ*cutX* mutant, 10 ± 1 and 20 ± 15 nm · min^{-1} · mg^{-1}; Δ*cutW* mutant, 10 ± 6 and 20 ± 5 nm · min^{-1} · mg^{-1}, and Δ*cutY* mutant, 4 ± 3 and 6 ± 4 nm · min^{-1} · mg^{-1}. Controls showed that the CutF ALD was stable in the presence of oxygen (within experimental error, it retained >95% activity after ~24 h in air). Hence, we infer that the *cut* operon is not induced substantially in the presence of oxygen and that *cutW*, *cutX*, and *cutY* deletions do not substantially affect aerobic expression.

Complementation studies. Complementation studies were performed for four key *cut* mutants, Δ*cutC*, Δ*cutF*, Δ*cutX*, and Δ*cutY*. For these studies, we tested whether expression of the corresponding gene from plasmid pLac22 would restore growth simulation by choline to wild-type levels. For all four mutants, full complementation was observed (Fig. 4). These results established that the phenotypes observed for the *cutC* (choline TMA lyase), *cutF* (aldehyde dehydrogenase), *cutX* (transcriptional regulator), and *cutY* deletions were a consequence of that mutation and not an unexpected mutation inadvertently introduced during strain construction. For these tests, growth curves were performed using a microplate reader inside an anaerobic chamber, as described in Materials and Methods.

DISCUSSION

A study by Craciun and Balskus identified a glycyl-radical choline TMA lyase and a *cut* gene cluster used for bacterial choline degradation (58). Further studies determined that *cut* clusters are widely but unevenly distributed across *Proteobacteria*, *Firmicutes*,

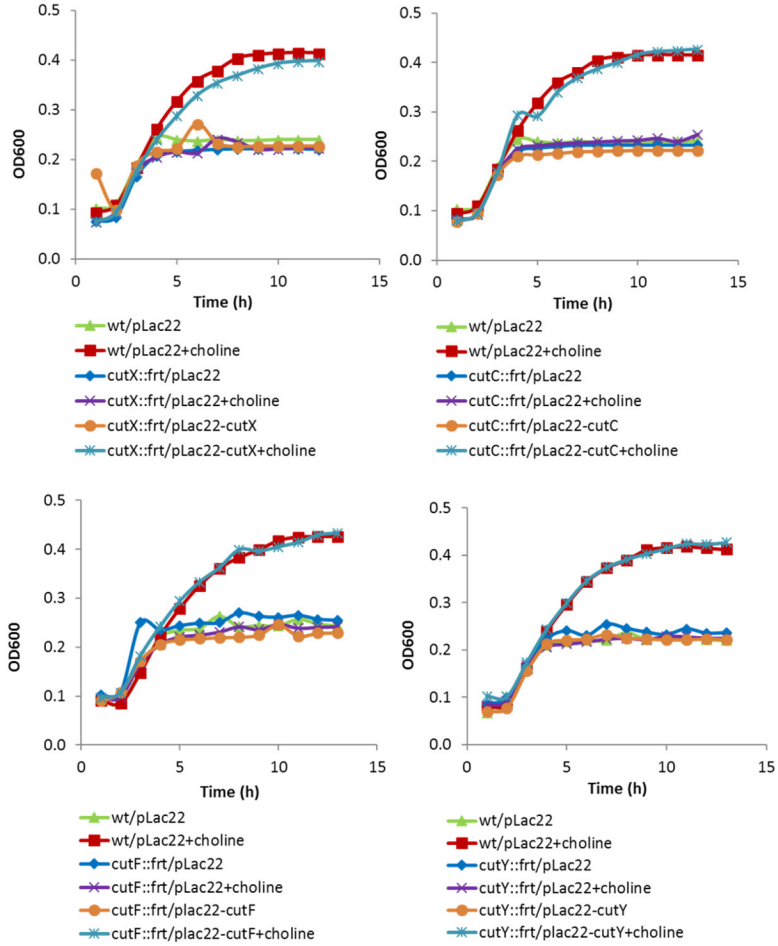

FIG 4 Complementation of key choline mutants used in this study (*cutX*, *cutC*, *cutF*. and *cutY* mutants). These growth tests were performed using a microplate reader. The growth medium used for complementation studies was NCE supplemented with 0.75% yeast extract, 175 mM NaCl, 50 μM ferric citrate, and 10 mM $MgCl_2$, with or without 1% choline chloride (pH 7.0). Media also contained 50 μM IPTG to induce gene expression from pLac22.

Actinobacteria, and *Fusobacteria* (61). Two types of *cut* clusters were identified: one that has ~20 genes (type I), and a second that has ~16 genes (type II) (61). The two types include a similar complement of choline degradative enzymes, suggesting a common pathway of choline catabolism. Both also include genes for the formation of bacterial MCPs, although these genes vary substantially (58–61). Based on the gene content of the *cut* cluster, as well as biochemical studies, a pathway for choline degradation has been proposed (Fig. 5) (58, 59, 61). Degradation begins with choline entering the lumen of the Cut MCP, where it is converted to TMA and acetaldehyde by choline TMA lyase (CutC), supported by its activating enzyme (CutD) (58). TMA is likely excreted and not used as a nitrogen source (59). Acetaldehyde is converted to acetate and ethanol. This pathway generates 1 ATP, an electron sink (acetaldehyde), and a source of acetyl coenzyme A (acetyl-CoA). Based on analogy with the 1,2-propanediol, ethanolamine, and carboxysome MCPs, the likely function of the choline MCP is to increase pathway flux and to sequester acetaldehyde to prevent toxicity and carbon loss (11, 12, 15).

In this study, we investigated choline degradation in *E. coli* 536. This organism has a type II *cut* gene cluster. Studies showed that choline stimulated the growth of *E. coli* 536 on glucose, glycerol, fumarate, or small amounts of yeast extract, similar to the results obtained with *P. mirabilis* (59). Here, we also tested for anaerobic respiration of choline with nitrate, DMSO, and TMAO, three compounds *E. coli* is known to use as terminal electron acceptors (70); however, the results were negative. The results were

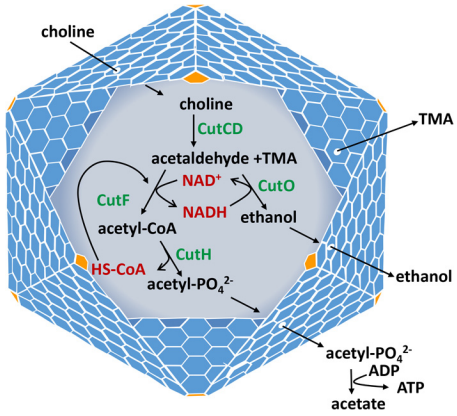

FIG 5 Model for the bacterial MCP used for choline degradation by *E. coli* 536. Choline diffuses through the protein shell and enters the lumen of the Cut MCP, where it is converted to acetaldehyde and TMA. The acetaldehyde is further metabolized to ethanol and acetate, generating 1 ATP to support growth. Based on analogy with other MCPs, its proposed function is to sequester acetaldehyde to prevent toxicity and/or diffusive loss through the cell envelope. CutCD, choline TMA lyase and its activating enzyme; CutF, acetaldehyde dehydrogenase; CutO, alcohol dehydrogenase; CutH, phosphotransacetylase; TMA, trimethylamine.

also negative for aerobic growth on choline as a sole carbon source and for anaerobic respiration of choline with tetrathionate as terminal electron acceptor. Tetrathionate was of interest because it can serve as a terminal electron acceptor for the anaerobic respiration of ethanolamine and 1,2-propanediol, and both of these processes involve a bacterial MCP (15). Overall, the growth studies conducted here show that *E. coli* 536 degrades choline by fermentation and possibly by anaerobic respiration with fumarate as a terminal electron acceptor, although the latter possibility will need to be substantiated by further studies to demonstrate electron transport phosphorylation.

For this report, the organization of the *cut* gene cluster of *E. coli* 536 was examined (Fig. 3). The *cut* cluster then begins with two genes that encode proteins with homology to transcriptional regulators, *cutW* and *cutX*, and a third gene (*cutY*) that encodes a hypothetical protein. Given that *cutW*, *cutX*, and *cutY* coding regions are separated by 22 bp or overlap by 1 bp, respectively, we propose that the *cutW*, *cutX*, and *cutY* genes form an operon (Fig. 3). Downstream of *cutY* is a 519-bp intergenic region, followed by the *cmcA-cmcB-cmcC-cutF-cmcD-cutO-cmcC-cutD-cmcE-cutH-cutT-cutU-cutV* genes, which likely form the main *cut* operon, since the largest intergenic region here is only 52 bp (Fig. 3).

Regulation of the *cut* cluster was investigated using the *cutF* gene as a transcriptional reporter for the main *cut* operon. The results indicated that the main *cut* operon was induced by choline supplementation (Table 2). A similar result was obtained for *P. mirabilis* by transcriptomics, which showed that each gene in the main *cut* operon was induced by choline supplementation (59). Here, we expanded on prior work by looking at possible regulatory roles for CutW, CutX, and CutY (CutW and CutX both have homology to transcriptional regulators). The results showed that choline did not induce the *cut* operon in a Δ*cutX* mutant. In contrast, a Δ*cutW* mutant had no effect on choline induction under similar conditions. Based on these studies, we hypothesize that CutX is a positive transcriptional regulator that binds choline (or a downstream metabolite) and induces transcription of the main *cut* operon by binding to the intergenic region between *cutY* and *cmcA* (although no binding studies were performed here). We also found that a *cutY* deletion prevented induction of the main *cut* operon. This result was somewhat unexpected, since CutY lacks recognizable homology to transcriptional regulators. However, since both CutY and CutX are required for induction of the *cut* operon, these proteins might work together to mediate operon induction. The results also showed that choline does not induce the *cut* operon under aerobic conditions; however, the regulatory systems involved were not identified. CutW, CutX, or CutY

might respond to or be inactivated by oxygen. Alternatively, oxygen control might be mediated by global regulators, such as Arc or Fnr (71, 72).

Each gene in the *cut* cluster of *E. coli* 536 was deleted individually by lambda Red recombineering (68). For each mutant, choline degradation was tested by growth on liquid medium and color formation on MacConkey-choline indicator plates (Table 1; see Fig. S2 and S3 in the supplemental material). Deletions of *cutX*, *cutY*, IGR1, *cutF*, *cutO*, *cutC*, *cutD*, *cutU*, and *cutV* eliminated growth stimulation by choline in liquid culture, indicating that these genes are required for choline degradation. The *cutF*, *cutO*, *cutC*, and *cutD* genes all encode metabolic enzymes (or homologs thereof) used to degrade choline. CutX and CutY are required for induction of the main *cut* operon, as described above. IGR1 likely includes *cis*-acting elements needed for operon induction. CutU and CutV are homologs of efflux pumps that extrude amines (73). Presumably, CutU and CutV are needed to pump TMA out of the cell to prevent inhibition, although the reasons behind TMA inhibition are currently unknown to us. A deletion of the *cutH* gene did not significantly reduce choline degradation. CutH has homology to phosphotransacetylase enzymes. *E. coli* is known to express the Pta phosphotransacetylase constitutively (74). This enzyme might be able to replace the CutH enzyme for choline degradation. However, this finding was somewhat surprising, since MCP-associated Pta enzymes are required for internal cofactor recycling in the *eut* and *pdu* MCPs, and the deletion of their encoding genes results in reduced growth on MCP substrates (75).

Deletion of each of the five MCP shell genes of the *cut* operon individually (*cmcA*, *cmcB*, *cmcC*, *cmcD*, and *cmcE*) did not noticeably impair choline degradation under the conditions used here. Based on analogy with other MCPs, the *cut* MCP is presumed to sequester acetaldehyde to prevent toxicity or diffusive loss (11, 12). For this study, choline degradation was measured under anaerobic conditions in sealed tubes, which would prevent diffusive loss of acetaldehyde. Furthermore, aldehyde toxicity has primarily been observed as DNA damage (12, 14), which was not measured here. Thus, it was not particularly surprising to us that the deletion of *cut* MCP shell genes lacked a phenotype in the studies performed here. Further work will be needed to verify the specific physiological function of the *cut* MCP.

Electron microscopy was used to test whether *E. coli* 536 forms MCPs during growth on choline. The results indicated that this organism forms MCPs in the presence of choline but not in its absence (Fig. 2). The Cut MCPs formed by *E. coli* 536 were similar in size and shape to the Pdu MCPs formed by *S. enterica* during growth on 1,2-propanediol (Fig. 2). Prior studies showed that *P. mirabilis* (which also has a type I gene *cut* gene cluster) forms MCPs during growth on choline (59). Hence, two studies have indicated that a bacterial MCP is produced for choline degradation.

To better evaluate the distribution of choline degradation among *E. coli* strains, we screened the ECOR collection using MacConkey agar supplemented with choline. The ECOR collection consists of 72 *E. coli* strains that are representative of the genetic variation of the species as a whole (64). Five of 72 ECOR strains were found to degrade choline, and all five strains also tested positive for choline TMA lyase by PCR. Two of the choline-positive ECOR strains were isolated from patients with urinary tract infections (UTIs), and three strains were from feces of healthy individuals. We know of no specific connection between choline degradation and *E. coli* 536 pathogenesis, although choline does induce virulence factors in enterohemorrhagic *E coli* (76).

MATERIALS AND METHODS

Chemicals and reagents. Antibiotics, NAD^+, and coenzyme A were from the Sigma Chemical Company (St. Louis, MO). Choice *Taq* Blue master mix was from Denville Scientific (Holliston, MA). *KOD* Hot Start master mix was from EMD Millipore (Billerica, MA). Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was from Diagnostic Chemicals Limited (Charlottetown, Prince Edward Island, Canada). Restriction enzymes and T4 DNA ligase were from New England BioLabs (Beverly, MA). Bacterial protein extraction reagent (B-PERII), 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), DNase, choline chloride, and other reagents were from Fisher Scientific (Pittsburgh, PA).

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 3. Biosafety level 2 (BSL2) precautions were used for all growth studies. The rich media used were lysogeny broth (LB), also known as Luria-Bertani medium (Becton, Dickinson and Company, Franklin

TABLE 3 Strains used in this study

Strain[a]	Genotype	Source
E. coli 536 (BE2278)	Wild type	Gift from Harry L. T. Mobley
BE2346	Δ*cutW*::frt	This study
BE2309	Δ*cutX*::frt	This study
BE2348	Δ*cutY*::frt	This study
BE2310	ΔIGR1::frt	This study
BE2354	Δ*cmcA*::frt	This study
BE2306	Δ*cmcB*::frt	This study
BE2308	Δ*cmcC*::frt	This study
BE2307	Δ*cutF*::frt	This study
BE2352	Δ*cmcD*::frt	This study
BE2305	Δ*cutO*::frt	This study
BE2433	Δ*cutC*::frt	This study
BE2362	Δ*cutD*::frt	This study
BE2350	Δ*cmcE*::frt	This study
BE2358	Δ*cutH*::frt	This study
BE2356	Δ*cutT*::frt	This study
BE2364	Δ*cutU*::frt	This study
BE2360	Δ*cutV*::frt	This study
BE2531	*E. coli* 536/pLac22	This study
BE2532	Δ*cutX*::frt/pLac22-*cutX*	This study
BE2533	Δ*cutX*::frt/pLac22	This study
BE2534	Δ*cutC*::frt/pLac22-*cutC*	This study
BE2535	Δ*cutC*::frt/pLac22	This study
BE2556	Δ*cutF*::frt/pLac22-*cutF*	This study
BE2557	Δ*cutF*::frt/pLac22	This study

[a]All mutants used in this study are derivatives of *E. coli* 536.

Lakes, NJ) (77), and Terrific broth (MP Biomedicals, Solon, OH). MacConkey-choline indicator plates were prepared using a Difco MacConkey agar base supplemented with 0.8% choline chloride from a 50% stock solution that was adjusted to pH 7.0. The liquid medium used was no-carbon-E (NCE) medium (78) supplemented with 1 mM $MgSO_4$, 50 μM ferric citrate, and one or more of the following: 1% choline chloride (pH 7.0), 0.2% yeast extract, 50 mM disodium fumarate (pH 7.0), 10 mM glucose, and/or 10 mM glycerol. Anaerobic growth curves were performed using 18 by 150-mm serum tubes sealed with butyl rubber stoppers. Serum tubes (containing 6 ml of medium) were inoculated to 120 μl of overnight culture grown aerobically on LB supplemented with 10 mM $MgSO_4$. Tubes were moved inside an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) allowed to degas, sealed, and removed. The sealed tubes were incubated at 37°C with shaking at 275 rpm in an Innova 2400 shaker (New Brunswick Scientific). Optical density was measured using a Spectronic 20D+ spectrophotometer (Thermo Fisher Scientific). For complementation studies, growth curves were determined using a Synergy HT microplate reader (BioTek, Winooski, VT) as previously described (79), with the modification that the microplate reader was placed inside an anaerobic chamber. The growth medium used for complementation studies was NCE supplemented with 0.75% yeast extract, 175 mM NaCl, 50 μM ferric citrate, and 10 mM $MgCl_2$, with or without 1% choline chloride (pH 7.0).

Construction of chromosomal mutations. Chromosomal deletions were made using the lambda Red recombinase, as described previously (68, 79). Chloramphenicol resistance was selected (kanamycin selection gave many false-positive colonies). Chloramphenicol resistance markers were removed using the FLP recombinase, as described previously (68). This leaves behind an 82-nucleotide scar (an FRT site). All mutations were verified by colony PCR amplification across the scar, followed by DNA sequencing.

Electron microscopy. Double-strength LB medium (40 g/liter) supplemented with 10 mM $MgSO_4$ and 50 μM ferric citrate was dispensed into two sterile test tubes (2 ml/tube), and one tube was supplemented with 1% choline chloride. Both tubes were inoculated with 20 μl of an aerobic LB overnight culture of *E. coli* 536 and incubated statically for 8 h in an anaerobic chamber. Inside an anaerobic chamber, 300 μl of this starter culture was used to inoculate 30 ml of similar medium in 50-ml sterile tubes. These cultures were incubated in the anaerobic chamber for ~16 h at 37°C, statically. While still working inside the anaerobic chamber, cells were dispensed into 50-ml centrifuge tubes, capped tightly, and centrifuged at 10,000 × *g* using a Beckman JA-10 rotor. After centrifugation, the supernatants were decanted inside that anaerobic chamber. Cells were removed from the chamber, quickly resuspended in 2% paraformaldehyde plus 0.3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), and incubated for 30 min at room temperature. After fixation, cells were washed three times with 0.1 M cacodylate buffer (pH 7.2). Imbedding, sectioning, and electron microscopy were carried out as described previously (79).

Enzyme assays. Overnight cultures (aerobic) were prepared in 3 ml of LB and 10 mM $MgSO_4$ and incubated overnight at 37°C with shaking at 275 rpm. This culture (500 μl) was used to inoculate 50 ml of LB, 1 mM $MgSO_4$, and 50 μM ferric citrate, with and without 1% choline chloride. Cells were incubated anaerobically at 37°C for ~5 h to an optical density at 600 nm (OD_{600}) of ~1.0 or aerobically at 37°C for ~2.5 h to an OD_{600} of ~1.0. Cells were harvested by centrifugation at 5,000 × *g* for 10 min using a JA7.5

rotor and a Beckman Avanti J-25 centrifuge. The cells were washed with 10 ml of 50 mM Tris (pH 8.0), 300 mM NaCl, and 5% glycerol. Cells were lysed with 1 ml of 50% B-PERII in 100 mM Tris (pH 8.0), 300 mM KCl, 10 mM $MgCl_2$, 1 mg/ml lysozyme, 0.04 mM AEBSF, and 0.05 mg/ml DNase. The lysate was centrifuged at 15,000 × *g* for 10 min at 4°C in a 5417R microcentrifuge (Eppendorf). The supernatant was collected, and its protein concentration was determined using Bio-Rad protein assay reagent and used for enzyme assays. Aldehyde dehydrogenase assays were performed by following the increase in absorbance at 340 nM in assay mixtures containing 1 mm NAD^+, 0.5 mM coenzyme A, 1 mM dithiothreitol, 35 mM potassium phosphate (pH 8.0), 50 mM KCl, and an appropriate amount of enzyme, as previously described (69). Quantification was based on ε340 of 6.22 $mM^{-1} \cdot cm^{-1}$.

Molecular biology methods. Agarose gel electrophoresis, plasmid purification, PCR, restriction digestion, ligation reactions, and electroporation were carried out using standard protocols, as described previously (16, 80). *Taq* or Phusion DNA polymerase (New England BioLabs) was used for amplification of chromosomal DNA and colony PCR. *KOD* DNA polymerase was used for amplification of plasmid templates. Plasmid DNA was purified using Qiagen products (Chatsworth, CA), according to the manufacturer's instructions. Following restriction digestion or PCR amplification, DNA was purified using Promega Wizard PCR preps (Madison, WI) or Qiagen gel extraction kits. For ligation of DNA fragments, T4 DNA ligase or NEBuilder HiFi DNA assembly master mix was used according to the manufacturer's instructions (New England BioLabs).

Complementation studies. Genes used for complementation were cloned into plasmid pLac22, which allows for tight regulation of cloned genes by IPTG (81). Chromosomal genes were amplified using Phusion DNA polymerase and cloned between the BglII and HinDIII sites of pLac22. Cloning was accomplished either by designing PCR primers with BglII and HinDIII sites or ~20 bp of homology for ligation with T4 DNA ligase or by Gibson assembly, respectively. Positive clones were identified by colony PCR, and the DNA sequences of all clones were verified.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00764-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

This work was supported by grant AI081146 from the National Institutes of Health to T.A.B.

We thank Harry L. T. Mobley and Stephanie Himpsl for kindly providing *E. coli* 536. We also thank the ISU DNA Sequencing and Synthesis Facility for assistance with DNA analyses and the ISU Microscopy and Nanoimaging facility for help with electron microscopy.

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