© Med Sci Monit, 2012; 18(11): BR427-434
PMID: 23111733

Received: 2012.02.24
Accepted: 2012.07.03
Published: 2012.11.01

Comparative kinetic analysis of glycerol 3-phosphate cytidylyltransferase from *Enterococcus faecalis* and *Listeria monocytogenes*

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Ashley N. Mericl[ABCDEF], **Jon A. Friesen**[ACDEG]

Department of Chemistry, Illinois State University, Normal, IL, U.S.A.

Source of support: Departmental sources

Summary

Background: Glycerol 3-phosphate cytidylyltransferase (GCT) is an enzyme central to the synthesis of teichoic acids, components of the cell wall in gram positive bacteria. Catalysis by GCT from *Enterococcus faecalis* and *Listeria monocytogenes* has been investigated and catalytic properties compared.

Material/Methods: The genes encoding GCT were cloned from genomic DNA and recombinant proteins expressed in *E. coli* and purified. Enzyme assays were used to determine kinetic constants k_{cat} and K_m. Chemical crosslinking provided a means to assess quaternary structure of each GCT.

Results: Recombinant *Enterococcus faecalis* GCT had an apparent k_{cat} value of 1.51 s^{-1} and apparent K_m values of 2.42 mM and 4.03 mM with respect to substrates cytidine 5'-triphosphate (CTP) and glycerol phosphate. *Listeria monocytogenes* GCT had an apparent k_{cat} value of 4.15 s^{-1} and apparent K_m values of 1.52 mM and 6.56 mM with respect to CTP and glycerol phosphate. This resulted in k_{cat}/K_m values of 0.62 $s^{-1}mM^{-1}$ and 0.37 $s^{-1}mM^{-1}$ for *E. faecalis* GCT and 2.73 $s^{-1}mM^{-1}$ and 0.63 $s^{-1}mM^{-1}$ for *L. monocytogenes* GCT with respect to CTP and glycerol phosphate, respectively.

Conclusions: The genome of both *Enterococcus faecalis* and *Listeria monocytogenes* contain a gene that encodes a functional GCT. The genes are 67% identical at the nucleotide level and the encoded proteins exhibit a 63% amino acid identity. The purified, recombinant enzymes each appear to be dimeric and display similar kinetic characteristics. Studying the catalytic characteristics of GCT isoforms from pathogenic bacteria provides information important for the future development of potential antibacterial agents.

key words: **catalysis • cytidylyltransferase • kinetics**

Full-text PDF: http://www.medscimonit.com/fulltxt.php?ICID=883535

Word count: 3430
Tables: 1
Figures: 8
References: 24

Author's address: Jon A. Friesen, Department of Chemistry, 4160 Chemistry, Illinois State University, Normal, IL 61790-4160, U.S.A., e-mail: jfriese@ilstu.edu

Background

Entercoccus faecalis is a Gram-positive bacterium found in the gastrointestinal tract, oral cavity, and heart lining of humans. This fermentative, facultatively anaerobic bacterium is found in birds, reptiles, insects, plants, water, and soil in addition to mammals [1]. *E. faecalis* is known to cause endocarditis, an inflammation of the heart lining. Vancomycin-resistant *Enterococci* (VRE), first isolated in Europe in 1988, are encountered in the hospital setting and are often treated with drugs such as linezolid, daptomycin, and tigecycline [2,3]. *Listeria monocytogenes*, also a Gram-positive, facultative anaerobic bacterium, is commonly found in foods and ingestion can cause the food borne illness listeriosis, potentially resulting in a blood infection or meningitis and miscarriage in pregnant women [4,5]. The treatment for *Listeria* infections is usually ampicillin or penicillin G combined with an aminoglycoside [6].

Growth of most Gram-positive bacteria is dependent on the synthesis of teichoic acids, major components of the bacterial cell wall. Glycerol-3-phosphate cytidylyltransferase (GCT, EC 2.7.7.39) catalyzes the transfer of the cytidyl group of cytidine 5'-triphosphate (CTP) to glycerol 3-phosphate (Figure 1) and is part of a larger pathway that results in the synthesis of teichoic acid poly (glycerol phosphate). The genes concerned with the synthesis of teichoic acid poly (glycerol phosphate) in *Bacillus subtilis* are organized into two divergently transcribed operons, tagAB and tagDEF [7]. A gene of the tagDEF operon, tagD, encodes GCT [8]. GCT is part of a larger family of cytidylyltransferases, enzymes that catalyze reversible reactions in which an alcohol and CTP are the substrates, and pyrophosphate and a cytidylyl ester are the products. There are three principal members in the cytidylyltransferase family, CTP: phosphocholine cytidylyltransferase (CCT), CTP: phosphoethanolamine cytidylyltransferase (ECT), and GCT [9]. CCT is a major regulatory enzyme in the CDP-choline pathway, which results in the synthesis of phosphatidylcholine in eukaryotes. In addition to its catalytic domain, CCT contains a phosphorylated carboxy terminus and is regulated by activation following association of a lipid binding domain with the membrane [10]. ECT is part of the CDP-ethanolamine pathway in eukaryotes and is involved in the synthesis of phosphatidylethanolamine. Inspection of the primary sequence of ECT suggests it is comprised of two catalytic domains [11]. GCT is the smallest member of the cytidylyltransferase family and appears to function as a homodimer. The genes encoding GCT from *Bacillus subtilis* and *Staphylococcus aureus* have previously been cloned and each protein expressed in *E. coli*. The three-dimensional structure of GCT has been solved for both of these recombinant forms of the enzyme using X-ray crystallography and serve as model structures for the catalytic domain of cytidylyltransferases [9,12,13].

The goal of the research presented here was to clone the genes encoding GCT from the pathogenic Gram-positive bacteria *Enterococcus faecalis* and *Listeria monocytogenes*, express the recombinant proteins in *E. coli*, and characterize the kinetic properties of each enzyme. Kinetic parameters of the two recombinant GCT isoforms were compared to each other and with the previously reported values for other isoforms of this important enzyme.

Material and Methods

Chemicals

Activated charcoal, sn-Glycerol 3-phosphate bis(cyclohexylammonium) salt, cytidine 5'-triphosphate (CTP) disodium salt, T7 forward primer (5'-TAATACGACTCACTATAGGG-3'), and T7 reverse primer (5'-GCTAGTTATTGCTCAGCGG-3') were obtained from Sigma Aldrich. Deoxyribonucleotide triphosphate (dNTP) mix, Luria-Bertani (LB) broth, isopropyl β-D-1-thiogalactopyranoside (IPTG), ammonium persulfate (APS), *N,N,N',N'*-Tetramethylethylenediamine (TEMED), acrylamide, imidazole, trichloroacetic acid (TCA), and ScintiSafe® scintillation fluid were purchased from Fisher Scientific. *Pfx* DNA polymerase, SYBR® Safe DNA gel stain, and DH5α competent cells were purchased from Invitrogen. *Enterococcus faecalis* genomic DNA (ATCC® 700802D™) was obtained from the American Type Culture Collection. *Listeria monocytogenes EGD-e* genomic DNA was purified from a bacterial cell culture obtained from Dr. Brian Wilkinson, School of Biological Sciences, Illinois State University. New England Biolabs was the source of BamHI, XhoI, and T4 DNA ligase and BL21(DE3)RIL competent cells were obtained from Agilent Technologies. The pET45b vector was from Novagen and BigDye® Terminator sequencing kit was obtained from Applied Biosystems. Low Range SDS-PAGE protein standards and BioRad™ Protein Assay Concentrate were purchased from BioRad. TALON® Metal Affinity Resin was obtained from Clontech. Disuccinimidyl glutarate (DSG) and Dimethyl Suberimidate (DMS) were purchased from Pierce. [^{14}C] glycerol 3-phosphate was obtained from Amersham Biosciences.

Amplification of the genes encoding GCT

PCR was used to amplify genes encoding GCT using *Enterococcus faecalis* or *Listeria monocytogenes* genomic DNA as a template. For amplification of the gene encoding *E. faecalis* GCT the 5' oligonucleotide had the sequence 5'-TACTGGATCCCAAAAAAATACTTACTTACG-3' and the 3' oligonucleotide was 5'-TACTCTCGAGTTATTCAC TATAATATAATTC-3'. Restriction enzyme sites BamHI and XhoI (underlined) were added for cloning into pET45b. For amplification of the gene encoding *L. monocytogenes* GCT the 5' and 3' oligonucleotides were 5'-TGAA CCATGGATCCCGGGAAAAAGGTTATTACATATGG-3' and 5'-GCGATTACGCGGCCGCGTCGACTTATT TTAG TTCATCTTTTATTTG-3'. Restriction enzyme sites BamHI and NotI (underlined) were added for cloning into pET45b. In addition to approximately 100 ng of template DNA, the following were added to each PCR reaction in a final volume of 100 µl: 0.2 mM of each dNTP, 0.1 µM of each oligonucleotide, and 3 units of *Pfx* DNA polymerase. $MgSO_4$ concentration was varied from 0.5 mM to 6 mM. Thermocycler parameters were 30 cycles of 94°C for 15 seconds, 45°C for 30 seconds, and 68°C for 60 seconds. Following PCR the DNA was digested with BamHI and XhoI (*E. faecalis* GCT) or BamHI and NotI (*L. monocytogenes* GCT) and cloned into pET45b. The nucleotide sequence of each gene was verified by sequencing using an Applied Biosystems 3130 Genetic Analyzer and the BigDye® Terminator sequencing kit. The resulting pET45b vectors containing either the gene encoding *E. faecalis* or *L. monocytogenes* GCT were termed pET45b-Ef-GCT and pET45b-LmGCT. Cloning appended an additional

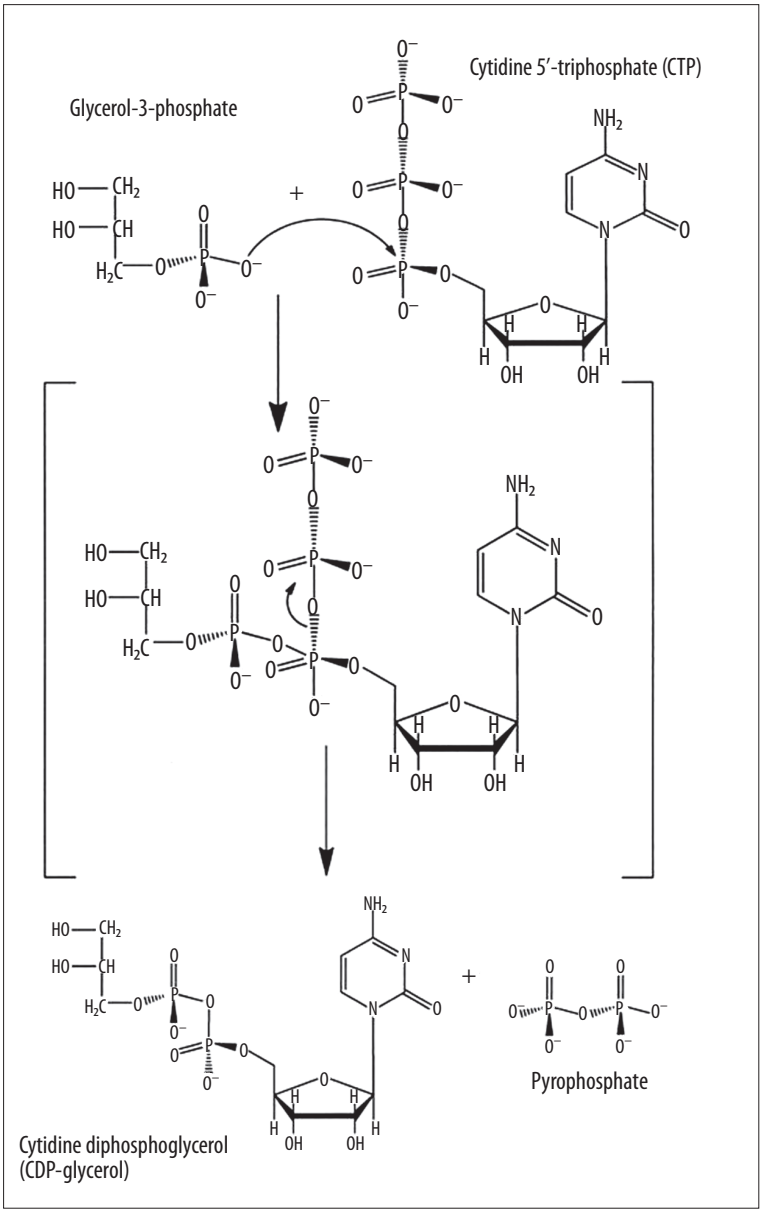

Figure 1. The proposed mechanism of the reaction for production of CDP-glycerol and pyrophosphate by GCT. Glycerol phosphate serves as a nucleophile, attacking the phosphorous of the alpha phosphate in CTP. Shown in brackets is the putative pentacoordinate phosphate intermediate. Release of pyrophosphate generates the CDP-glycerol product.

24 amino acids at the amino terminus (MAHHHHHHVGT GSNDDDDKSPDPH) that contained a 6x-His-tag.

Expression of GCT in *E. coli*

The BL21(DE3)RIL strain of *E. coli* was transformed with recombinant pET45b-EfGCT or pET45b-LmGCT and grown at 37°C in LB media containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. One liter cultures were grown at 37°C while shaking at 250 rpm until an optical density of 0.8 was reached at 600 nm. Induction of protein production was accomplished by the addition of IPTG to a final concentration of 1 mM. Cells were collected by centrifugation at 5,000 × g for 5 minutes and resuspended in 20 ml lysis buffer (20 mM Tris-Cl, 100 mM NaCl, pH 7.5). Protease inhibitor cocktail (Sigma Aldrich) was added so that the final concentration of protease inhibitors were 20 µM AEBSF, 0.003 µM aprotinin, 1.3 µM bestatin, 10 µM EDTA, 0.14 µM E-64, and 0.01 µM leupeptin. Cells were lysed at 10,000 psi using a French Press, the cell extract was centrifuged (27,000 × g, 20 minutes, 4°C), and the supernatant, which contains recombinant GCT, was collected. A 15% SDS-PAGE gel was used to analyze protein expression. After electrophoresis at 150 mV for 70 minutes the gel was stained with a solution containing 0.1% Coomassie Blue, 10% methanol, and 45% acetic acid for 20 minutes. The gel was destained using a solution of 10% methanol and 45% acetic acid until the regions of the gel not containing protein were sufficiently devoid of stain.

Purification of GCT using affinity chromatography

Purification utilized the incorporated 6x-His-tag and a 3 mL column of TALON® Metal Affinity Resin equilibrated with lysis buffer. The supernatant was passed through the column followed by 50 mL of lysis buffer and a second wash with 50 mL of lysis buffer containing 10 mM imidazole. Recombinant GCT was eluted from the column

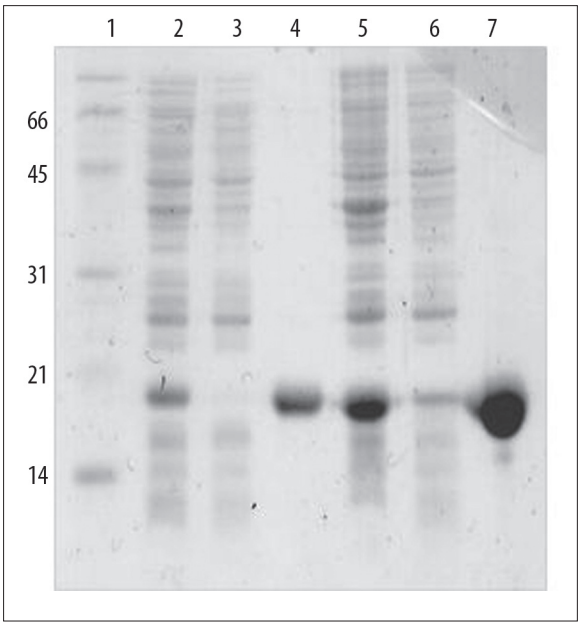

Figure 2. Analysis of LmGCT and EfGCT purification using 15% SDS-PAGE. Purification was conducted as described in material and methods. Lane 1 contains the protein standards, with molecular mass indicated in kDa. Lanes 2–4 are samples from the purification of *E. faecalis* GCT and lanes 5–7 are samples from the purification of *L. monocytogenes* GCT. Lanes 2 and 5 contain the cellular supernatant liquid after lysis of bacterial cells and centrifugation. Lanes 3 and 6 contains the fraction that did not bind to the metal affinity column. Lanes 4 and 7 contain the purified GCT protein eluted from the column.

using 10 mL of lysis buffer containing 150 mM imidazole. Ten 1 mL fractions were collected from the column and protein concentration determined using the BioRad™ Protein Assay kit and bovine serum albumin (BSA) as standard. SDS-PAGE was used to assess the purity of the protein samples.

Enzymatic assay of purified GCT

The GCT enzyme assay was conducted in 20 mM Tris-HCl pH 8 that contained 6 mM magnesium chloride and indicated concentrations of the substrates CTP and [^{14}C]glycerol phosphate in a final volume of 100 µL. A control tube containing substrates and buffer, but no enzyme, served as background. Assays were conducted at 37°C for 15 minutes, terminated by the addition of 100 µL of 10% (v/v) TCA, placed on ice, and 0.5 mL of a 10 mg/mL activated charcoal suspension was added. After incubation on ice for 15 minutes, tubes were centrifuged for 3 minutes at 16,000 × g, the supernatant was removed, and the charcoal was washed twice with 600 µL of H_2O. After washing, the bound [^{14}C] CDP-glycerol product was eluted from the charcoal by incubation in 0.5 mL of 10% (v/v) acetic acid at 37°C for 5 minutes. Following incubation, the tubes were centrifuged at 16,000 × g for 3 minutes, the supernatant was removed and placed in a scintillation vial along with 5 mL of scintillation fluid, and radioactivity determined in a Beckman LS6500 scintillation counter. For kinetic analysis CTP concentration was varied while glycerol phosphate was held constant at 20 mM and glycerol phosphate concentration was varied while CTP was held constant at 10 mM.

Crosslinking of GCT

Crosslinking reactions were conducted using Disuccinimidyl Glutarate (DSG) and Dimethyl Suberimidate (DMS). Reaction tubes contained 0.25 mM purified GCT and either 0 mM, 0.4 mM, or 2 mM crosslinker in a total volume of 100 µl. Following incubation at 22°C for 30 minutes 15% SDS-PAGE was used to analyze the crosslinking reactions.

Results

Expression and purification of GCT using metal affinity chromatography

The pET45b plasmid encodes a 6-histidine (6x-His) tag that was added to the amino terminus of *E. faecalis* GCT (EfGCT) and *L. monocytogenes* GCT (LmGCT), affording the use of metal affinity column chromatography to purify each recombinant GCT. Expression of each GCT was robust in *E. coli*, with an overexpressed band evident on SDS-PAGE at the expected molecular mass of 16.4 kDa for EfGCT and 17.2 kDa for LmGCT (Figure 2, lanes 2 and 5). Each protein was soluble in the cell supernatant liquid following expression and centrifugation. Purification yielded ample amounts of protein, with over 50 mg of purified protein produced per liter culture of *E. coli*. To assess purity of the protein eluted from the column, purification fractions were analyzed via SDS-PAGE. The gel indicates each GCT was successfully purified (Figure 2, lanes 4 and 7).

Catalysis by EfGCT and LmGCT

A radioisotope assay monitoring conversion of [^{14}C]glycerol phosphate to [^{14}C]CDP-glycerol was used to assess catalytic activity of EfGCT and LmGCT [8]. During catalysis, glycerol 3-phosphate acts as a nucleophile, attacking the α-phosphorous of the CTP, which leads to a putative pentacoordinate phosphorous transition state (Figure 1). To obtain apparent k_{cat} and K_m values for EfGCT and LmGCT the concentrations of glycerol phosphate and CTP were each varied and specific activity determined at each concentration. Data were analyzed using the Michaelis-Menten equation and nonlinear regression analysis (Figures 3, 4). Apparent V_{max} and K_m values were obtained from the curve fitting. V_{max} values were then converted to k_{cat} values for comparison to k_{cat} values reported for other bacterial GCT isoforms. Analysis revealed an apparent k_{cat} of 1.51 s^{-1} for EfGCT and an apparent K_m values of 2.42 mM with respect to CTP and 4.03 mM with respect to glycerol phosphate. LmGCT exhibited an apparent k_{cat} of 4.15 s^{-1} and apparent K_m values of 1.52 mM and 6.56 mM with respect to CTP and glycerol phosphate, respectively (Table 1).

Quaternary structure of GCT isoforms

Cross linking reactions using two different chemical crosslinkers were employed to indicate quaternary structure of EfGCT and LmGCT. Disuccinimidyl glutarate (DSG) contains a *N*-hydroxysuccinimide (NHS ester) and creates 7.7 Å (5 atom) cross links between amino groups in proteins. Dimethyl suberimidate (DMS) creates a 11.0 Å (8 atom

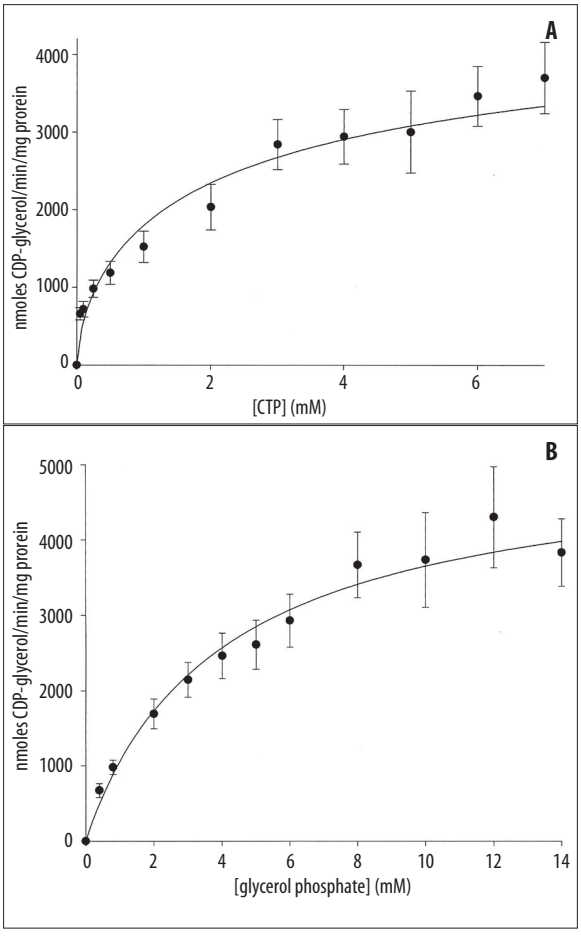

Figure 3. Plots of EfGCT activity as a function of substrate concentration for kinetic analysis. The initial reaction velocity (specific activity) as a function of the substrate concentration CTP (**A**) or glycerol phosphate (**B**). Data were fit to the Michaelis Menten equation using nonlinear regression analysis and the apparent K_m and k_{cat} values obtained from the curve fitting. The units of specific activity are nmoles CDP-glycerol formed per minute per mg of enzyme.

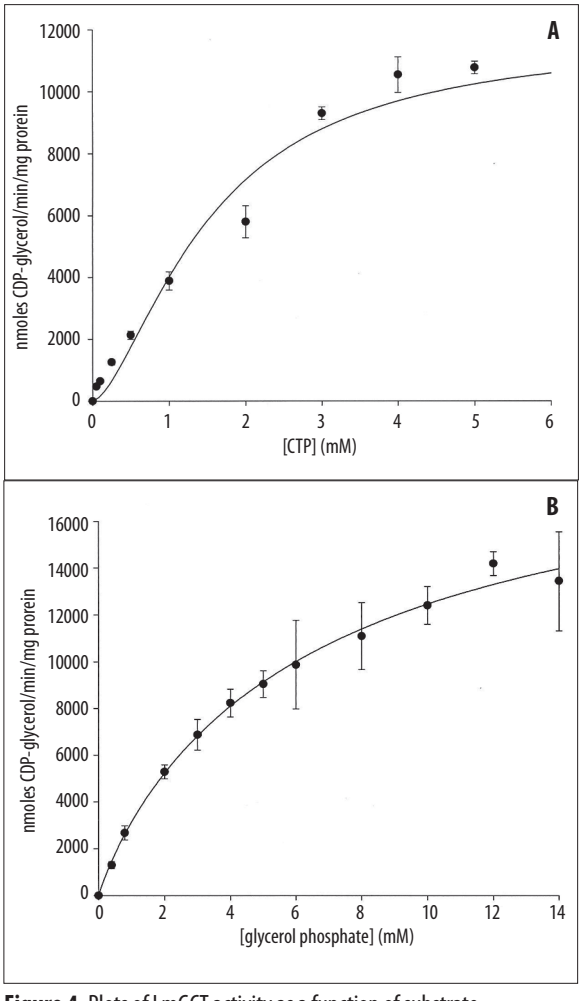

Figure 4. Plots of LmGCT activity as a function of substrate concentration for kinetic analysis. The initial reaction velocity (specific activity) as a function of the substrate concentration CTP (**A**) or glycerol phosphate (**B**). Data were fit to the Michaelis-Menten equation using nonlinear regression analysis and the apparent K_m and k_{cat} values obtained from the curve fitting. The units of specific activity are nmoles CDP-choline formed per minute per mg of enzyme.

long) cross link between amino groups in proteins via an imidoester group at each end of the spacer arm. Analysis of cross-linking reaction using SDS-PAGE indicated the presence of a band at approximately twice the monomeric molecular weight of either EfGCT or LmGCT in the presence, but not the absence, of crosslinkers (Figure 5).

Discussion

E. faecalis and *L. monocytogenes* GCT gene sequences

The nucleotide sequence of the genomes of both *E. faecalis* (accession AE016830) and *L. monocytogenes* (accession NC_003210) have been determined and sequences that encode a putative GCT have been annotated for each organism. In our study the nucleotide sequence of the genes cloned agreed with the database sequences. Comparison of the 399 bp *E. faecalis* GCT gene with the 384 bp *L. monocytogenes* GCT gene reveals 256 of the 384 nucleotides in the *L. monocytogenes* GCT gene sequence match the corresponding nucleotide in the *E. faecalis* GCT gene sequence for a 67% identity. GCT isoforms from *Bacillus subtilis* (accession AM260209) and *Staphylococcus aureus* (accession NC_009632) have been

Table 1. Comparison of kinetic parameters for GCT isoforms.

Organism	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Enterococcus faecalis	CTP: 2.42 GP: 4.03	1.51	CTP: 0.62 GP: 0.37
Listeria monocytogenes	CTP: 1.52 GP: 6.56	4.15	CTP: 2.73 GP: 0.63
Staphylococcus aureus [22]	CTP: 0.036 GP: 0.021	2.6	CTP: 72 GP: 130
Bacillus subtilis [15]	CTP: 1.39 GP: 1.09	18.8	CTP: 13.5 GP: 17.2

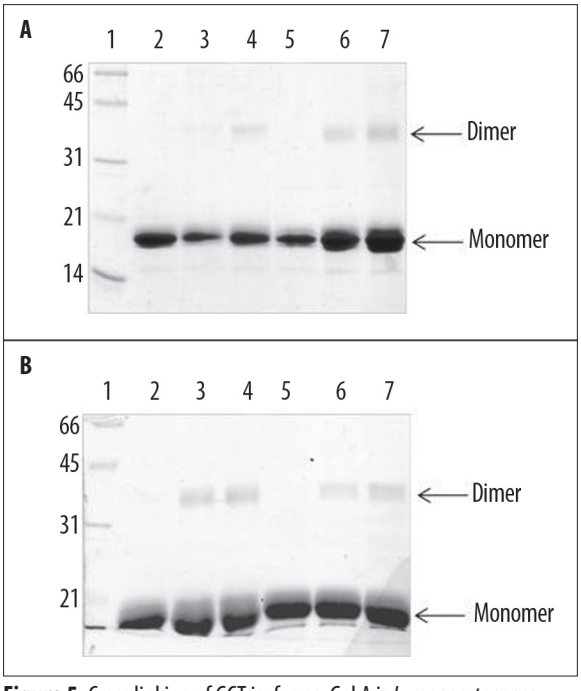

Figure 5. Cross linking of GCT isoforms. Gel A is *L. monocytogenes* GCT and B is *E. faecalis* GCT. Lane 1 contains the protein standards, molecular mass in kDa indicated. Lanes 2 and 5 contain purified protein without crosslinker. Lanes 3 and 4 contain protein crosslinked with DSG. Lanes 6 and 7 contain protein crosslinked with DMS.

Amino Acid Identify	LmGCT	EfGCT	BsGCT	SaGCT
LmGCT		63	69	72
EfGCT	63		62	59
BsGCT	69	62		63
SaGCT	72	59	63	

Figure 6. Amino acid identity between GCT sequences from *Listeria monocytogenes*, *Enterococcus faecalis*, *Bacillus subtilis*, and *Staphylococcus aureus*.

the most extensively studied to date [8,9,12–16]. The *L. monocytogenes* GCT gene sequence has greater identity to both *S. aureus* and *B. subtilis* than to *E. faecalis* while the *E. faecalis* GCT gene sequence is 66–67% identical to each of the other three GCT genes. In an alignment of all four GCT gene sequences there is an overall identity of 50% at the nucleotide level. A notable difference between the four gene sequences is the insertion of six nucleotides in the *E. faecalis* gene sequence, TTGAA found at positions 311–315 and G at position 328. The five nucleotides from 311–315 shift the reading frame temporarily, with the additional G inserted at position 328 restoring the reading frame relative to the other three GCT gene sequences.

Comparison of EfGCT and LmGCT catalysis to other GCT isoforms

Both recombinant EfGCT and LmGCT exhibited significant catalytic activity, indicating each *tagD* gene did, in fact, encode glycerol 3-phosphate cytidylyltransferase. This provides experimental evidence that the genome of both *Enterococcus faecalis* and *Listeria monocytogenes* contain a gene that encodes a functional GCT. Kinetic parameters for EfGCT and LmGCT were compared to previously characterized GCT isoforms from *B. subtilis* and *S. aureus* (Table 1). Analysis reveals that EfGCT and LmGCT are quite similar to each other with respect to kinetic parameters, with only a 1.6-fold greater CTP K_m for EfGCT and 1.6-fold greater glycerol phosphate K_m for LmGCT. When compared to kinetic parameters reported for GCT from *B. subtilis*, K_m values for EfGCT and LmGCT are comparable, however, *S. aureus* GCT has been reported to have K_m values 50-fold to 300-fold lower than those determined for EfGCT or LmGCT. With respect to k_{cat}, *B. subtilis* GCT has the highest reported value, approximately 5-fold to 12-fold greater than *S. aureus* GCT, EfGCT, or LmGCT. Since the apparent k_{cat} and K_m values for EfGCT and LmGCT are similar, the ratio of k_{cat}/K_m, a measure of catalytic efficiency, is similar. *B. subtilis* GCT, due primarily to its greater k_{cat}, has a catalytic efficiency 5-fold to 50-fold greater than either EfGCT or LmGCT. *S. aureus* GCT, due primarily to its low K_m values, exhibits catalytic efficiencies 25-fold to 350-fold greater than either EfGCT or LmGCT.

EfGCT and LmGCT are dimeric

Cross linking reactions indicated the presence of a band on SDS-PAGE of approximately 35 kDa in the presence, but not the absence, of crosslinkers (Figure 5). Both EfGCT and LmGCT are about 15 kDa, with an extra 3 kDa contributed by the N-terminal sequence containing the 6x-His-tag. Therefore, a band of approximately 35 kDa indicates the crosslinking of two monomers. These data indicate both EfGCT and LmGCT are dimeric. Three-dimensional structures of *B. subtilis* and *S. aureus* GCT indicate these forms of GCT are also dimeric [12,13]. The homodimeric forms of the enzyme contain two active sites per dimer. In addition, binding constants have been determined for *B. subtilis* GCT and indicate negative cooperativity of substrate binding [17]. The data from this previous study indicate that efficient catalysis will only occur when both active sites of a dimeric form of GCT are bound to substrate. Since these data indicate a monomer will be catalytically impaired, we infer that dimerization of LmGCT and EfGCT is likely necessary for efficient catalysis to occur as well.

Analysis of the *E. faecalis* and *L. monocytogenes* GCT amino acid sequences

Comparison of the 132 residue *E. faecalis* GCT amino acid sequence with the 127 residue *L. monocytogenes* GCT amino acid sequence reveals a 63% identity between the two sequences

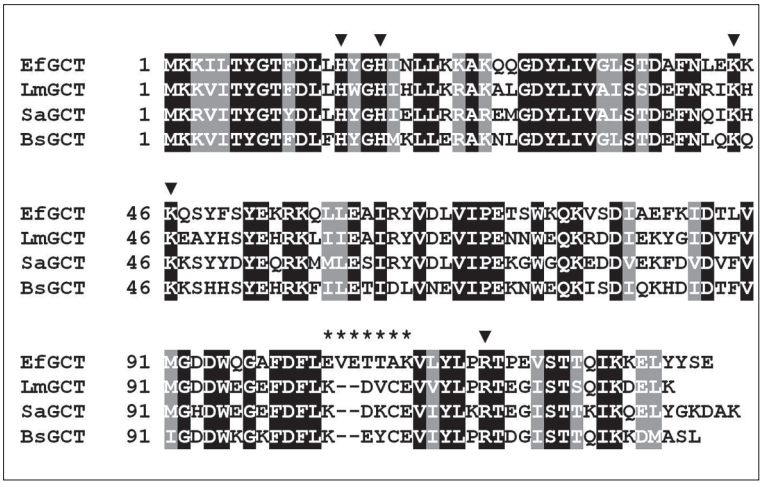

Figure 7. Amino acid sequence alignment of glycerol-3-phosphate cytidylyltransferase from *Entercoccus faecalis* (EfGCT), *Listeria monocytogenase* (LmGCT), *Staphylococcus aureus* (SaGCT, accession YP_001315823), and *Bacillus subtilis* (BsGCT, accession CAC86112). Amino acids known to be critical for catalysis in studies conducted with BsGCT are indicated by a triangle (▼). Amino acids indicated by an asterisk are encoded by nucleotides 310–330 of the EfGCT gene. Amino acids highlighted in black are identical in all four GCTs. Amino acids highlighted in grey are similar (D=E, R=K, A=G, S=T, F=Y=W, M=L=I=V).

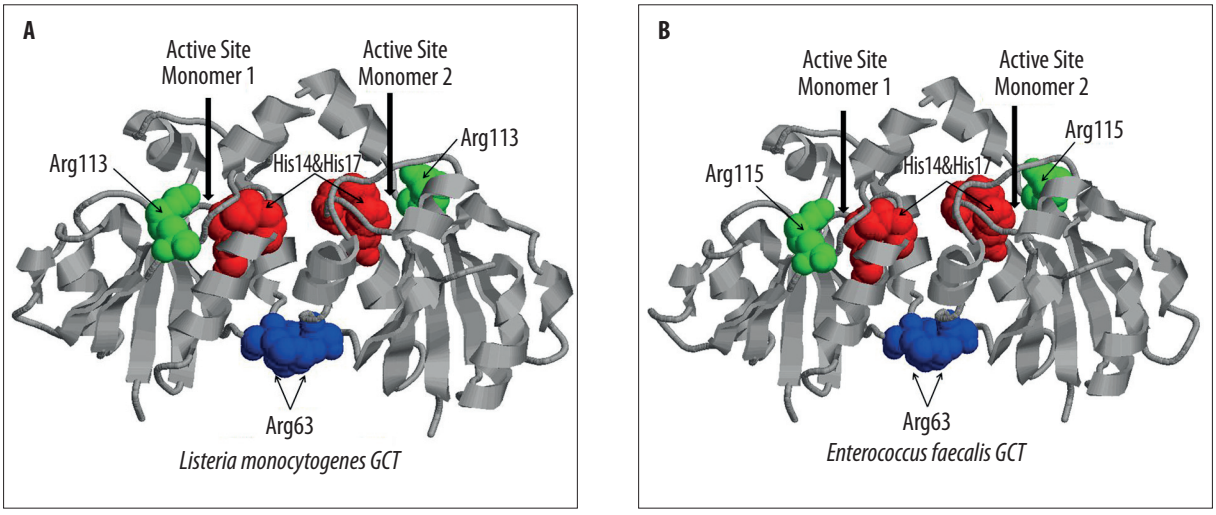

Figure 8. Three-dimensional structural model of *L. monocytogenes* GCT (**A**) and *E. faecalis* GCT (**B**). Models were generated using SWISS-MODEL (*http://swissmodel.expasy.org*) with PDB ID 1N1D as a template. Active site histidines 14 and 17 are indicated in red, an active site arginine (113 in LmGCT and 115 in EfGCT) in green, and arginine 63 in blue.

(Figure 6). *L. monocytogenes* GCT has greater identity to both *S. aureus* and *B. subtilis* GCT, 69% and 72% identity, respectively, than to *E. faecalis* GCT. *E. faecalis* GCT, however, has a lower identity with respect to *S. aureus* and *B. subtilis* GCT, 59% and 62%, respectively, than to *L. monocytogenes* GCT (Figure 6). In an alignment of all four GCT amino acid sequences there is an overall identity of 46% (Figure 7).

The molecular mass of the two forms of GCT studied here differs slightly with the 132 amino acid EfGCT having a calculated molecular mass of 15,440 Daltons and the 127 amino acid LmGCT a mass of 15,060 Daltons. EfGCT contains an additional 3 amino acids at the carboxy terminus and two inserted amino acids when aligned with LmGCT, found in the region between amino acids 103 and 109 due to the insertion of nucleotides 311-315 and 328 (Figure 7). There are three regions of sequence that have been found to be highly conserved across nearly all of the known GCT sequences and are also present in LmGCT and EfGCT (Figure 7). The first is the sequence ^{8}GX(Y/F)DXXHXGH^{17} (where X is any amino acid) that contains catalytically important histidines. Previous studies showed that mutating histidine 14 or histidine 17 to alanine in *B. subtilis* GCT caused a decrease in V_{max} values of more than three orders of magnitude [14]. A second conserved sequence is ^{113}RTXGISTT^{120}, a region in the structure of *B. subtilis* GCT that wraps around the nucleotide portion of CTP to form interactions that contribute to the specificity of the enzyme. These two sequences comprise one side of the active site bowl. The conserved ^{63}RYVDEVI^{69}sequence, distant from the CTP-binding site, is found towards the bottom of the *B. subtilis* GCT monomer and is part of the dimer interface [13].

Two other amino acids shown by site directed mutagenesis to be important for catalysis in *B. subtilis* GCT are lysine 44 and lysine 46, also present in LmGCT and EfGCT (Figure 7). In the crystal structure of *B. subtilis* GCT with bound CDP-glycerol, the positively charged side chains of the lysines interact with the negatively charged phosphates of CDP-glycerol. When these two lysines were experimentally mutated to alanines, there was a threefold increase in the K_m values for the substrates CTP and glycerol phosphate and the V_{max} value decreased to approximately 10% of the wild-type value [9].

Modeling the three-dimensional structure of *E. faecalis* and *L. monocytogenes* GCT

The three-dimensional structure of *Bacillus subtilis* GCT has been solved and serves as a model structure for the catalytic domain of cytidylyltransferases [9,13]. To assess the potential similarity of *L. monocytogenes* GCT and *E. faecalis* GCT to *B. subtilis* GCT, SWISS-MODEL (*http://swissmodel.expasy.org*), an automated comparative protein modeling tool [18–22], was used to model the structure of each protein (Figure 8). The amino acid sequences of LmGCT and EfGCT were submitted and the three-dimensional structure modeled using *Bacillus subtilis* GCT (PDB ID 1N1D) as a template. Models suggest both LmGCT and EfGCT likely adopt a dimeric three-dimensional structure similar *to B. subtilis* GCT. Active site histidines 14 and 17, as well as an active site arginine (113 in LmGCT and 115 in EfGCT), are present at the putative active sites in each enzyme. This suggests the active site architecture and catalytic mechanism are similar to the well characterized *B. subtilis* GCT. In addition, arginine 63, known to be at the interface between the two monomers, is also present at the dimer interface in each modeled structure.

Conclusions

Since GCT is an enzyme central to teichoic acid synthesis in Gram-positive bacteria such as *E. faecalis* and *L. monocytogenes*, inhibition of GCT in these potentially pathogenic bacteria could halt infection in humans. GCT expression may be related to pathogenicity, as indicated by a genomic profiling study of *Staphylococcus epidermidis* strains from individuals with ocular infections. Pathogenic *S. epidermidis* isolates from endophthalmitis showed amplification of the genomic region containing tagD, suggesting GCT expression may be a factor for the development of infection [23]. The potential of GCT as a potential drug target is validated by research showing a tagD gene deletion in *Bacillus subtilis* exhibited a lethal phenotype. Gene deletion resulted in altered cell morphology and lysis. Rescue of the tagD mutant was accomplished by expression of GCT [24]. Studying GCT catalysis and structure provides information vital to the future design and synthesis of compounds with the potential to inhibit the production of teichoic acids in bacteria.

References:

1. Rocas IN, Siquiera JF Jr, Santos KR: Association of *Enterococcus faecalis* with different forms of periradicular diseases. J Endod, 2004; 30: 315–20
2. Courvalin P: Vancomycin resistance in Gram-positive cocci. Clin Infect Dis, 2006; 42: S25–34
3. Amyes S: Enterococci and streptococci. Int J Antimicrob Agents, 2007; 29: S43–52
4. Ramaswamy V, Cresence VM, Rejitha JS et al: Listeria-review of epidemiology and pathogenesis. J Microbiol Immunol Infect, 2007; 40: 4–13
5. Schlech WF III: Foodborne listeriosis. Clin Infect Dis, 2000; 31: 770–75
6. Charpentier E, Courvalin P: Antibiotic resistance in *Listeria* spp. Antimicrob. Agents Chemother, 1999; 43: 2103–8
7. Lazarevic V, Abellan FX, Möller SB et al: Comparison of ribitol and glycerol teichoic acid genes in *Bacillus subtilis* W23 and 168: identical function, similar divergent organization, but different regulation. Microbiology, 2002; 148: 815–24
8. Park YS, Sweitzer TD, Dixon JE, Kent C: Expression, purification, and characterization of CTP: Glycerol-3-phosphate cytidylyltransferase from *Bacillus subtilis*. J Biol Chem, 1993; 268: 16648–54
9. Pattridge KA, Weber CH, Friesen JA et al: Glycerol 3-phosphate cytidylyltransferase: Structural changes induced by binding of CDP-glycerol and the role of lysine residues in catalysis. J Biol Chem, 2003; 278: 51863–71
10. Kent C: CTP: phosphocholine cytidylyltransferase. Biochim Biophys Acta, 1997; 1348: 79–90
11. Bladergroen BA, Houweling M, Geelen MJ et al: Cloning and expression of CTP: phosphoethanolamine cytidylyltransferase cDNA from rat liver. Biochem J, 1999; 343: 107–14
12. Fong DH, Yim VC-N, D'Elia MA et al: Crystal structure of CTP: glycerol-3-phosphate cytidylyltransferase from *Staphylococcus aureus*: Examination of structural basis for kinetic mechanism. Biochim Biophys Acta, 2005; 1764: 63–69
13. Weber CH, Park SP, Sanker S et al: A prototypical cytidylyltransferase: CTP: glycerol-3-phosphate cytidylyltransferase from *Bacillus subtilis*. Structure 1999;9: 1113-24.
14. Park YS, Gee P, Sanker S et al: Identification of functional conserved residues of CTP: glycerol-3-phosphate cytidylyltransferase: role of histidines in conserved HXGH in catalysis. J Biol Chem, 1997; 272: 15161–66
15. Stevens SY, Sanker S, Kent C, Zuiderweg ERP: Delineation of the allosteric mechanism of a cytidylyltransferase exhibiting negative cooperativity. Nature Struct Biol, 2001; 8: 947–52
16. Badurina DS, Solli-Juran M, Brown ED: CTP: glycerol 3-phosphate cytidylyltransferase (TarD) from *Staphylococcus aureus* catalyzes the cytidylyl transfer via an ordered Bi-Bi reaction mechanism with micromolar Km values. Biochim Biophys Acta, 2003; 1646: 196–206
17. Sanker S, Campbell HA, Kent C: Negative cooperativity of substrate binding but not enzyme activity in wild-type and mutant forms of CTP: glycerol-3-phosphate cytidylyltransferase. J Biol Chem, 2001; 41: 37922–28
18. Arnold K, Bordoli L, Kopp J, Schwede T: The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. Bioinformatics, 2006; 22: 195–201
19. Kiefer F, Arnold K, Künzli M et al: The SWISS-MODEL Repository and associated resources. Nucleic Acids Res, 2009; 37: D387–92
20. Schwede T, Kopp J, Guex N, Peitsch MC: SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res, 2003; 31: 3381–85
21. Guex N, Peitsch MC: SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modelling. Electrophoresis, 1997; 18: 2714–23
22. Peitsch MC: Protein modeling by E-mail. Bio/Technology, 1995; 13: 658–60
23. Duggirala A, Kenchappa P, Sharma S et al: High-resolution genome profiling differentiated *Staphylococcus* epidermidis isolated from patients with ocular infections and normal individuals. Invest Ophthalmol Vis Sci 2007; 48: 3239–45
24. Bhavsar AP, Beveridge TJ, Brown ED: Precise deletion of tagD and controlled depletion of its product, glycerol 3-phosphate cytidylyltransferase, leads to irregular morphology and lysis of *Bacillus subtilis* grown at physiological temperature. J Bacteriol, 2001; 183: 6688–93