Bile acid oxidation by *Eggerthella lenta* strains C592 and DSM 2243\textsuperscript{T}

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**ABSTRACT**

Strains of *Eggerthella lenta* are capable of oxidation-reduction reactions capable of oxidizing and epimerizing bile acid hydroxyl groups. Several genes encoding these enzymes, known as hydroxysteroid dehydrogenases (HSDH) have yet to be identified. It is also uncertain whether the products of *E. lenta* bile acid metabolism are further metabolized by other members of the gut microbiota. We characterized a novel human fecal isolate identified as *E. lenta* strain C592. The complete genome of *E. lenta* strain C592 was sequenced and comparative genomics with the type strain (DSM 2243) revealed high conservation, but some notable differences. *E. lenta* strain C592 falls into group III, possessing 3α, 3β, 7α, and 12α-hydroxysteroid dehydrogenase (HSDH) activity, as determined by mass spectrometry of thin layer chromatography (TLC) separated metabolites of primary and secondary bile acids. Incubation of *E. lenta* oxo-bile acid and iso-bile acid metabolites with whole-cells of the high-activity bile acid 7α-dehydroxylating bacterium, *Clostridium scindens* VPI 12708, resulted in minimal conversion of o xo-derivatives to lithocholic acid (LCA). Further, Isochenodeoxycholic acid (iso-CDCa: 3β,7α-dihydroxy-5β-cholan-24-oic acid) was not metabolized by *C. scindens*. We then located a gene encoding a novel 12α-HSDH in *E. lenta* DSM 2243, also encoded by strain C592, and the recombinant purified enzyme was characterized and substrate-specificity determined. Genomic analysis revealed genes encoding an Rnf complex (rnfABCDEG), an energy conserving hydrogenase (echABCDEF) complex, as well as what appears to be a complete Wood-Ljungdahl pathway. Our prediction that by changing the gas atmosphere from nitrogen to hydrogen, bile acid oxidation would be inhibited, was confirmed. These results suggest that *E. lenta* is an important bile acid metabolizing gut microbe and that the gas atmosphere may be an important and overlooked regulator of bile acid metabolism in the gut.

**Introduction**

Once considered mere detergent molecules aiding and abetting digestion of dietary lipids and cholesterol, bile acids are now regarded as important digestive hormones that regulate numerous physiological processes in the host.\textsuperscript{1–3} Bile acids are C-24 steroids generated from cholesterol in the liver via side-chain modification, and hydroxylation at the C-3, C-7 position, and in the case of cholic acid (CA; 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid), also the C-12 position. Several grams of bile acids, conjugated to glycine or taurine, are released from the gallbladder daily where they aid in lipid absorption in the small bowel. Once reaching the terminal ileum, bile salts are actively transported into enterocytes, and after being transported across the basolateral membrane into the portal vein, bile salts are returned to the liver and recycled in a process known as the enterohepatic circulation. Several hundred milligrams of bile salts escape the high affinity active transporters in the terminal ileum, entering the large bowel.\textsuperscript{4,5} Bile salts act as important top-down selective forces, regulating microbial colonization and microbiome structure\textsuperscript{6} in the small and large intestines due to their detergent
properties, and through induction of farnesoid-X-receptor (FXR)-dependent anti-microbial peptide synthesis in the ileum.\(^7\)

In response, microbes have evolved a suite of genes, that we refer to as the human gut sterolome, which encode enzymes that modify bile acids and other steroid molecules.\(^8\) These genes include the “gateway” bile salt hydrolase (BSH), a reaction which hydrolyzes the amide bond linking the bile acid side-chain to glycine or taurine.\(^9\) Deconjugation is a pre-requisite for the conversion of CA to deoxycholic acid (DCA; 3α,12α-dihydroxy-5β-cholan-24-oic acid) or chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid) to lithocholic acid (LCA; an activity previously demonstrated in bile acid 7α-dehydroxylating bacteria\(^10,20\) (Figure 1). However, this metabolite ran higher than ADCA on TLC plates. The pattern of metabolites suggested oxidation of bile acid hydroxyl groups rather than 7α-dehydroxylation. To simplify our analysis, we chose two dihydroxy bile acids to further evaluate bile acid metabolism by strain C592. We then incubated the dihydroxy primary bile acid CDCA (3α, 7α-dihydroxy) or the secondary bile acid DCA (3α, 12α-dihydroxy) with strain C592 and determined the identity of the reaction products. Formation of mono- and dioxo-bile acid products was evident when incubated with either CDCA or DCA (Figure 2A & 2B). We did not observe the 7α-dehydroxylation of CDCA by C592, suggesting this strain is not a bile acid 7α-dehydroxylation bacterium. Incubation of [24\(^-\)C] CDCA resulted in the formation of three products, CDCA-A (R\(_f\) 0.50), CDCA-B (R\(_f\) 0.62), and CDCA-C (R\(_f\) 0.98). We also ran non-labeled CDCA under identical conditions and scraped these metabolites from the TLC for UPLC-IT-TOF-MS under negative ion mode. CDCA-A (389.2701 m/z) and CDCA-B (389.2702 m/z) were 2 amu less than the parent compound CDCA (MW 392). CDCA-A, but not CDCA-B, could be oxidized by commercial 3α-HSDH in the presence of NAD\(^+\) (data not shown), which co-migrated with CDCA-C (387.2539 m/z). These results suggest that CDCA-A is 7-oxoLCA (3α-hydroxy-7-oxo-5β-cholan-24-oic acid) and that

**Results**

**Characterization of bile acid oxidation by anaerobic fecal isolate, strain C592**

Strain C592 is a gram-positive, obligate anaerobe originally isolated from a centenarian stool sample at the University of Ryukyus in Okinawa, Japan. Based on visual examination of the relative migration of bile acid metabolites on thin layer chromatography (TLC), strain C592 appeared to convert CA to allodeoxycholic acid (ADCA), a 5α-epimer of DCA; an activity previously demonstrated in bile acid 7α-dehydroxylating bacteria\(^10,20\) (Figure 1).
CDCA-B is 7-oxo-isoLCA (3β-hydroxy-7-oxo-5β-cholan-24-oic acid). The loss of 4 amu from CDCA-C, and the conversion of CDCA-B to CDCA-C by oxidation of a 3α-hydroxyl group suggested that CDCA-C is 3,7-dioxocholanoic acid (3,7-dioxo-5β-cholan-24-oic acid) (Figure 2A).

To determine the ability of strain C592 to metabolize the 12α-hydroxyl of DCA, we separated DCA metabolites after 24 hr incubation in brain heart infusion broth (BHI). Two major metabolites were detected on TLC and characterized, DCA-A (RF 0.6) and DCA-B (RF 0.95) (Figure 2B). Compared with DCA (MW 392), the products DCA-B lost 4 amu (387.2528 m/z) suggesting oxidation of the 3α-hydroxyl and 12α-hydroxyl forming 3,12-dioxocholanoic acid (3,12-dioxo-5β-cholan-24-oic acid). DCA-A (389.2687 m/z) lost 2 amu, forming either 3-dehydroDCA or 12-oxoLCA. Because these results confirmed oxidation of the 12α-hydroxyl, we did not further pursue differentiation of these two possibilities.

Taken together, these results indicated that strain C592 expresses 3α-HSDH, 7α-HSDH, and 12α-HSDH enzymes. 3β-hydroxyl metabolites (so called “iso-bile acids”) were also identified, indicating the expression of 3β-HSDH, and confirmed previously reported activities in strains of Eggerthella lenta and Ruminococcus gnavus. Indeed, 16S rDNA gene sequencing placed the taxonomic identity of strain C592 as Eggerthella lenta (99% sequence ID), clustering closely with the type strain (DSM 2243). Sequencing of the 16S rDNA of the other strains represented in Figure 1 resulted in identification of several strains of C. scindens reported previously.

**E. lenta CDCA end-product metabolism by Clostridium scindens VPI 12708**

Bile acid 7α-dehydroxylation is quantitatively the most important bile acid biotransformation in the gut, and is implicated in disease of the GI tract including cholesterol gallstone disease and colorectal cancer. Therefore, we wanted to determine whether bile acid 7α-dehydroxylating bacterium Clostridium scindens VPI 12708 is capable of converting oxo- and iso-derivatives of CDCA generated by E. lenta strain C592 to the secondary bile acid LCA. We purified and quantified [24-14C] CDCA metabolites from E. lenta C592 culture by TLC and incubated each metabolite in cultures of C. scindens VPI 12708 grown in BHI.

Two major observations were gathered from these experiments. First, only metabolites with 3-oxo or 3α-hydroxyl groups were 7α-dehydroxylated, while iso-CDCA metabolites were not converted to LCA.
Figure 2. Characterization of bile acid metabolism and metabolites by *Eggerthella lenta* strain C592. (Upper Panel) Representative TLC showing formation of oxo-bile acids and iso-bile acid derivatives of CDCA as identified by LC-MS following TLC separation. CDCA metabolites were separated as previously described on TLC both with and without [24-14C]-radiolabel. Isolated unlabeled substrates corresponding to CDCA-A, CDCA-B, and CDCA-C then underwent MS analysis as described in the Materials and Methods. (Lower Panel). Identification of metabolites formed following growth of C592 in the presence of [24-14C] DCA. Autoradiograph of representative TLC shows relative migration of DCA control and formation of DCA-A and DCA-B by strain C592. Regions of the TLC corresponding to DCA-A and DCA-B were subjected to MS analysis resulting in identification of DCA-B and possible products of DCA-A. Three biological replicates of this experiment were performed.
Figure 3. Metabolism of E. lenta CDCA metabolites by CA-induced vs. un-induced cells of Clostridium scindens VPI 12708. (Upper Panel) Conversion of E. lenta bile acid metabolites by induced (+ CA) and uninduced cultures of lithocholic acid (LCA)-forming gut bacterium, C. scindens VPI 12708. Experiments were repeated in triplicate ± standard errors and data were analyzed by two-tailed T-test *p < 0.01, **p < 0.001, ***p < 0.0001. (Lower Panel) Schematic representation of alternative 7α-dehydroxylation and isobilic acid (3α-hydroxy ↔ 3-oxo ↔ 3β-hydroxy) pathways between C. scindens and E. lenta, respectively.
derivatives (Figure 3A). This observation can be explained by the sequence of reactions in the bile acid 7α-dehydroxylation pathway, particularly the step that requires oxidation of the 3α-hydroxyl group\textsuperscript{27,28} prior to formation of a C\textsubscript{4}-C\textsubscript{5} double bond\textsuperscript{22} which is then 7α-dehydroxylated, forming a stable 3-dehydro-4,6-intermediate.\textsuperscript{23,24} 3β-HSDH activity has not been observed in C. scindens VPI 12708. Thus, isoCDCA or isoCA formation is expected to preclude formation of LCA and DCA, respectively (Figure 3B). The iso-bile acid pathway may therefore be useful in shifting the bile acid pool from hydrophobic, and thus toxic, to hydrophilic and health-promoting. Iso-bile acids returning to the liver during enterohepatic circulation will then be converted to the 3α-hydroxyl orientation and re-secreted into bile.\textsuperscript{25} Second, o xo-bile acid metabolites are only 7α-dehydroxylated by C. scindens VPI 12708 if pre-cultured in the presence of CA, which induces the bai regulon.\textsuperscript{26}

Characterization of a recombinant gene product encoded by E. lenta DSM 2243 involved in bile acid oxidation

Early studies\textsuperscript{14,17,19} demonstrated that strains of E. lenta vary with respect to the expression of HSDHs capable of metabolizing bile acids at the 3α, 7α, and 12α-hydroxyl groups, with some strains capable of epimerizing the 3α-hydroxyl to 3β-hydroxyl group, forming iso-bile acids. E. lenta strain C592 falls into group III, possessing 3α, 3β, 7α, and 12α-HSDH activity, along with the type strain, E. lenta DSM 2243.\textsuperscript{12} Genes encoding 3α-HSDH and 7α-HSDH have been characterized in gut bacteria\textsuperscript{10,29-32} and soil isolates\textsuperscript{33}; however, there is a paucity of characterized 12α-HSDH genes. 12α-HSDH is an enzyme important in industrial synthesis of therapeutic ursodeoxycholic acid from cholic acid,\textsuperscript{34} and the first step in conversion of host bile acids to 12β-hydroxyl bile acid epimers.\textsuperscript{35} The only bacterial gene demonstrated to encode a 12α-

Table 1. Enzyme Kinetic Data for Elen_2515.

| Substrate   | Co-substrate   | Vmax (μmoles min\textsuperscript{-1} mg\textsuperscript{-1}) | Km (μM)         | Kcat (min\textsuperscript{-1}) | Kcat/Km |
|-------------|----------------|------------------------------------------------|----------------|--------------------------------|---------|
| DCA         | NAD\textsuperscript{+} | 62.18 ± 3.54                          | 96.78 ± 3.89    | 1,685.11 ± 95.89               | 17.41 ± 1.40 |
| 12-oxoLCA   | NADH           | 17.98 ± 4.05                            | 191.62 ± 14.61  | 121.82 ± 27.47                | 0.63 ± 0.17 |

Values reported as standard error of the mean (S.E.M) of four independent replicates.
HSDH (ERJ00208.1) was reported in Clostridium sp. ATCC 29733. In order to locate a 12α-HSDH in E. lenta strains, we searched the deduced amino acid sequence of the 12α-HSDH reported in Clostridium sp. ATCC 29733 (ERJ00208.1) against the E. lenta DSM 2243 genome. The 12α-HSDH from Clostridium sp. ATCC 29733 is a member of the SDR-family with conserved canonical pyridine-nucleotide binding site (GGGX₅GXG) and conserved catalytic triad (S160, Y173, K177). A tBlastn of ERJ00208.1 against the E. lenta DSM 2243 genome resulted in identification of Elen_2515 as a probable candidate, sharing 56% amino acid sequence identity (70% similarity; E value 2e⁻⁸¹). Elen_2515 was previously overexpressed in E. coli and screened for metabolism of DCA; however, bile acid metabolites were not detected, perhaps due to screening against undialyzed clarified cell lysate without the addition of oxidized pyridine nucleotide.

We cloned, overexpressed and Talon-affinity purified recombinant N-terminal hexa-histidine tagged enzyme (rElen_2515) (27.57 ± 1.0 kDa) (Figure 4). The pH optimum for rElen_2515 in the oxidative direction with NAD⁺ as co-factor was 9.0, with a broad optimum between 7.0-7.5 in the reductive direction with NADH as co-factor (Figure S1). Purified rElen_2515 converted 12-oxoLCA to DCA, which was confirmed by mass spectrometry (Figure 4). rElen_2515 shows greater activity in the oxidative direction as evidenced by a higher $V_{max}$, lower $K_m$, higher $K_{cat}$, and a catalytic efficiency ($K_{cat}/K_m$) two orders of magnitude greater in the oxidative than the reductive direction.

### Table 2. Substrate-specificity of bile acid and pyridine nucleotides for rElen_2515.

| Steroid                      | Coenzyme | Relative Activity (%) |
|------------------------------|----------|-----------------------|
| deoxycholic acid*            | NAD⁺     | 100.00⁰               |
| glycodeoxycholic acid        | NAD⁺     | 97.80 ± 3.44          |
| taurodeoxycholic acid        | NAD⁺     | 90.84 ± 1.51          |
| cholic acid                  | NAD⁺     | 98.50 ± 3.41          |
| glycocholic acid             | NAD⁺     | 81.08 ± 3.34          |
| taurocholic acid             | NAD⁺     | 98.95 ± 1.32          |
| chenodeoxycholic acid        | NAD⁺     | NA                    |
| glycodeoxycholic acid        | NAD⁺     | NA                    |
| taurocholic acid             | NAD⁺     | NA                    |
| deoxycholic acid             | NADP⁺    | 3.14 ± 0.66           |
| 12oxolithocholic acid        | NADH     | 19.60 ± 1.39          |
| 12oxochenodeoxycholic acid   | NADH     | NA                    |
| 3oxodeoxycholic acid         | NADH     | NA                    |
| 3oxocholic acid              | NADH     | NA                    |

*Substrate-specificity was determined with 25 nM enzyme, 50 μM bile acid substrates, and 150 μM co-factor.

bValues reported as standard error of the mean (S.E.M) of four independent replicates.

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![Figure 5](image-url)  
**Figure 5.** Comparative genomic analysis between *Eggerthella lenta* strain C592 and *Eggerthella lenta* DSM 2243 (type strain). Circular and linear Mauve alignment representation are displayed. BlastKOALA pie chart depicts abundances within color-coded functional gene categories.
reductive direction at optimal pH (Table 1). In the oxidative direction with NAD\(^+\) as co-factor, rElen_2515 had similar substrate-specificity for DCA and CA (100 ± 2.53\% vs. 98.50 ± 1.32\%, respectively) and glycine and taurine conjugates were also substrates (Table 2).

Previous reports suggest *E. lenta* strains have bile salt hydrolase activity,\(^3\) suggesting that oxidation of bile acid hydroxyl groups may precede conversion of conjugated to free bile acids. Lacking a 12α-hydroxyl group, CDCA and its glycine and taurine conjugates were not substrates. NAD\(^+\) is the preferred cofactor, as oxidation of DCA in the presence of NADP\(^+\) resulted in only 3.14 ± 0.66\% relative maximal activity. Reduction of 12-oxoLCA to DCA in the presence of NADH occurred at only 19.60 ± 1.39\% maximal activity relative to the oxidation of DCA. Interestingly, activity with 12-oxoCDCCA was not detected in the reductive direction. As expected, reduction of 3-dehydroDCA or 3-dehydroCA in the presence of NADH was not detected, demonstrating regio-specificity of this enzyme for C-12.

We attempted to locate a gene encoding 7α-HSDH by cloning into pET46 and overexpressing 7 additional SDR family enzymes (Figure S2) predicted in the genome of *E. lenta* DSM 2243, but excluded Elen_0198, Elen_0690, and Elen_1325, which were previously shown to recognize the C3 position.\(^3\) Surprisingly, we did not detect 7α-HSDH activity in any of the TALON\(^®\)-purified enzymes, perhaps suggesting that *E. lenta* DSM 2243 expresses a novel 7α-HSDH in a different family of pyridine nucleotide-dependent enzymes (See Table S3 for *E. lenta* dehydrogenases). Further studies will be necessary to identify 7α-HSDH(s) from *E. lenta*.

**Identification of Rnf complex, Energy-conserving hydrogenase complex (Ech), and Wood-Ljungdahl Pathway genes**

Because we observed some notable steroid metabolic potential in *E. lenta* strain C592, such as the presence of steroid 17β-HSDH activity (Figure S3), we sequenced the complete 3,593,230 bp genome of *E. lenta* strain C592. (Figure 5; Tables S1 & S2). Mauve alignments (Figure 5) between *E. lenta* strain C592 and *E. lenta* DSM 2243 reveal substantial synteny; however, we observed additional notable differences including the absence of the cgr locus predicted to be involved in metabolism of cardiac glycosides such as digoxin\(^3\) as well as the absence of a “baiE” homolog in a “bai-like operon” of unknown function.\(^4\) Consistent with the formation of 3-dehydro and 3β-hydroxyl bile acid products (Figure 2), we identified an ORF in strain C592 corresponding to 3α-HSDH Elen_0690 (CAB18_RS04320) from *E. lenta* DSM 2243, as well as ORFs corresponding to 3β-HSDHs Elen_0198 (CAB18_RS014915) and Elen_1325 (CAB18_RS09005).\(^4\) A homolog of new characterized 12α-HSDH, Elen_2515, was also identified in the genome of *E. lenta* strain C592 (CAB18_RS03605).

In order to try to elucidate the role of bile acid oxidation in the physiology of *E. lenta*, we searched the genome of *E. lenta* strain C592 for metabolic pathways that may suggest the fate of reducing equivalents generated during bile acid oxidation. Genetic information obtained for *E. lenta* strain C592 was used to populate KEGG maps using Blast KOALA and pathway mapping tools. Of the entirety of predicted protein-encoding sequences in C592, only 1340 (42%) matched KEGG annotations. When carbon metabolism potential was analyzed, genes involved in the Wood-Ljungdahl pathway were found and putative clusters encoding the hydrogenase subunits were determined in both *E. lenta* strains C592 and DSM 2243.

Prior surveys of the gut of wood-feeding cockroaches\(^5\) and acidic fen\(^6\) resulted in identification of *Eggerthella* as encoding a formyl-tetrahydrofolate synthase (*fhs*) gene, suggesting that *Eggerthella* may have acetogenic potential. Common to all acetogens are genes encoding acetyl-CoA synthase (ACS)/carbon monoxide dehydrogenase (CODH).\(^7\) We located a conserved cluster of genes (Elen_3026-3030; CAB18_RS02000-2010) in both *E. lenta* DSM 2243 and *E. lenta* C592 which encode 4Fe-4S hybrid cluster proteins which include ACS and CODH. *Eggerthella* sp. strain YY7918 was found to harbor a different gene cluster, annotated as encoding *acsA* (EGGY_24090), *ascB/cdhC* (EGGY_24100), *acsF* (BAK45480) providing further genomic evidence that *Eggerthella* isolates encode WLP genes.

Genes encoding enzymes in the methyl branch of the WLP were also identified in the genomes of *E. lenta* DSM 2243 and *E. lenta* sp. strain C592 including formate dehydrogenase (*fdh*) (Elen_3031; CAB18_RS01995), which flanks the ACS/CODH cluster, formyl-tetrahydrofolate synthase (*fsh*) (Elen_2864; CAB18_RS01970), and methylene-tetrahydrofolate
dehydrogenase/cyclohydrolase (folD) (Elen_2861; CAB18_RS01985), bifunctional homocysteine S-methyltransferase/5,10-methylene-tetrahydrofolate reductase (yitJ) (Elen_2573; CAB18_RS03325). These results provide a plausible explanation for why *E. lenta* oxidizes bile acids in a reducing environment: oxidation of bile acids provides reducing equivalents for the fixation of CO₂ to acetate. Genes encoding enzymes involved in the energy conservation by substrate-level phosphorylation resulting in the conversion of acetyl-CoA to acetyl-PO₄ (eutD) (Elen_1728, and acetate kinase (ackA) (Elen_1729; CAB18_RS07170) were also identified. We also located pyruvate:ferredoxin oxidoreductase (PFOR) in both *E. lenta* DSM 2243 (Elen_2140) and several annotated genes for PFOR in *E. lenta* strain C592 (CAB18_RS00220; CAB18_RS05160; CAB18_RS07295; CAB18_RS07920; CAB18_RS13765). PFOR links the Wood-Ljungdahl pathway to the reductive tricarboxylic acid cycle, allowing autotrophic biosynthesis of complex macromolecules. Additional gene clusters encoded by model acetogens, including membrane-spanning electron transport chains such as the *Clostridium ljungdahlii* proton-translocating ferrodoxin:NAD⁺ oxidoreductase (Rnf) were found in both *E. lenta* DSM 2243 (Elen_2140) and several annotated genes for PFOR in *E. lenta* strain C592 (CAB18_RS00220; CAB18_RS05160; CAB18_RS07295; CAB18_RS07920; CAB18_RS13765). PFOR links the Wood-Ljungdahl pathway to the reductive tricarboxylic acid cycle, allowing autotrophic biosynthesis of complex macromolecules. An energy-conserving hydrogenase (Ech) was also located in *E. lenta* DSM 2243 (Elen_1570-1575) and *E. lenta* strain C592 (CAB18_RS07980-7960). In addition, both strains harbor ATP synthase transmembrane complexes, able to utilize this proton gradient generated from Rnf and Ech complexes to generate ATP from ADP.

**Molecular hydrogen inhibits bile acid oxidation by *E. lenta* strains**

The Rnf complex, as well as other electron-bifurcating enzymes, can shuttle electrons from reduced ferrodoxin to oxidized NAD⁺, thereby generating reduced NADH, which we predict might ultimately inhibit bile acid oxidation. Both *E. lenta* strain C592 and *E. lenta* DSM 2243 encode NiFe Group 4e (Ech) predicted to utilize hydrogen to reduce oxidized ferredoxin, we tested the effect of gas atmosphere on bile acid oxidation. When CDCA was added to an *E. lenta* sp. strain C592 growth culture, CDCA accounted for only 1.62 ± 0.11% of the total remaining bile acids after 24 hours growth under a N₂ atmosphere (0.68 atm) vs. 74.4 ± 2.02% under a H₂ atmosphere (0.68 atm) (p < 0.001), indicating that H₂ strongly inhibits bile acid oxidation. A similar observation was made with *E. lenta* DSM 2243 (Figure 6). N₂ atmosphere favored the formation of the most oxidized bile acid metabolite, 3,7-

![Figure 6](image_url) Effect of gas atmosphere on the formation of bile acid metabolites by *E. lenta* strain C592. Relative amount of CDCA metabolites formed by cultures of *E. lenta* C592 inoculated in Balch tubes in which headspace contained either H₂ (red) or N₂ (blue) (0.68 Atm). Bile acids were extracted after 24hrs growth in BHI + 1% arginine at 37°C. Experiments were repeated in triplicate ± standard errors and data were analyzed by two-tailed T-test ***p < 0.0001.
dioxocholanoic acid, which represented 47.55 ± 1.71% of the CDCA metabolites in E. lenta C592 vs. 0.38 ± 0.33% under H₂ atmosphere (p < 0.001). 7-oxoLCA was the second most abundant metabolite in both strains representing 36.82 ± 0.24% from E. lenta C592 and 52.12 ± 1.92% under N₂, compared with 14.03 ± 1.04% and 12.75 ± 0.99%, respectively, under H₂ atmosphere. The formation of isoCDCA derivatives (3β-hydroxyl), which requires oxidation of 3α-hydroxyl forming a stable 3-dehydro-intermediate followed by reduction, was essentially ablated under H₂. Similar experiments with C. scindens VPI 12708 demonstrate that bile acid 7α-dehydroxylation is not affected by the gas atmosphere (Figure S4). These findings may indicate that E. lenta can utilize hydrogenases and reverse electron flow to generate NADH from H₂ and that the gas atmosphere in the gut may be an important regulator of bile acid metabolism by particular intestinal bacteria (Figure 6).

Discussion

The current work is a key step toward understanding the physiological role for bile acid oxidation by E. lenta. Our initial screening of E. lenta strain C592, among a number of bile acid 7α-dehydroxylation bacteria such as C. scindens sp. strain I10, C. scindens sp. strain SO96, and C. scindens sp. strain SO77 revealed a distinct pattern of bile acid metabolites (Figure 1). Characterization of C592 metabolites of dihydroxy bile acids such as CDCA (3α-, 7α-hydroxyl), and DCA (3α-, 12α-hydroxyl), resulted in identification of oxo-derivatives and iso-bile acid metabolites (Figure 2). Sequencing of the 16S rDNA gene and WGS (Oxford nanopore & Illumina) resulted in identification of strain C592 as a novel strain of Eggerthella lenta (Figure 5). Taken together, E. lenta sp. strain C592 is categorized as a Type III strain because it expressed 3α-, 3β-, 7α-, and 12α-HSDHs (Figure 2), but it lacks the cgr locus and a “baiE” homolog in the “bai-like” cluster identified previously.

Previous work characterizing dozens of E. lenta strains, most of which were not deposited in culture collections, demonstrated 12α-HSDH activity. Here, we present a novel gene encoding 12α-HSDH in E. lenta (Figure 4). Purified rElen_2515 converted 12-oxoLCA to a product co-migrating with DCA, whose major mass ion was identical to authentic DCA (Figure 4). Kinetic analysis of the enzyme clearly shows that the oxidative direction is favored with a Kₘ in the oxidative direction ~100 μM lower than the reductive direction, and a turnover number (K₉Cat) an order of magnitude higher in the oxidative direction (Table 1). This is consistent with the observed complete oxidation of hydroxyl groups of CA, CDCA and DCA under a nitrogen atmosphere by E. lenta whole cells (Figures 1 & 2). rElen_2515 was NAD(H)-dependent (Table 2) with broad specificity, recognizing unconjugated primary (CA) and secondary bile acids (DCA), as well as taurine-conjugated (TCA, TDCA) and glycine-conjugated bile acids (GDCA, GCA) possessing a 12α-hydroxyl group. CDCA, which lacks a 12α-hydroxyl group was not a substrate (Table 2). This is in contrast to the only other gene reported that encodes 12α-HSDH from Clostridium group P strain C48-50/ATCC 29733, which had greatest specificity for CA and required NADP(H) as co-enzyme. The germinant receptor (CspC) in C. difficile was reported to specifically recognizes 12α-hydroxylated bile acids. A complex relationship exists between bile acid structure and C. difficile growth and sporulation, and oxidation or epimerization of the 12α-hydroxyl group may thus affect C. difficile germination. Nosocomial antibiotic-associated diarrhea by C. difficile results in nearly $5 billion in annual health care costs, with approximately 500,000 cases resulting in 29,000 deaths. Future experimentation should look to the effects that 12-oxoLCA has on both C. difficile growth and spore germination, as well as its ability to be recognized by the CspC receptor. The effects these oxo-bile acid metabolites have are not limited to other members of the gut microbiome.

The extent of epimerization and the accumulation of oxo-bile acids appears to be influenced by the oxidation/reduction potential of the local cellular environment. For example, the formation of oxo-bile acids in the gut may be more favorable in bacteria associated closer to the mucosal edges, where there is a higher redox potential than further inside the lumen of the intestines. In culture, anaerobic bacteria that reduce bile acids under strict anaerobic conditions, shift to oxidation of bile acid hydroxyl groups when introduced to aerobic conditions, as is the case with C. hiranonis. Oxidized or epimerized bile acids have differing effects on host physiology. 3-dehydroLCA has been shown to be the most potent agonist for the vitamin D receptor (VDR). Epimerization of the 7α-hydroxyl group on CDCA yields a much more
hydrophilic and therefore less toxic metabolite ursodeoxycholic acid, which has been shown to be protective against CRC-inducing effects of DCA. Recent studies reported that 7-oxoLCA acts as a competitive inhibitor of human hepatic 11β-HSDH-1. 11β-HSDH-1 is responsible for converting 7-oxoLCA back to CDCA; however, it also catalyzes the activation of cortisol from cortisone. When 7-oxoLCA is in high enough concentrations, it acts as a competitive inhibitor preventing production of active cortisol. 7-oxoLCA and ursodeoxycholic acid are both less potent agonists of FXR than the endogenous bile acid they are formed from, CDCA. Since the expression of the antimicrobial peptide cathelicidin is controlled by FXR in enterocytes, it follows that by lessening the affinity of bile acids for FXR, an otherwise susceptible microbe could increase its fitness in the lumen of the large intestine. The full extent to which alteration in bile acid hydroxyl oxidation or epimerization effects host metabolism is a field that requires more significant study.

Bile acids are known to activate host G-protein-coupled and nuclear receptors to varying extents based on the regio- and stereo-positioning of the functional groups attached to their steroid cores and side chains. 3-dehydroLCA, the most likely product of \textit{E. lenta} C592 LCA metabolism, has been shown to be the most potent vitamin D receptor agonist. Additionally, 7-oxoLCA and 3,7-dioxocholanoic acid have both been shown to be less potent agonists for FXR than their α-reduced counterparts, but more potent than β-reduced epimers. Oxo-bile acids, therefore, already have an established role in host physiology unique from their α- and β-reduced counterparts. However, the full spectrum of primary and secondary oxo-bile acid derivatives has not been tested for agonist properties on various bile acid-sensitive receptors. Of particular interest would be to determine the ability of an oxo-bile to activate TGR-5, as this is implicated in significantly altering host metabolism.

Intriguingly, genomic analysis of \textit{E. lenta} strains suggests that this gut microbial species encodes the Wood-Ljungdahl pathway, consistent with previous end-product analysis demonstrating that \textit{E. lenta} generates acetate and succinate. In addition, the partial pressures of molecular hydrogen may be a previously unrecognized factor in colonic bile acid metabolism. \textit{E. lenta} strains, through generation of dihydro- and trihydroxole acid, can be thought of as representing a bile acid hydroxyl “reset” affording the opportunity to drive bile acid metabolism in particular directions. However, hydrogen metabolism appears to be an important aspect of regulating bile acid oxidation by \textit{E. lenta} and requires further study (Figure 6). At present, we cannot say that bile acid oxidation is linked to reductive acetogenesis. Development of a minimal medium capable of supporting growth of \textit{E. lenta} in addition to genetic tools to knock out particular genes is currently lacking.

The production of oxo-bile acids, while substrates for intestinal microbial species expressing various HSDHs, may reduce the rate of 7α-dehydroxylation in the intestine. (Figure 3A). Additionally, iso-bile acids are not substrates for early key enzymatic steps prior to rate-limiting 7α-dehydration (Figure 3B). Thus, our data suggests that oxo-bile acid and iso-bile acid primary bile acid formation by \textit{E. lenta} may reduce formation of toxic, cancer-promoting secondary bile acids such as DCA.

\textbf{Materials and methods}

\textbf{Bacterial strains, culture conditions, materials}

\textit{Clostridium scindens} VPI 12708 obtained from Virginia Polytechnic Institute, and human fecal isolates C592, I10, SA14, 19BHI, K511, SO96, SO77 from collaborators at Ryukyu University in Okinawa, Japan are maintained as −80°C glycerol stocks in our laboratory. \textit{Eggerthella lenta} DSM 2243 was acquired commercially (DSMZ). Before further analysis, strains were propagated on brain heart infusion (BHI) agar plates and grown under anaerobic conditions in Brewer jar with AnaeroPack (Mitsubishi) for 48 hours at 37°C, and colonies were picked and grown individually. Unless otherwise noted, bacterial strains were grown in liquid BHI broth (Becton, Dickinson) in round bottom flasks anaerobically under 100% N\textsubscript{2} gas atmosphere (Airgas), supplemented with 5g L\textsuperscript{−1} yeast extract (Becton, Dickinson), 1g L\textsuperscript{−1} cysteine HCl (Sigma) and 40mL/L of a salt solution containing 0.2g CaCl\textsubscript{2}, 0.2g MgSO\textsubscript{4}, 1g K\textsubscript{2}HPO\textsubscript{4}, 1g KH\textsubscript{2}PO\textsubscript{4}, 10g NaHCO\textsubscript{3} per liter. When arginine (Sigma) was used, it was added separately to the media to a final concentration of 5g L\textsuperscript{−1} (0.5% wt/volume) or 10g L\textsuperscript{−1} (1% wt/volume). For gas atmosphere experiments, BHI tubes were flushed with either H\textsubscript{2} or N\textsubscript{2} at 0.68 atm to replace the atmosphere, using a double-needle system in which sterile needles were inserted with input gas.
filtered with 0.2 \( \mu \text{M} \) filter. Oxygen was removed from commercial gases by running through a heated manifold containing reduced copper shavings. *Escherichia coli* DH5α and *E. coli* BL21-CodonPlus(DE3) RIPL competent cells were used for cloning and overexpression, respectively. The pET-46 Ek/LIC vector kit was obtained from Novagen (San Diego, CA). The QIAprep Spin Miniprep kit was obtained from Qiagen (Valencia, CA). Isopropyl \( \beta \)-D-1-thiogalactopyranoside (IPTG) was purchased from Gold Biotechnology (St. Louis, MO). Bile acid substrates were purchased from Steraloids, Inc (Newport, RI, USA). Before addition to culture, bile acids were suspended in methanol to a concentration of 10 mM before being diluted to their final concentration in culture media.

### Whole cell bile acid conversion assays

Screening of putative bile acid 7α-dehydroxylation bacteria was carried out by cultivation in BHI containing 25 \( \mu \text{M} \) CA (0.1\( \mu \text{Ci/\mu mole} \) [24\(^{14}\text{C}] \) CA) for 24 hrs. [24\(^{14}\text{C}] \) allodeoxycholic acid (ADCA; 3\( \alpha,12\alpha \)-dihydroxy-5\( \alpha \)-cholan-24-oic acid) was produced as previously reported. Generation of metabolites for whole-cell bile acid conversion assay were generated by cultivating *E. lenta* C592 in BHI containing 25 \( \mu \text{M} \) CDCA (0.1\( \mu \text{Ci/\mu mole} \) [24\(^{14}\text{C}] \) CDCA) for 24 hrs. Metabolites were extracted twice with two volumes ethyl acetate and separated on BakerFlex Silica B gel TLC plates (J.T. Baker, LLC) with solvent system of 75:20:2 (v/v/v) toluene:dioxane:glacial acetic acid. Kodak Biomax MS film was exposed to the TLC for 24 hrs and developed. Metabolites were then extract from the TLC plate and quantified by liquid scintillation spectrometry. Metabolites were identified by mass spectrometry and compared to mass spectra from standards.

### HPLC and mass spectrometry of bile acid metabolites

For MS analysis, 100 mL *E. lenta* strain C592 cultures were grown to stationary phase as stated above in the presence of 25 \( \mu \text{M} \) CDCA or DCA with and without [24\(^{14}\text{C}] \) radiolabel. Bile acid metabolites were extracted, separated, and isolated as stated above and then underwent LC-MS analysis. LC-MS analysis was run on a Shimadzu UPLC coupled with a Shimadzu LCMS-IT-TOF System (Shimadzu Corporation, Kyoto, Japan). The LC operating conditions were as follows: LC column, C-18 analytical column (Capcell Pak C18, Shiseido, Japan), 250 mm \( \times \) 2 mm

### Table 3. Cloning primers used in this study.

| Gene ID   | 5'-Forward Primer-3' | 5'-Reverse Primer-3' | Mw (kDa) | Extinction coefficient (M\(^{-1}\).cm\(^{-1}\)) |
|-----------|----------------------|----------------------|----------|---------------------------------------------|
| Elen_0360 | GACGACGACAAGATGGAAGAGAGAGCCGTTTGGAC | GAGGAGAAGCCGCTCTAGTGCACTGAAAGCGCGGGGCTCTTT | 27.166   | 17,670                                      |
| Elen_0359 | GACGACGACAAGATGGAAGAGAGAGCCGTTTGGAC | GAGGAGAAGCCGCTCTAGTGCACTGAAAGCG | 27.660   | 16,180                                      |
| Elen_0358 | GACGACGACAAGATGGAAGAGAGAGCCGTTTGGAC | GAGGAGAAGCCGCTCTAGTGCACTGAAAGCG | 31.589   | 30,160                                      |
| Elen_1208 | GACGACGACAAGATGGAAGAGAGAGCCGTTTGGAC | GAGGAGAAGCCGCTCTAGTGCACTGAAAGCG | 29.729   | 28,795                                      |
| Elen_1325 | GACGACGACAAGATGGAAGAGAGAGCCGTTTGGAC | GAGGAGAAGCCGCTCTAGTGCACTGAAAGCG | 27.310   | 21,680                                      |
| Elen_1987 | GACGACGACAAGATGGAAGAGAGAGCCGTTTGGAC | GAGGAGAAGCCGCTCTAGTGCACTGAAAGCG | 26.837   | 4,845                                       |
| Elen_2188 | GACGACGACAAGATGGAAGAGAGAGCCGTTTGGAC | GAGGAGAAGCCGCTCTAGTGCACTGAAAGCG | 30.963   | 27,390                                      |
| Elen_2515 | GACGACGACAAGATGGAAGAGAGAGCCGTTTGGAC | GAGGAGAAGCCGCTCTAGTGCACTGAAAGCG | 27.078   | 20,065                                      |
| CDD59474 | GACGACGACAAGATGGAAGAGAGAGCCGTTTGGAC | GAGGAGAAGCCGCTCTAGTGCACTGAAAGCG | 26.355   | 6,085                                       |
| CDD59473 | GACGACGACAAGATGGAAGAGAGAGCCGTTTGGAC | GAGGAGAAGCCGCTCTAGTGCACTGAAAGCG | 31.590   | 46,785                                      |
| CDD59475 | GACGACGACAAGATGGAAGAGAGAGCCGTTTGGAC | GAGGAGAAGCCGCTCTAGTGCACTGAAAGCG | 28.330   | 27,640                                      |
E. lenta strain C592 genomic sequencing and comparative genomics

Generic 16S primers (16s357F, 16s1392R) and ExTaq polymerase kit (Takara-Bio, Kusatsu, Japan) were used for initial 16S screening of E. lenta strain C592. Genomic DNA (1.5 μg) was sheared in a gTube (Covaris, Woburn, MA) for 1 minute at 6,000 rpm in an Eppendorf MiniSpin plus microcentrifuge (Eppendorf, Hauppauge, NY). The sheared DNA was converted into a Nanopore library with the Nanopore Sequencing kit SQK-NSK007 (Oxford Nanopore, UK). The library was sequenced on a SpotON Flowcell MK I (R9) flowcell for 48 hours, using a MinION MK 1B sequencer. Basecalling was performed in real time with the software Metrichor version 2.40.17. Poretools v-0.5.1 software was used to extract sequences from Oxford Nanopore MinION output file folder, and then converted to fastq format. FastQC v-0.11.2 software was also used to further access quality scores and other attributes of the data set. A Perl script was then used to trim adaptors from the raw nanopore reads. The adapter trimmed reads were used to blast against NCBI Ecoli_strK12_MG1655 genome. Reads with greater than 95% alignment to this genome were removed. 2,113,230 reads from the Illumina paired end MiSeq run and 14,023 reads from Oxford Nanopore sequencing platform were used for de novo hybrid assembly with SPAdes-v3.9.0. The assembly produced 245 contigs, five of which were 500 base pairs and longer. The top five contigs were selected to blast NCBI NT database. Nucleotide level comparisons between E. lenta DSM 2243 genome and the longest contig from the assembly were done with the dnadiff program from MUMmer v-3.23. Annotation comparisons between E. lenta DSM 2243 genome and the longest contig were made with Prokka v-1.11. Annotated CDS file for the longest E. lenta strain C592 contig were then imported into Geneious v9.1.3 for Mauve alignment and further analysis, as well as utilized to form KEGG maps via BlastKOALA. The genome data was deposited to NCBI (Reference Sequence NZ_CP021140.1).

Cloning, expression and purification of recombinant proteins

Sequences corresponding to E. lenta genomic DNA regions was synthesized by Integrated DNA Technologies (IDT) (Coralville, IA, USA). Gene fragments were amplified with primers synthesized by IDT (Table 3), using the Phusion high fidelity polymerase (Strategene, La Jolla, CA) and cloned into pET-46b vector (Novagen), as described by the manufacturer’s protocol. Cultivation, plasmid isolation and sequence confirmation were as previously described. 12α-HSDH were expressed as previously described. The recombinant proteins were then purified using TALON® Metal Affinity Resin (Clontech Laboratories, Mountain View, CA) as per manufacturing protocol. The recombinant protein was eluted using an elution buffer composed of 20 mM Tris-HCl, 150 mM NaCl, 20% glycerol, 10 mM 2-mercaptoethanol pH 7.9 and 250 mM imidazole. The protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein bands were visualized by staining with Coomassie brilliant blue G-250. Recombinant protein concentration was calculated based on their molecular mass and extinction coefficients. Subunit molecular mass was calculated using three independent SDS-PAGE gels with Biorad...
Enzyme assays

12α-HSDH enzyme pH optima in both reductive and oxidative direction was determined essentially as described previously with bile acid substrates DCA and 12-oxoLCA and pyridine-nucleotide co-factor. The reactions were initiated by addition of enzyme. Linearity of enzyme activity with respect to time and enzyme concentration was determined aerobically by monitoring the oxidation/reduction of NAD(P)(H) at 340 nM (ε = 6,220 M⁻¹·cm⁻¹) in the presence of bile acid and steroid substrates. Kinetic parameters were estimated by fitting the data to the Michaelis-Menten equation by non-linear regression method using the enzyme kinetics module in GraphPad Prism (GraphPad Software, La Jolla, CA). Substrate-specificity was determined with 25 nM enzyme, 50 µM bile acid substrates, and 150 µM co-factor.

Statistics

Descriptive statistics are provided, where appropriate, in the figure legends. Three biological replicates from bile acid biotransformation experiments were analyzed for significance by GraphPad Prism using two-tailed student T-test.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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