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1,2-Diacylglycerol choline phosphotransferase catalyzes the final step in the unique *Treponema denticola* phosphatidylcholine biosynthesis pathway

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Summary

Treponema denticola **synthesizes phosphatidylcholine through a *licCA*-dependent CDP-choline pathway identified only in the genus *Treponema*. However, the mechanism of conversion of CDP-choline to phosphatidylcholine remained unclear. We report here characterization of TDE0021 (herein designated *cpt*) encoding a 1,2-diacylglycerol choline phosphotransferase homologous to choline phosphotransferases that catalyze the final step of the highly conserved Kennedy pathway for phosphatidylcholine synthesis in eukaryotes. *T. denticola* Cpt catalyzed *in vitro* phosphatidylcholine formation from CDP-choline and diacylglycerol, and full activity required divalent manganese. Allelic replacement mutagenesis of *cpt* in *T. denticola* resulted in abrogation of phosphatidylcholine synthesis. *T. denticola* Cpt complemented a *Saccharomyces cerevisiae* CPT1 mutant, and expression of the entire *T. denticola* LicCA-Cpt pathway in *E. coli* resulted in phosphatidylcholine biosynthesis. Our findings show that *T. denticola* possesses a unique phosphatidylcholine synthesis pathway combining conserved prokaryotic choline kinase and CTP:phosphocholine cytidylyltransferase activities with a 1,2-diacylglycerol choline phosphotransferase that is common in eukaryotes. Other than in a subset of mammalian host-associated *Treponema* that includes *T. pallidum*, this pathway is found in neither bacteria nor Archaea. Molecular dating analysis of the Cpt gene family suggests that a horizontal gene transfer event introduced this gene into an ancestral *Treponema* well after its divergence from other spirochetes.**

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Introduction

Phosphatidylcholine (PC) is a major component of eukaryotic cell membranes, in which it accounts for 40–60% of total phospholipids. It is a major structural component of membrane bilayers and serum lipoproteins, and participates in several signal transduction pathways (Cui and Houweling, 2002; Wright *et al.*, 2004). PC is also found in an estimated 10–15% of bacterial taxa (Sohlenkamp *et al.*, 2003; Geiger *et al.*, 2013), where it may be the major membrane phospholipid as in *Acetobacter aceti* (Hanada *et al.*, 2001) or constitute only a few percent of total membrane lipids as in *Pseudomonas aeruginosa* (Albelo and Domenech, 1997). *E. coli*, *Bacillus* spp. and *Streptococcus* spp. do not make PC. Interestingly, bacterial symbionts and pathogens of eukaryotes appear most likely to contain PC (reviewed in (Sohlenkamp *et al.*, 2003; Aktas *et al.*, 2010)). In eukaryotes, PC can be synthesized by either one of two pathways. The CDP-choline (Kennedy) pathway (Fig. 1, left side), present in all mammalian cells and in most eukaryotic cells (Lykidis, 2007; Gibellini and Smith, 2010), consists of three enzymatic steps catalyzed by choline kinase (CK), CTP:phosphocholine cytidylyltransferase (CCT), and 1,2-diacylglycerol choline phosphotransferase (CPT or CPT/EPT) (Kennedy and Weiss,

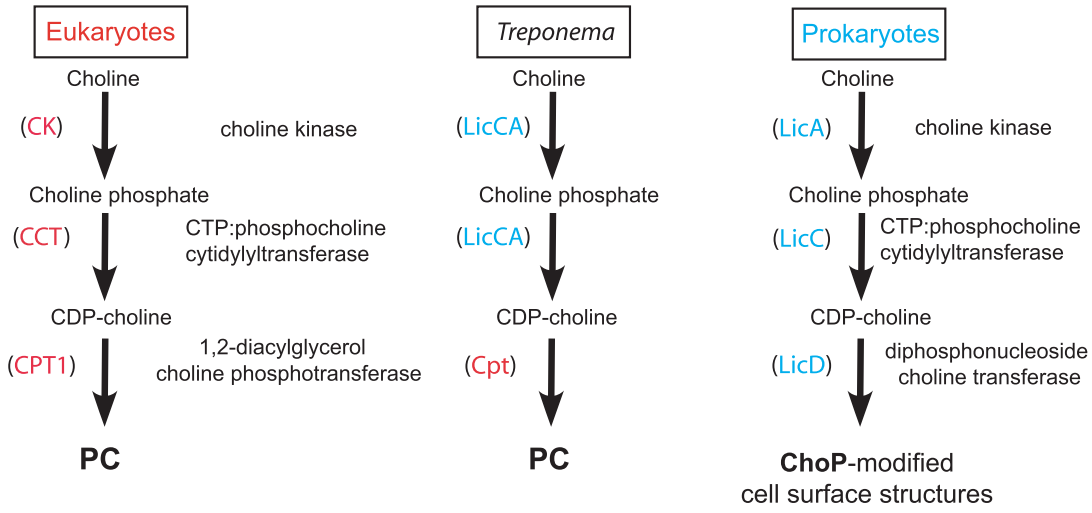

Fig. 1. CDP-choline pathways for biosynthesis of phosphatidylcholine and cell-surface glycoconjugate-linked phosphorylcholine (ChoP). CDP-choline pathways for PC biosynthesis are found in eukaryotes and in the prokaryotic genus *Treponema*. The CDP-choline pathway for addition of ChoP to cell surface glycoconjugates is found in numerous commensal and pathogenic bacteria. In prokaryotes containing the *lic* genes, LicA is the choline kinase and LicC is the CTP:phosphocholine cytidylyltransferase, while the analogous eukaryotic enzymes are CK and CCT. The diphosphonucleoside choline transferase that transfers ChoP to the complex polysaccharide is encoded by *licD*. The 1,2-diacylglycerol choline phosphotransferase of eukaryotes (CPT1) and its *T. denticola* homologue (Cpt) catalyze the final step in CDP-choline-dependent PC biosynthesis.

1956). In the liver, PC is also synthesized by three stepwise methylations of phosphatidylethanolamine (PE) to PC by phosphatidylethanolamine *N*-methyltransferase (Pmt) (Bremer *et al.*, 1960). Two PC pathways are well characterized in prokaryotes: **[1]** the stepwise methylation of PE by Pmt, with *S*-adenosylmethionine serving as methyl group donor (Kaneshiro and Law, 1964), and **[2]** the phosphatidylcholine synthase (Pcs) pathway (de Rudder *et al.*, 1999). In the Pcs pathway, which is unique to bacteria, Pcs catalyzes a single step reaction between host-derived choline and CDP-diacylglycerol to form PC (de Rudder *et al.*, 1999). Most bacteria that make PC probably have both pathways, though some, including *Borrelia*, *Pseudomonas* and *Burkholderia* spp., possess only one of these pathways (reviewed in (Sohlenkamp *et al.*, 2003)). Recently, a third pathway for PC biosynthesis has been described in *Xanthomonas campestris* in which glycerophosphocholine is acylated twice to obtain PC as final product (Moser *et al.*, 2014).

While it was long assumed that bacteria do not have the eukaryote-like CDP-choline-dependent pathway for PC synthesis (Lopez-Lara and Geiger, 2001), recent reviews (Sohlenkamp *et al.*, 2003; Geiger *et al.*, 2013; Sohlenkamp and Geiger, 2016) speculated that certain bacteria might utilize a distinct bacterial CDP-choline pathway as a means to synthesize PC. Genes encoding the first two enzyme activities in the pathway (LicA, encoding CK activity; and LicC, encoding CCT activity) are present in many species that colonize mucosal tissue. A wide range of Gram-positive and Gram-negative species that colonize mucosal tissue produce LicC-LicA dependent phosphocholine (ChoP) glycoconjugate modifications of various surface structures: lipopolysaccharide (*Haemophilus influenzae*) (Lysenko *et al.*, 2000), pili (pathogenic *Neisseria* spp.) (Weiser *et al.*, 1998), lipoteichoic acid (pneumococcus) (Zhang *et al.*, 1999), a 43-kDa surface protein (*P. aeruginosa*) (Weiser *et al.*, 1998), and surface components of diverse commensal oral species (Schenkein *et al.*, 2001). As shown in Fig. 1, these bacteria use homologues of the first two enzymes of the CDP-choline PC synthesis pathway to make CDP-choline. The gene encoding diphosphonucleoside choline phosphotransferase that donates ChoP to a cell surface glycoconjugate has not been definitively identified in many ChoP-producing microbes (Young *et al.*, 2013), but is proposed to be *licD* in *H. influenzae* (Lysenko *et al.*, 2000) and *S. pneumoniae* (Zhang *et al.*, 1999).

PC comprises approximately one third of total *Treponema denticola* membrane phospholipids (Livermore and Johnson, 1974; Smibert, 1976; Kent *et al.*, 2004). Genes required for the Pmt pathway, the PC synthase pathway or the yeast-like acylation pathway are absent in the *T. denticola* ATCC35405 genome (Seshadri *et al.*, 2004), supporting the idea that a novel PC synthesis pathway exists in this genus. We previously reported that PC synthesis in *T. denticola* is dependent on CDP-choline produced via LicCA, evidence strongly suggestive of presence of a novel CDP-choline-dependent PC synthesis pathway in *Treponema denticola* (Kent *et al.*, 2004). Recombinant *T. denticola* LicCA was shown to have both CK and CCT activity, and a defined *T.*

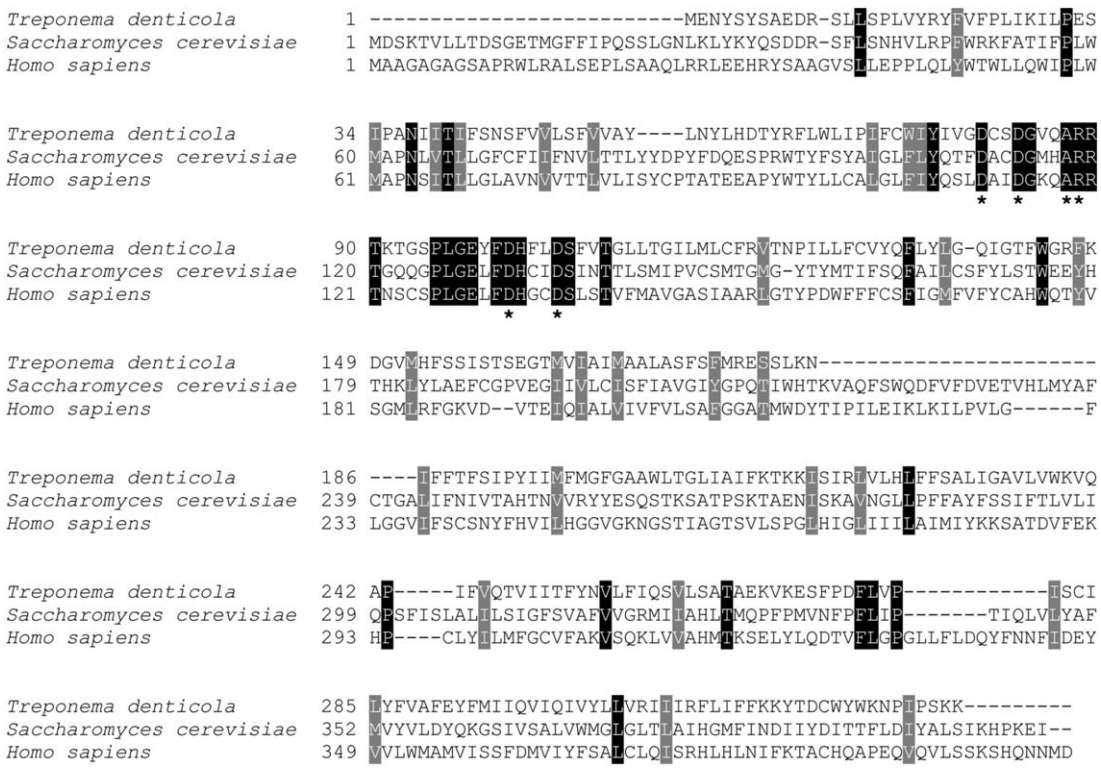

Fig. 2. Comparison of the amino acid sequences of the putative *T. denticola* choline phosphotransferase with representative eukaryotic choline phosphotransferases.
Optimized alignment of deduced amino acid sequences of *T. denticola* TDE0021 (NP_970638), *S. cerevisiae* CPT1 (AAA63571) and *Homo sapiens* CHPT1 (NP_064629). Identical residues are marked in black, similar residues are shaded in grey. Amino acid residues forming part of the conserved CDP-alcohol phosphotransferase motif are labeled with asterisks (Williams and McMaster, 1998; Solis-Oviedo *et al.*, 2012).

denticola licCA mutant lacked PC. Our analyses of the *T. denticola* and *T. pallidum* genomes revealed the presence of a gene encoding a putative 1,2-diacylglycerol choline/ethanolamine phosphotransferase. This gene is most closely related to eukaryotic genes encoding CPT and EPT activity required for the final step of PC and PE synthesis in the Kennedy pathway. In this study, we report identification, characterization and mutagenesis of the gene encoding the choline phosphotransferase activity that completes the CDP-choline-dependent PC biosynthesis pathway in *Treponema spp*. Finally, we present a bioinformatics analysis supporting the hypothesis that the archetypal *Treponema* choline phosphotransferase was acquired by horizontal gene transfer (HGT) from a eukaryotic host organism.

Results

Identification of TDE0021 as a putative CDP-choline:1,2-diacylglycerol choline phosphotransferase with homology to the eukaryotic CPT/EPT family

Genes encoding key enzymes in pathways for synthesis of several phospholipids have not yet been identified in the annotated genomes of *T. pallidum* and *T. denticola* (Fraser *et al.*, 1998; Seshadri *et al.*, 2004). In the case of *T. pallidum*, this reflects the absence of numerous biosynthetic pathways in its small 1 Mb genome and its apparent reliance on unknown factors supplied by its host. However, this is less likely to be the case in *T. denticola*, which has a 2.8 Mb genome and can be grown in culture. Our prior studies indicated that *T. denticola* and, by inference, *T. pallidum* utilize a CDP-choline-dependent pathway for biosynthesis of PC, producing CDP-choline by means of LicC/LicA activity. The final step of this pathway requires transfer of the choline moiety to diacylglycerol (DAG).

In *S. cerevisiae* and other eukaryotes CPT1, encoding CDP-choline:1,2-diacylglycerol choline phosphotransferase (EC 2.7.8.2), represents the final step in the CDP-choline-dependent Kennedy PC pathway. CPT1 is a member of the CDP-alcohol phosphotransferase superfamily that includes several other enzymes involved in phospholipid biosynthesis such as type II phosphatidylserine synthase (PssA), phosphatidylglycerol phosphate synthase (PgsA), phosphatidylinositol synthase (Pis), eukaryotic type cardiolipin synthase (type II Cls), and phosphatidylcholine synthase (Pcs). We hypothesized

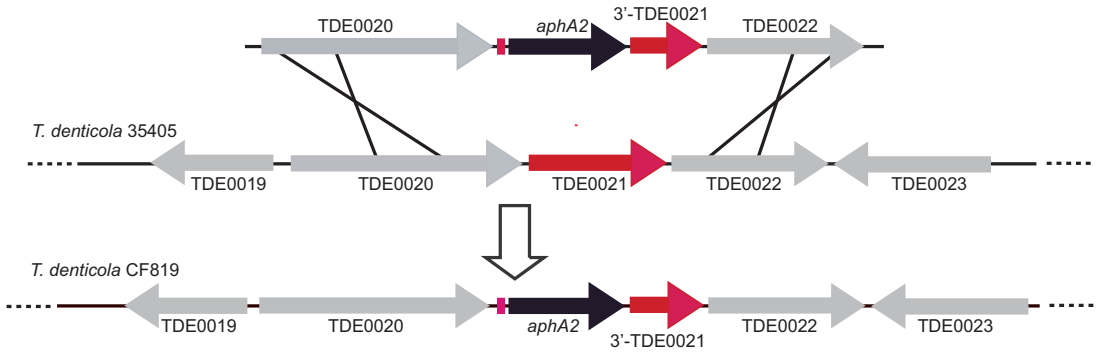

Fig. 3. Strategy for *T. denticola* TDE0021 mutagenesis.
An isogenic TDE0021 mutant was constructed as described in the text by electroporating *T. denticola* 35405 with linear DNA consisting of an engineered TDE0020-TDE0022, in which the *aphA2* cassette replaced bp 38-504 of TDE0021. The expected mutation in *T. denticola* CF819 resulting from double crossover homologous recombination in TDE0020 and TDE0022 was validated by PCR and DNA sequencing. Black arrows: *aphA2* cassette. Red arrows: TDE0021 and 5′- and 3′-end fragments. Light grey arrows: flanking genes including TDE0020 (*glyS*) and TDE0022 (hypothetical protein).

that the unknown *T. denticola* choline phosphotransferase would belong to this protein superfamily. Four genes encoding putative CDP-alcohol phosphotransferases (TDE0021, TDE1366, TDE2572 and TDE2669) are annotated in *T. denticola* genome (Seshadri *et al.*, 2004). Of these, TDE0021 was the most promising as a candidate choline phosphotransferase involved in PC synthesis, because its deduced amino acid sequence shares homology with CPT1 proteins from eukaryotes, particularly the conserved CDP-alcohol phosphotransferase domain within the hydrophilic N-terminal region (Fig. 2).

Mutagenesis of TDE0021 results in loss of PC in T. denticola

As *licCA* is required for biosynthesis of PC in *T. denticola*, we hypothesized that allelic replacement mutagenesis of the putative choline phosphotransferase TDE0021 would yield a similar phenotype. *T. denticola* 35405 was transformed with a linear DNA fragment of pCF789 in which a the *aphA2* cassette encoding kanamycin resistance replaced DNA encoding amino acid residues 13-167 of the annotated TDE0021 open reading frame (Fig. 3), including the conserved CDP-alcohol phosphotransferase motif (Fig. 2) (Williams and McMaster, 1998; Solis-Oviedo *et al.*, 2012). The identity and homogeneity of the mutant strain was confirmed both by DNA sequencing of the mutated region and by PCR analyses using oligonucleotide primer sets specific for the *aphA2* gene, the DNA flanking the insertion and the deleted region of TDE0021 (data not shown). Interestingly, mutant colonies isolated in soft agar medium could not be grown directly in broth medium, but required at least two passages in broth medium containing methylcellulose. Subsequently, the mutant grew at slightly slower rate than the parent in broth medium without methylcellulose, yet reached the same density at stationary phase (data not shown).

Consistent with prior reports, PC and PE were the predominant phospholipids detected in *T. denticola* 35405, accounting for approximately 70% of total phospholipid in a colorimetric TLC assay (Table 1). As expected, PC was not detected in *T. denticola* LBE3, a *licCA* mutant (Kent *et al.*, 2004). It should be noted that, in addition to defects in both CK and CCT activity, LBE3 is also defective in choline uptake due to the fact that the mutation in *licCA* blocks transcription of the downstream gene *cudT* (data not shown). The phospholipid profile of *T. denticola* CF819 (ΔTDE0021) revealed no PC, indicating that interruption of the PC synthesis pathway after the CDP-choline step results in abrogation of all PC production. *T. denticola* CF819, with no defect in either choline uptake or LicCA, is expected to produce wildtype levels of CDP-choline. Based on its homology with eukaryotic choline phosphotransferases and the phenotype of the *T. denticola* CF819, we here designate the TDE0021 locus as *cpt*.

Table 1. Phospholipid profiles of *T. denticola* parent and mutant strains.

T. denticola strain	Genotype	PC[a]	PE[a]	Other phospholipids[b]
35405	Parent	22.4	44.7	32.9
LBE3	35405Δ*licCA*	0	54.1	45.9
CF819	35405ΔTDE0021	0	54.3	45.7

a. Values shown are percentage of total cellular phospholipid, average of three samples per strain.
b. Other reported phospholipids of *T. denticola* are primarily phosphatidylglycerol and cardiolipin (Livermore and Johnson, 1975; Smibert, 1976; Kent *et al.*, 2004).

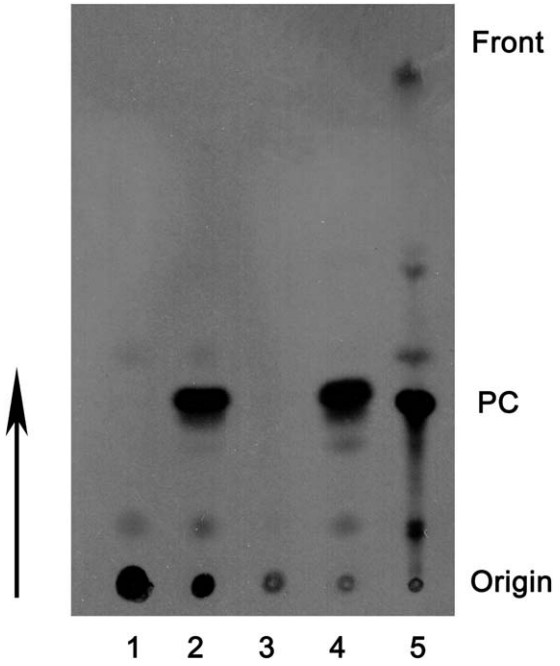

Fig. 4. *T. denticola* TDE0021 complements the *S. cerevisiae CPT1* mutant HJ091.
S. cerevisiae HJ091 cells harboring empty plasmid pSP-Gm2 (lane 1), pSP-Gm2-Trepo (lane 2), empty plasmid pYEp352 (lane 3), or pYEp352-CPT1 from yeast (lane 4) were labeled for 30 min with [^{14}C]choline. Lipids were extracted from the cells and separated by one-dimensional TLC. A commercial [^{14}C]phosphatidylcholine standard was run in lane 5. PC-phosphatidylcholine.

T. denticola *Cpt complements the PC deficiency in a* S. cerevisiae *CPT1 mutant*

To show that *T. denticola* TDE0021 (*cpt*) encodes a functional choline phosphotransferase *in vivo*, we expressed TDE0021 in *S. cerevisiae* HJ091. HJ091 is deficient in both *CPT1* and *EPT1* and is therefore unable to incorporate [^{14}C]choline into [^{14}C]PC by the Kennedy pathway (Hjelmstad *et al.*, 1994; McMaster *et al.*, 1996). TDE0021 was cloned into the yeast expression plasmid pSP-Gm2 (Rodríguez-Limas *et al.*, 2011), which allows expression of genes of interest under control of a constitutive yeast promoter. HJ091 complemented with the yeast *CPT1* gene under control of its endogenous promoter cloned into shuttle vector pYEp352 (pRH150), TDE0021 (pSP-Gm2-Trepo) and the respective vector controls were grown in SD-Ura medium in the presence of [^{14}C]choline. After 30 minutes of labeling, cells were extracted according to Bligh and Dyer (Bligh and Dyer, 1959). As shown in Fig. 4, PC was detected in the organic phase of extracts from both the complemented yeast mutant and the yeast mutant expressing TDE0021 (Fig. 4, lanes 2 and 4). Only minor amounts of radioactive material were present in the organic phases from the Bligh and Dyer extractions of the vector control strains and they did not migrate as PC in the TLC system (Fig. 4, lanes 1 and 3). When aqueous phases of the extracts were subjected to one-dimensional TLC three radiolabeled compounds were detected in all four strains, presumably representing [^{14}C]choline, [^{14}C]choline phosphate and CDP-[^{14}C]choline. A fourth water-soluble compound (possibly glycerophosphocholine) was only present in aqueous extracts from the yeast mutant harboring plasmid pRH150 (Supporting Information Fig. S1). *T. denticola* Cpt encoded by TDE0021 therefore complements the yeast mutant HJ091 with respect to PC formation. To determine whether *T. denticola* Cpt also has ethanolamine phosphotransferase activity, the same experiment was conducted using [^{14}C]ethanolamine. Minor amounts of PE were detected only in *S. cerevisiae* HJ091 carrying pRH150, indicating that *T. denticola* TDE0021 does not have EPT activity (Supporting Information Fig. S2).

Expression of the Treponema *Kennedy pathway for PC biosynthesis in* E. coli

The membrane lipid composition of *E. coli* has been studied in detail and it is known that *E. coli* forms PE, phosphatidylglycerol (PG) and cardiolipin (CL) as major phospholipids, but not PC. To determine whether LicCA and Cpt were sufficient to convert *E. coli* into a PC-forming organism, we co-expressed TDE0021 and LicCA (TDE1260) in *E. coli* BL21(DE3)/pLysS, similarly as has been described for expression of bacterial phospholipid *N*-methyltransferase and phosphatidylcholine synthase in *E. coli* (Arondel *et al.*, 1993; de Rudder *et al.*, 1999; de Rudder *et al.*, 2000; Sohlenkamp *et al.*, 2000; Hacker *et al.*, 2008). *E. coli* strains carrying TDE0021, *licCA*, both genes or both empty plasmid vectors were labeled with [^{14}C]acetate. In parallel, cell-free protein extracts were subjected to SDS-PAGE and stained with Coomassie Blue (Supporting Information Fig. S3). In all cases, lipids migrating as PG, PE and CL were observed in TLC (Fig. 5A, lanes 1-4). A lipid spot migrating below PE was detected in all four *E. coli* strains and was greatly increased in the strain expressing both TDE0021 and LicCA (Fig. 5A, lane 4). All four strains were again labeled, but this time with the more specific [^{14}C]choline label (Fig. 5B, lanes 5-8). Under these conditions, a lipid migrating as [^{14}C]PC was detected only in *E. coli* expressing both LicCA and TDE0021 (Fig 5B, lane 8), indicating that TDE0021 is a choline phosphotransferase responsible for PC formation. The [^{14}C]PC formed by the *E. coli* strain expressing both LicCA and TDE0021 co-migrated with a commercially available [^{14}C]PC standard (Fig. 5B, lane 9). To confirm that the [^{14}C]choline labeled lipid indeed was PC, total lipids from unlabeled *E. coli* expressing both *Treponema* genes were extracted and compared to lipids from *E. coli* expressing only one *Treponema* gene by

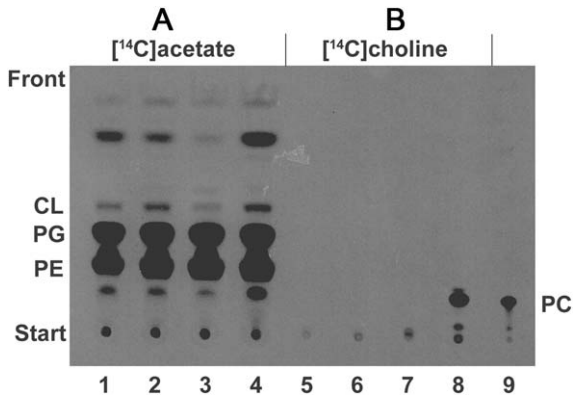

Fig. 5. Formation of PC by co-expression of *T. denticola* LicCA and Cpt in *E. coli*.
E. coli expression strain carrying empty plasmid vectors (lanes 1, 5), TDE0021 alone (lanes 2, 6), LicCA alone (lanes 3, 7), or both genes together (lanes 4, 8) were grown in liquid cultures, induced with IPTG and labeled with [^{14}C]acetate (lanes 1-4) or [^{14}C]choline (lane 5-8). Lipids were extracted using the method of Bligh and Dyer and separated by one-dimensional TLC. Lane 9 shows a commercial [^{14}C]PC standard. PG- phosphatidylglycerol; PE- phosphatidylethanolamine; CL- cardiolipin; PC- phosphatidylcholine.

normal phase liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS). A major total ion chromatogram peak is observed at 20.82 min in the chromatogram of the *E. coli* expressing TDE0021 and LicCA but is absent in the other samples (Fig. 6, A–C). Positive ion mass spectra acquired during the retention times between 20 and 22 min show the presence of a series of PC species with various chain length combinations in the lipid sample derived from *E. coli* carrying both pCDF-Duet-Trepo and pET17-LicCA (Fig. 6F), but not in similar samples. MS/MS of the *m/z* 706.5 ion produced a major fragment at *m/z* 184, corresponding to the phosphocholine head group, thus further confirming the presence of PC in this sample (Supporting Information Fig. S4). The three other *T. denticola* genes annotated as CDP-alcohol phosphotransferases (TDE1366, TDE2572 and TDE2669) were also co-expressed with LicCA in *E. coli*, but no formation of PC could be detected (data not shown).

T. denticola *cpt requires* Mn^{2+} *for optimal enzymatic activity*

To quantify choline phosphotransferase activity of *T. denticola* Cpt expressed in *S. cerevisiae*, we used the standard assay for the yeast enzyme. Microsomal membranes were prepared from the above-described derivatives of the *S. cerevisiae* strain HJ091 expressing yeast CPT1 or TDE0021, both of which CPT1 and TDE0021 complemented the CPT1 defect in HJ091 (Fig. 4). Choline phosphotransferase activity (and PC formation) was determined by measuring incorporation of [^{14}C]CDP-choline into the organic phase of a standard reaction mixture by both scintillation counting and by TLC. Using the standard assay, [^{14}C]CDP-choline-dependent [^{14}C]PC formation was detected in the *S. cerevisiae* membrane extract containing yeast CPT1 but no PC formation was observed when TDE0021 was present under identical conditions (data not shown). We tested a wider range of bivalent ions for stimulation of TDE0021 activity (Mn^{2+}, Ni^{2+}, Co^{2+}, Ca^{2+}, Cu^{2+} and Mg^{2+}). Mn^{2+} or Co^{2+} supported the highest activities (Table 2). Much less activity was observed in the presence of Mg^{2+} and only minor PC formation was observed in the presence of Ni^{2+}. The other tested ions did not support PC formation. TDE0021 CDP-choline phosphotransferase activity was detectable from pH 6.0 to 8.5 with the maximum activity observed at pH 7.0 in Tris/HCl buffer (Supporting Information Fig. S5).

Conservation of TDE0021 in the genus Treponema

Using the TDE0021 amino acid sequence as query, BLASTP and TBLASTN searches identified significant homologues (BLAST E-value $< 1 \times e^{-40}$) in eight members of the genus *Treponema* whose genome sequences are available. As shown in Fig. 7, the species with TDE0021 homologues form a distinct group within the *Treponema* genus when examined by 16S rRNA phylogeny. These include all sequenced strains of human-associated *T. denticola*, *T. pallidum, T. phagedenis* and *T. vincentii* as well as porcine digital dermatitis-associated *T. pedis*. Interestingly, TDE0021 homologues were not identified in the genomes of other less closely related *Treponema* spp. These include the human oral isolates *T. socranskii, T. lecithinolyticum* and *T. maltophilum* (which inhabit the same subgingival oral environment as *T. denticola*), bovine digital dermatitis-associated *T. brennaborense*, porcine intestinal *T. succinifaciens* and termite gut-associated treponemes. All treponemes with TDE0021 homologues also contained homologues of genes encoding LicCA and the choline transporter CudT (data not shown). While phospholipid profiles are not available for many species, all treponemes reported to produce phosphatidylcholine possess genes encoding LicCA and Cpt homologues.

TDE0021 represents a sparsely distributed gene family seeded in bacteria by an HGT event

Having first focused on TDE0021 as encoding a potential choline phosphotransferase due to the presence of conserved phosphotransferase domains similar to those in eukaryotic CPTs, we then examined its global

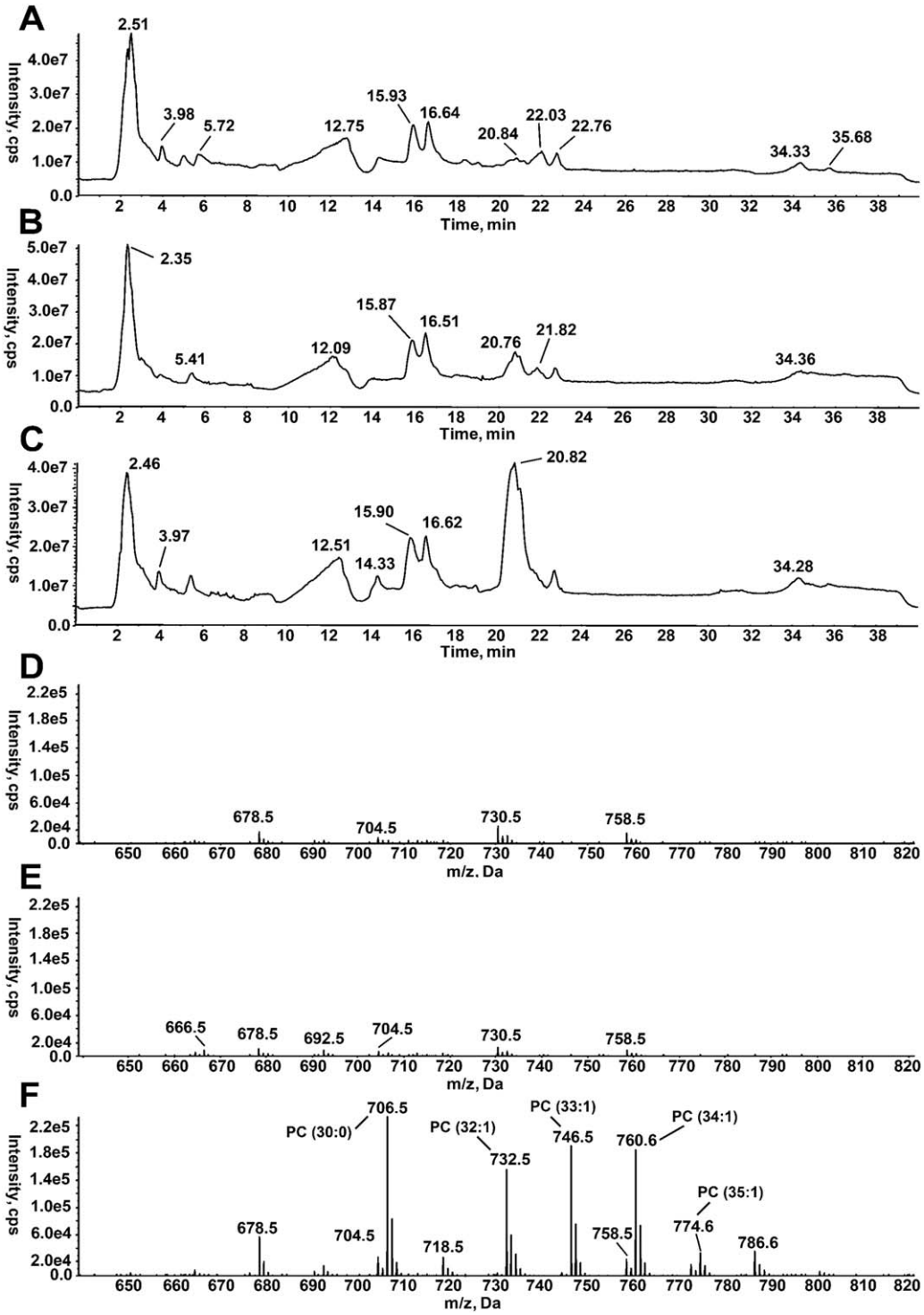

Fig. 6. LC-MS/MS analysis of *E. coli* total lipid extracts from *E. coli* expression strain producing LicCA and TDE0021 forms PC. Total ion chromatograms of the LC-MS analysis of total lipid extracts from an *E. coli* expression strain carrying both pET17b-TDE0021 and pCDF-Duet1 (panel A, TDE0021 only), *E. coli* expression strain carrying pET17b andpCDF-Duet1-LicCA (panel B, LicCA only), and *E. coli* expression strain carrying pET17b-TDE0021 and pCDF-Duet1-LicCA (panel C, TDE0021 and LicCA). Positive ion mode mass spectra of the fraction of samples shown in panels A to C with retention times between 20 and 22 min where PC would be expected to be eluted off the column and detected by MS (panels D, E, and F respectively). One of the major ion species, PC(30:0) at *m/z* 706.5, from panel F was subjected to MS/MS analysis. The presence of a characteristic *m/z* 184 phosphocholine head group peak in the product ion spectrum confirms its identity as PC (Fig. S4).

Table 2. Cation requirement for TDE0021 *in vitro* activity.

Metal ion added	Enzyme activity[a] (nmol PC/min/mg protein)
Mg^{2+}	0.33
Mn^{2+}	3.45
Co^{2+}	1.77
Ca^{2+}	ND[b]
Cu^{2+}	ND
Ni^{2+}	0.11
Mn^{2+}, EDTA	ND

a. Effect of divalent cations on incorporation of choline derived from [^{14}C]CDP-choline into [^{14}C]PC by membrane fractions of *E. coli* expressing TDE0021. Values represent the average of two independent experiments.
b. ND: no radioactivity above background detected.

distribution by conducting a targeted protein BLAST search (see Methods) to identify and quantify the number of homologues in other Bacteria, Archaea and Eukaryotes. Interestingly, we found only a few homologous genes given our stringent search criteria: a total of 77 genes including 55 of Eukaryotic origin, 22 from Bacteria (all but two in *Treponema*) and none from Archaea. Of note, all of these genes have the CDP-alcohol phosphatidyltransferase domain previously described in the NCBI's conserved domain database (Marchler-Bauer *et al*., 2015). Figure 8 depicts the phylogenetic relationships of these homologous genes. Notably, in Bacteria we found homologous genes only in the genus *Treponema*. In contrast, identified Eukaryotic genes were from different Phyla and Kingdoms These include Microsporidia and Ascomyceta Phyla from Fungi, and Chordata and Nematoda Phyla from the Animalia. We also found some other major taxonomic groups such as Amoebozoa and Euglenozoa. We note that both the clade support as measured by bootstrap values for the Bacterial clade and the main Eukaryotic groups were very high (data not shown).

All significant Eukaryotic BLAST hits to the TDE0021 product are to the ubiquitous CPT/EPT family of proteins, with *Entamoeba dispar* (E-score 1 e^{-14}) and *Homo sapiens* CPT/EPT (E-score 2 e^{-10}) among the highest scoring organisms. The homology scores for *S. cerevisiae* CPT/EPT are approximately 2 e^{-9}. Taken together with the observation that all *Treponema* carrying TDE0021 homologues are mucosal tissue isolates from humans or domesticated animals, this suggests that the choline phosphotransferase-encoding gene present in a closely related group of *Treponema* spp. may have been acquired by HGT from a eukaryotic organism, resulting in a variation of the Kennedy pathway for PC biosynthesis unique both in its genetic composition and in the cell type in which it occurs.

The fact that the identified Bacterial sequences were confined to a single genus, whereas the Eukaryotic versions were much more widely distributed, is suggestive that a horizontal gene transfer (HGT) event brought this gene to an ancestral *Treponema* species. Therefore, to further explore this, we conducted a molecular dating analysis of this gene family (see Methods and Fig. 8). Remarkably, the divergence time of the bacterial sequences is some 1,143 million years ago (MYA), whereas the divergence time of the Eukaryotic sequences is considerably earlier (1,474 MYA). Furthermore, the time of divergence of all the sequences of this family (both Eukaryotic and Bacterial) is some 2,090 MYA, which is much more recent than what is expected from the time of divergence of Eukaryotes versus Prokaryotes (4,290 MYA, according to the TimeTree database). The rather young age of the common ancestor of the bacterial sequences of this gene family and the fact that they are essentially restricted to only one genus -

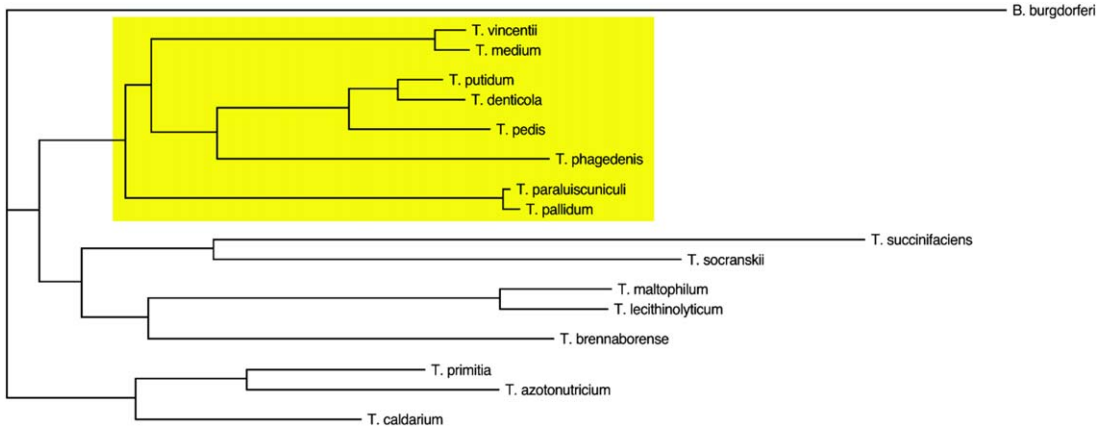

Fig. 7. 16S rRNA phylogenetic tree of representative *Treponema* spp. with sequenced genomes.
TDE0021 homologues are found only in the genomes of the *Treponema* species within the yellow box. *Borrelia burgdorferi* is included as an outgroup for tree construction.

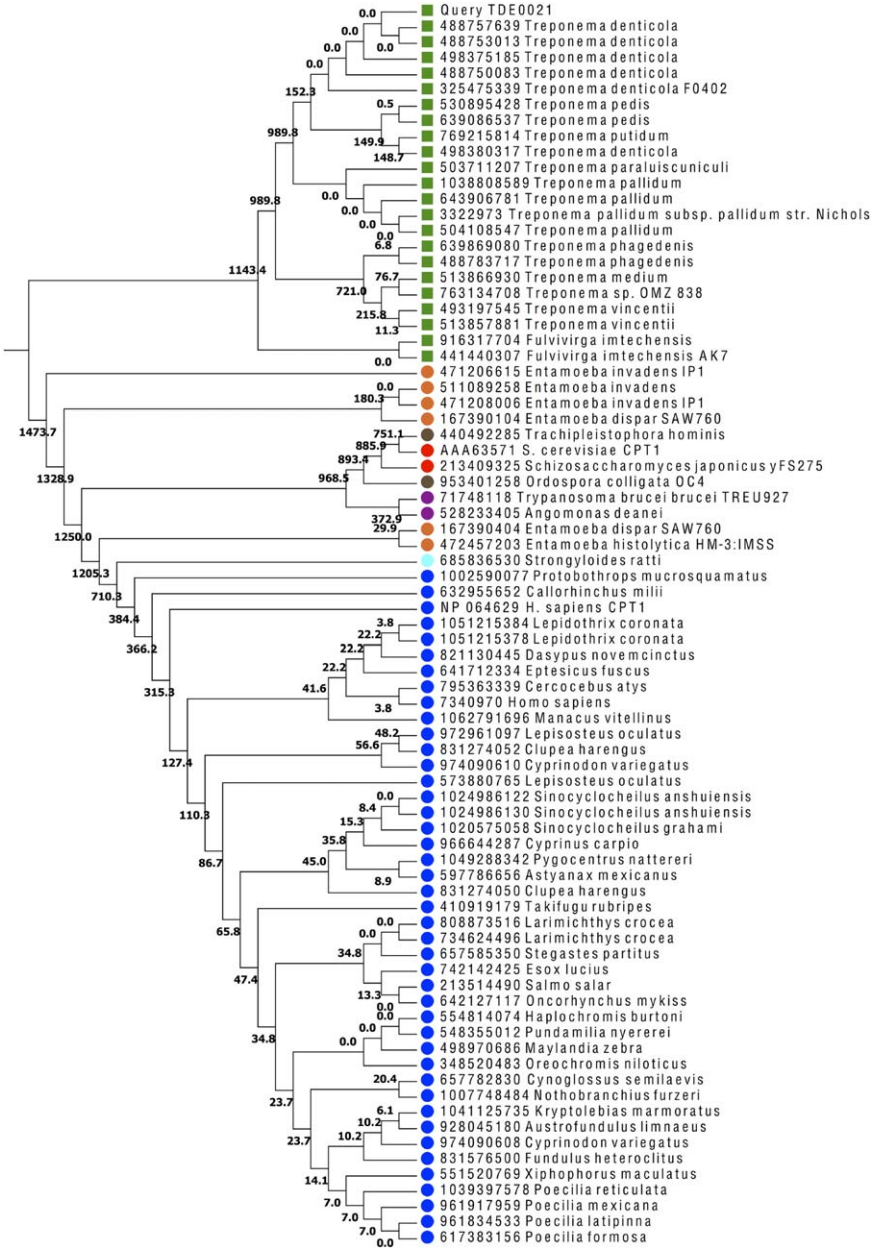

Fig. 8. Molecular dating analysis of TDE0021 homologues.
The molecular dating analysis was carried out on an alignment generated by Maximum Likelihood (ML) phylogeny analysis as described in Experimental Methods. The bacterial sequences are marked with small circles before the tip labels (red: TDE0021 BLAST query; green: homologous sequences from other *Treponema*.) The numbers at the branches are the divergence time estimates for the nodes in millions of years. The color coding is as follows: green squares, Bacteria sequences; orange circles, Amoebozoa; brown circles, Microsporidia; purple circles, Euglenozoa; red circles, Ascomyceta; cyan (light blue) circles, Nematoda; blue circles, Chordata.

compared with the more diverse Eukaryotic equivalents - suggests that the most parsimonious explanation for the distribution of the bacterial sequences is a HGT event into an ancestral *Treponema*.

Discussion

We report here characterization of *T. denticola* TDE0021 encoding Cpt, a 1,2-diacylglycerol choline phosphotransferase that, among prokaryotes, appears to be nearly limited to some closely related members of the genus *Treponema*. Outside of this group, *T. denticola* Cpt shares highest homology with eukaryotic choline- and ethanolamine-phosphotransferase enzymes responsible for the final step in the Kennedy pathway for PC biosynthesis. The *T. denticola* PC pathway includes a bifunctional LicCA with both CK and CCT activities required for synthesis of CDP-choline. LicC and LicA are present in a wide range of mucosa-associated bacteria and, along with LicD, are required for phosphorylcholine (ChoP) incorporation into cell surface glycoconjugates such as lipopolysaccharide and lipoteichoic acid (recently reviewed in (Young *et al.*, 2013)). Though it is present in a wide range of oral bacteria including both Gram-positive and Gram-negatives, ChoP

was not detected in *T. denticola* (Gmur *et al*., 1999). The *T. denticola* and *T. pallidum* genomes do not encode any protein containing the highly conserved LicD domain (residues 36-63) present in available LicD sequences (Young *et al*., 2013). Interestingly, preliminary genome annotations of some oral and intestinal treponemes, including *T. phagedenis*, *T. socranskii*, *T. pedis* and *T. succinifaciens*, reveal predicted LicD homologues (data not shown). To date there has been a single report, as yet unconfirmed, of a putative cell-surface ChoP epitope in *T. pallidum* (Blanco *et al*., 2005). Further studies are required to determine whether *T. denticola* LicCA contributes to a ChoP pathway as well as to the PC pathway.

In addition to TDE0021, at least three other *T. denticola* genes are annotated as encoding proteins with conserved CDP-alcohol phosphotransferase domains. We focused our attention on TDE0021 because it contains both significant homology to the catalytic domain in the conserved N-terminal hydrophilic region of eukaryotic CPTs and an extensive predicted hydrophobic C-terminal membrane region. Differences between the C-terminal regions of *Treponema* Cpt and eukaryotic CPT likely reflect substantial differences in membrane architecture between eukaryotes and spirochetes. Allelic replacement mutagenesis of TDE0021 in *T. denticola* resulted complete abrogation of PC synthesis. Based on the design and DNA sequence of the mutation in the *T. denticola* Δ*cpt* mutant (CF819), transcription should continue through *aphaA2* into the remaining portion of *cpt* 3′ to the CDP-alcohol phosphotransferase motif and TDE0022. This strategy was employed because we were unable to isolate mutants using genetic constructs in which transcription of any portions of either TDE0020 (encoding glycyl-tRNA synthetase) or TDE0022 (encoding a hypothetical protein) were disrupted (data not shown). The observation that potential Δ*cpt* mutants isolated as subsurface colonies on semisolid agar selection plates were not viable in NOS broth medium unless passaged at least twice in NOS broth medium containing 0.95% methylcellulose suggests that *T. denticola* undergoes adaptation to loss of PC production. Potential response and regulatory mechanisms involved in this process are subjects of ongoing investigation.

It should be noted that *T. denticola* LBE3 (defective in both CK and CCT activity), is also defective in choline uptake due to the fact that the mutation in *licCA* blocks transcription of the downstream gene *cudT* (data not shown). In contrast, *T. denticola* CF819, with no defect in either choline uptake or LicCA, should produce wild-type levels of CDP-choline but no Cpt activity. The phenotype of *T. denticola* CF819 indicates that none of the other *T. denticola* genes annotated as encoding proteins containing the CDP-alcohol phosphotransferase motif (TDE1366, TDE2572 and TDE2669) are capable of mediating synthesis of PC in the presence of CDP-choline. In contrast to TDE0021, neither TDE1366, TDE2572 nor TDE2669 mediated PC formation in *E. coli* when co-expressed with LicCA, further supporting the specificity of TDE0021 Cpt activity (data not shown).

The ability of *T. denticola* Cpt to complement a *S. cerevisiae* CPT1 mutant confirms its enzymatic activity *in vivo* and is consistent with its proposed eukaryotic origin. Interestingly, unlike some bifunctional eukaryotic CPT/EPT enzymes, *T. denticola* Cpt did not exhibit ethanolamine phosphotransferase activity when expressed in yeast and is therefore highly unlikely to participate in *T. denticola* PE biosynthesis. *T. denticola* most likely utilizes a typical prokaryotic phosphatidylserine-dependent PE pathway, though genes encoding putative phosphatidylserine synthase and phosphatidylserine decarboxylase remain to be identified.

The specific divalent cation requirement for optimal enzymatic activity is well established for eukaryotic CPT enzymes (Hjelmstad and Bell, 1992; McMaster *et al*., 1996). Interestingly, while *T. denticola* Cpt shares this general requirement, the cation specificity differed between *S. cerevisiae* (Mg^{2+}) and *T. denticola* (Mn^{2+}). The significance of the clear preference for Mn^{2+} in *T. denticola* over Mg^{2+} and other cations is not clear, but may reflect differences in intracellular environments between these unicellular microbes. Similar specific differences in metal cation requirements have been described even between very closely-related organisms, such as for the TroR transcriptional regulators of *T. denticola* and *T. pallidum* (Brett *et al*., 2008).

The *Treponema* CDP-choline-dependent PC pathway is limited to one branch of the genus. Genomes of all *Treponema* that have been shown to produce PC possess the genes encoding Cpt and LicCA, as well as a putative choline transporter directly adjacent to *licCA* (data not shown). The absence of identifiable genes encoding LicCA and Cpt in the genomes of human oral species *T. socranskii, T. lecithinolyticum* and *T. maltophilum* is consistent with their relative evolutionary distance from PC-containing *Treponema* (Fig. 7). The human oral microbiome alone includes more than fifty *Treponema* species and phylotypes, of which only a small minority have been cultured (Paster *et al*., 2001) and fewer whose genomes have been sequenced. Further studies of the genomes, phospholipid profiles and behaviors of diverse *Treponema* isolates are necessary to determine whether PC plays a specific role in the interaction between spirochetes and their mammalian hosts. Absence of genes encoding Cpt and some or all of the other PC pathway components in termite gut-associated *Treponema* and freshwater hotspring-associated *T. caldaria* likely reflects their considerable evolutionary

distance from mammalian mucosal tissue-associated *Treponema spp*.

A wide range of microbial genomes other than *Treponema* encode predicted proteins having some limited homology to TDE0021. All of these, which contain the conserved phosphatidyltransferase domain that is also present in TDE1366, TDE2572 and TDE2669, are likely members of phosphatidyltransferase families with cellular functions unrelated to PC. With the exception of a predicted homologue in the marine isolate *Fulvivirga imtechensis* (24% identity, E-score $4 \times e^{-14}$), all exhibit very limited homology to the TDE0021 gene product.

In addition to reporting genetic and functional characterization of this novel biosynthetic pathway, the present study contributes to ongoing discussion of the role of HGT in prokaryotic molecular evolution (Gupta, 2016). While HGT between prokaryotes and from prokaryotes to eukaryotes is well characterized, HGT from eukaryotes to prokaryotes has not been extensively studied and its importance in microbe-host interactions may be underestimated (Dunning Hotopp, 2011). Genes of probable eukaryotic origin can be identified in many bacterial genomes, but their importance to host-associated microbes is clear in few cases. Approximately 3% of the *Legionella pneumophila* genome consists of 'eukaryotic-like' proteins, several of which are likely to have important roles in the evolution and behavior of this intracellular pathogen (Lurie-Weinberger *et al*., 2010). Significant numbers of *Neisseria gonorrhoeae* isolates carry a fragment of the human long interspersed nuclear element L1 that is likely to be the result of a relatively recent HGT event (Anderson and Seifert, 2011). The exact mechanisms and significance of these and other HGT events are under study by several groups.

Based on molecular dating analysis, the appearance of the TDE0021 ancestral gene in *Treponema* occurred over 1,100 MYA. Similar molecular dating places the last common ancestor prior to the divergence of *Treponema* and *Borrelia* at approximately 1,800 MYA (Battistuzzi *et al*., 2004). This timescale is consistent with the presence of TDE0021 homologues in only one branch of *Treponema* and is strongly suggestive of a HGT from a eukaryote occurring long after establishment and further divergence of the *Treponema* genus from other spirochetes. All together our *in silico* analyses suggest this gene family is found throughout Eukaryota, is absent in Archaea, and was likely seeded into two genera of Bacteria by HGT events. Further unsampled sequences derived from culture-independent approaches such as metagenomics and single cell genomics may aid in resolving the actual number and order of these HGT events.

The importance of HGT in the evolution of host-associated spirochetes is well-recognized (Subramanian *et al*., 2000). *T. pallidum* genes reported as of likely eukaryotic origin include those encoding several tRNA synthases, a DNA repair helicase, von Willebrand factor domain A (vWA) proteins, ankyrin repeat proteins and a pyrophosphate-dependent phosphofructokinase (reviewed in (Subramanian *et al*., 2000)). Some of these, particularly vWA proteins and ankyrin repeat proteins, are signaling proteins likely to contribute to interactions between this microbe and its host (Ponting *et al*., 1999). Homologues of several of these *T. pallidum* genes are present in *T. denticola* (data not shown) and may have similar as yet undetermined roles in host interactions.

The biological role of PC in host-associated spirochetes is not clear. In some plant pathogens and symbionts, PC is believed to contribute to the host-microbe relationship (Sohlenkamp *et al*., 2003), while PC growth and temperature sensitivity in some free-living bacteria (reviewed in (Sohlenkamp *et al*., 2003)). PC in spirochetes is not restricted to *Treponema* spp. but is also found in *Borrelia spp*., including the causative agent of Lyme disease *B. burgdorferi. Borrelia* uses the single step Pcs pathway for synthesis of PC directly from choline (Martínez-Morales *et al*., 2003; Wang *et al*., 2004), While no *Borrelia* PC mutants have been reported, the Pcs pathway appears to be its sole means of PC formation. This is in contrast to several other Pcs-containing microbes that also contain a Pmt pathway for PC biosynthesis (Sohlenkamp *et al*., 2003). As in *Borrelia*, *Treponema* possesses only a single pathway for PC biosynthesis, thus both are dependent on exogenous (host) choline for PC biosynthesis. While there are relevant animal infection models for *T. pallidum* and *Borrelia* infections, animal models for *T. denticola* are problematic, largely due to its occurrence within a very complex oral microbial community and the chronic nature of periodontal disease. The fact that several oral *Treponema* spp. lack the PC pathway suggests that it is not crucial for survival in the oral mucosal environment. Our laboratories are currently exploring methods utilizing the isogenic PC mutant strains to determine the role of CDP-choline-dependent PC production in *T. denticola* interactions with host tissue and with other oral microbes.

Experimental procedures

Strains, media, and growth conditions

All strains and plasmids used in this study are described in Supporting Information Table S1. *T. denticola* ATCC 35405 and its isogenic mutants were grown as previously described (Fenno, 2005) under anaerobic conditions in NOS broth or semisolid medium containing 0.8% Noble agar, supplemented with erythromycin (40 μg ml^{-1}) and

kanamycin (Km; 25 μg ml^{-1}) as appropriate. In some experiments, NOS broth medium was supplemented with methylcellulose (0.95% w/v). All growth media were incubated under anaerobic conditions for at least 18 h prior to use (McMinn and Crawford, 1970). Purity of spirochete cultures was monitored by darkfield microscopy. *E. coli* strains were grown at 30°C in LB broth or agar medium (Sambrook and Russell, 2001). *Saccharomyces cerevisiae* strains harboring plasmids were grown at 30°C in SD-Ura medium (Kaiser *et al*., 1994) supplemented with 2% glucose (w/v). Antibiotics were added to the growth medium as appropriate to obtain the following final concentrations (mg/liter): chloramphenicol 20, spectinomycin 200, carbenicillin 100, kanamycin 50 (*E. coli*) and 25 (*T. denticola*), erythromycin 200 (*E. coli*) and 20 (*T. denticola*).

Bioinformatics analysis

The presence and conservation of genes of interest in *Treponema* genomes were analyzed using BLAST search tools (Altschul *et al*., 1990) on the NCBI, HOMD and ExPASY websites. Operon analysis of the TDE0021 region was done using the DOOR and ProOpDB prokaryotic operon analysis algorithms (Mao *et al*., 2009). Transcription promoter prediction was done using the BPROM algorithm (Taboada *et al*., 2010; Solovyev and Salamov, 2011). A *Treponema* 16S rRNA phylogeny tree was constructed using the Clustal-Omega tool (Soding, 2005) contained in the DNASTAR program suite.

To determine the potential relationship between TDE0021 and potential homologues in other organisms, we carried out a BLAST search (Altschul *et al*., 1990; Tamura *et al*., 2013) of the non-redundant protein database from NCBI with the TDE0021 amino acid sequence as query, using an E-value of 1e-08, query coverage of at least 30% and filtering for low-complexity segments as cutoffs. We adjusted the compositions of the sequences under comparison employing the universal compositional score matrix adjustment (Yu and Altschul, 2005). We used the constraint-based alignment tool for multiple alignment protein sequences (COBALT) program (Papadopoulos and Agarwala, 2007), which uses constraints obtained from the PROSITE protein-motif database and the conserved domain database to refine the alignment quality. We also included two other protein sequences, the CHTP1 (NP_064629) from *Homo sapiens* and *Saccharomyces cerevisiae* CTP1 (AAA63571). In order to do this, we first aligned the two CTPs using MUSCLE (Edgar, 2004). Then, to align the two multiple alignments (the two CPTs and the COBALT alignment) we used MUSCLE setting the option of profile-profile alignment. The final multiple alignment, manually edited to remove poorly aligned regions as well as columns with gaps of more than 30%, is available upon request. A Maximum Likelihood (ML) phylogeny analysis was generated with MEGA6 (Tamura *et al*., 2013) with the best mode model (LG + I+G + F) chosen using ProtTest (Darriba *et al*., 2011). To evaluate clade support we conducted a bootstrap analysis with 100 replicates. Finally, we conducted molecular clock analysis with RelTimeML from MEGA6 using the same substitution model as for the ML phylogeny. and set local clocks as the clock type as our data set did not conform to a global clock. To calibrate our molecular dating analysis within a range from 900 to 1600 MYA, we extracted the estimated time of divergence of *Lepisosteus oculatus* (spotted gar) and *Ordospora colligata* (microspordians) from the TimeTree database (Hedges *et al*., 2015). The times of divergence of two other major divisions (Eukaryotes and Prokaryotes; *Treponema* and *Bacteroides*) were also extracted from the TimeTree database.

Molecular biology methods

Recombinant DNA techniques were performed according to standard protocols (Sambrook and Russell, 2001). For expression studies in *E. coli*, the nucleotide fragments corresponding to *TDE0021* (*cpt*) and *TDE1260* (*licCA*) were synthesized by GeneArt (Invitrogen, Carlsbad, CA). In case of LicCA the version shown to be active by Kent *et al*. (Kent *et al*., 2004) lacking the N-terminal helix-turn-helix domain was synthesized. Both synthesized DNA fragments were designed to contain terminal NdeI and BamHI restriction sites to facilitate the subcloning of the inserts. After digestions with NdeI and BamHI, the *TDE0021* fragment was cloned into pET17b (EMD Biosciences, Billerica, MA) and the *TDE1260* fragment was cloned into pCDF-Duet1 (EMD Biosciences) to obtain the compatible expression plasmids pET17b-TDE0021 and pCDF-Duet1-LicCA respectively. The other three candidate genes TDE2669, TDE1366 and TDE2572 were PCR amplified from genomic DNA isolated from *T. denticola* using oligonucleotides incorporating NdeI and BamHI restriction sites (underlined) and cloned into pET17b (TDE1366F:5′-actgcatatgatatataataaaactttgatatacaa-3′; TDE1366R:5′-actgggatcctatatctaattttatatcgaattat-3′; TD EF2572F:5′-actgcatatgaagctatctaatatttttacatcatcg-3′; TDER 2572R:5′-actgggatcctattcatcagcagttctccttttatt-3′; TDEF2669 F:5′-actgcatatggaaaaaaaaatcggcagattggtttta-3′; TDER2669 R:5′-actgggatccctaagtttctatgtctctttgcg-3′). *E. coli* strains were transformed with plasmid DNA using a standard $CaCl_2$ method (Sambrook and Russell, 2001).

T. denticola TDE0021, annotated as encoding a choline phosphotransferase, was synthesized as described above and subcloned as a BglII-BamHI-fragment into the BamHI-site of the yeast expression plasmid pSP-Gm2 (Rodríguez-Limas *et al*., 2011) forming pSP-Gm2-Trepo. TDE0021 is expressed in this plasmid under the control of the constitutive TEF (translational elongation factor 1α) promoter. Plasmids were transformed into *S. cerevisiae* according to Becker and Lindblad (Becker and Lundblad, 2001).

Allelic replacement mutagenesis of T. denticola *TDE0021*

For construction of a *T. denticola* TDE0021 mutant, a 4,019 bp DNA fragment encompassing TDE0020 through TDE0022 was amplified from *T. denticola* genomic DNA using high fidelity Taq polymerase (Taq-HF, Invitrogen) and oligonucleotide primers CX554 (5′-ggcgcttatcatgttatgag-3′) and CX555 (5′-gattgaagatgcggaacttc-3′), then cloned in pGEM-Teasy (Promega, Madison, WI). The resulting plasmid was digested with BclI and BlpI to release a 261 bp fragment within the 5′end of TDE0021, which was then replaced by an antibiotic cassette consisting of a gene encoding kanamycin resistance (*aphA2*, (Li *et al*., 2015))

under transcriptional control of the *ermB* promoter from pVA2198 (Fletcher *et al.*, 1995; Goetting-Minesky and Fenno, 2010), yielding pCF744. Because TDE0020-TDE0021-TDE0022 are predicted to be cotranscribed as an operon, pCF744 was designed such that *aphA2* (in the same transcriptional orientation as TDE0021) is followed by stop codons in all three reading frames but no transcription terminator. To account for the possibility that TDE0022 transcription required an unidentified promoter element, the 3′ end of TDE0021 was retained in pCF744. TDE0020 encodes glycyl-tRNA synthetase while TDE0022 encodes a hypothetical protein. Further analysis of the resulting plasmid pCF744 revealed that it retained the last of the six residues comprising the conserved CDP-alcohol phosphotransferase motif (Fig. 2) (Williams and McMaster, 1998; Solis-Oviedo *et al.*, 2012). To remove the remnant of the predicted CDP-alcohol motif, pCF744 was amplified using oligonucleotide primers CX1051 (5′-tggtcctcgagat tatggctgcccttgc-3′) and CX1052 (5-ctagatgctcgagtcagaa gaactcgtcaag-3′), digested with XhoI (underlined in primer sequence) and self-ligated. The resulting plasmid pCF789 carries the first 37 bp and the last 498 bp of TDE0021 separated by the *aphA2* cassette (Fig. 3).

Plasmid pCF789 was digested with NotI to release a linear fragment consisting of the antibiotic cassette between DNA sequences flanking the target gene. *T. denticola* ATCC 35405 was transformed by electroporation with 10 μg of linearized DNA released from pCF789 and transformants were selected with resistance to kanamycin as described previously (Godovikova *et al.*, 2015), with the following modification. *T. denticola* colonies picked from NOS/Km/Noble agar selection plates were initially grown in NOS/Km broth supplemented with 0.95% methylcellulose for at least two passages before further growth in NOS/Km broth medium and subsequent characterization. Allelic replacement mutagenesis of TDE0021 was confirmed by PCR analysis as well as by DNA sequencing of the target region of genomic DNA. DNA sequencing was performed at the University of Michigan DNA Sequencing Core Facility and analyzed using DNASTAR sequence analysis software (DNASTAR Inc., Madison, WI).

PC determination in T. denticola *strains*

Phospholipid analysis of parent and mutant *T. denticola* strains was carried out at the University of Michigan Metabolomics Core Facility. To determine presence of PC in *T. denticola* wildtype and mutant strains, triplicate samples of 4-day broth cultures grown in the presence of antibiotics as appropriate were subjected to total lipid extraction (approximately 5×10^9 cells per sample) by the method of Bligh and Dyer (Bligh and Dyer, 1959). Presence and levels of PC and PE were determined using a modification of the method of Ames and Dubin (Ames and Dubin, 1960). Briefly total lipids isolated from each *T. denticola* strain were separated by thin-layer chromatography followed by phosphate determination via colorimetric measurement of phosphoammonium molybdate complexes, then quantitated by comparison with PC and PE standard curves.

Complementation of a *S. cerevisiae* mutant deficient in cholinephosphotransferase activity

The yeast CPT1 mutant HJ091 was transformed with plasmids shown in Supporting Information Table S1, as previously described (Becker and Lundblad, 2001). Transformants were selected in SD-Ura medium supplemented with 2% glucose. Cultures (20 mL) were grown to an optical density at 620 nm (OD_{620}) of 0.6 and 1 μCi [*methyl*-^{14}C]choline chloride (CFA.424, Amersham Life Science) or [2-^{14}C]ethanolamine hydrochloride (20 μM, 1×10^5 dpm/nmol; CFA.329 by Amersham Life Science) was added to the cultures. Following 30 min additional incubation, cells were harvested by centrifugation, washed once with ice-cold water and the pellets were resuspended in 1 mL $CHCl_3/CH_3OH$ (1:1, v/v). Acid-washed 0.5-mm glass beads (0.5 g) were added to each sample and the cells were broken by vortexing three times for 1 min. Samples were held on ice for 2 min between bursts. The supernatant was transferred to a new tube and the beads were washed once with 500 μL $CHCl_3/CH_3OH$ (2:1, v/v). Both supernatants were pooled, then water and $CHCl_3$ were added until a phase separation was obtained. Phospholipids in the organic phase were analyzed by TLC on Merck Silica 60 HPTLC plates using n-propanol/propionic acid/chloroform/water (3:2:2:1, v/v) as mobile phase. The aqueous phases were concentrated under vacuum and separated by TLC on Merck Silica 60 HPTLC plates in a solvent system consisting of CH_3OH/0.6% NaCl/NH_4OH (50:50:5, v/v).

Expression of the complete T. denticola *PC synthesis pathway in* E. coli

Plasmids pET17b-TDE0021 and pCDF-Duet1-LicCA were transformed into *E. coli* BL21(DE3)/pLysS. When the cultures reached an OD_{620} of 0.4, IPTG (0.2 mM) and 1 μCi [^{14}C]acetate or [^{14}C]choline were added, and cultures were incubated for a further 3 hours. Cells were harvested and the lipids extracted according to Bligh and Dyer (Bligh and Dyer, 1959). Aliquots of the lipid extracts were spotted on HPTLC silica gel 60 (Merck, Poole, U.K.) plates and were separated in two dimensions using $CHCl_3/CH_3OH/H_2O$ (140:60:10, v/v) as mobile phase for the first dimension and $CHCl_3/CH_3OH$/glacial acetic acid (130:50:20, v/v) for the second dimension. In some cases the organic phases were only analyzed by one-dimensional TLC using $CHCl_3/CH_3OH/H_2O$ (140:60:10, v/v) as mobile phase. The presence of phosphatidylcholine in the lipid extract from the *E. coli* strain expressing LicCA and TDE0021 was confirmed by LC-ESI-MS/MS.

In vitro *assays for TDE0021 choline phosphotransferase activity*

To establish an enzyme assay for TDE0021, microsomal membranes were prepared as described previously (Hjelmstad and Bell, 1992; Hjelmstad *et al.*, 1994; McMaster and Bell, 1994; McMaster *et al.*, 1996, Williams and McMaster, 1998) from *S. cerevisiae* HJ091 derivatives expressing

yeast CPT1 or TDE0021. Choline phosphotransferase activity was assayed using a variation on a standard assay for yeast CPT1 activity (Hjelmstad and Bell, 1992) as follows: 20 mM $MgCl_2$, 325 μM PC (egg, Sigma), 325 μM diacyglycerol (di1 6:1 DAG, Sigma), 0.45% Triton X-100 (w/v) and 500 μM [^{14}C]CDP- choline (American Radiolabeled Chemicals, 2000 dpm/nmol) as substrates. Assay conditions were further modified to optimize for *T. denticola* choline phosphotransferase activity expressed in *E. coli* as follows: 90 μg protein (membrane fraction), 50 mM Tris/HCl, pH 7.0, 20 mM $MnCl_2$, 325 μM PC (egg), 650 μM DAG (di1 6:1 DAG), 0.1% Triton X-100 (w/v) and 1 mM [^{14}C]CDP-choline (American Radiolabeled Chemicals, 2000 dpm/nmol) as substrates in a total volume of 100 μL. In some assays, $MnCl_2$ was replaced with $MgCl_2$, $CaCl_2$, $CoCl_2$, $NiCl_2$, or $CuCl_2$. EDTA was added at a final concentration of 20 mM in the corresponding assays. The reactions were started by the addition of CDP-choline. The pH dependency of the TDE0021 catalyzed reaction was tested using 50 mM BisTris/HCl (pH 6.0, 6.5, 7.0) or 50 mM Tris/HCl (pH 7.0, 7.5, 8.0, 8.5). The reaction mixtures were incubated in a water bath at 30°C for 30 min, and reactions were stopped by adding 375 μL $CH_3OH/CHCl_3$ (2:1, v/v). Phase separation was achieved by adding 125 μL water and 125 μL $CHCl_3$. Radioactivity in the organic phase was quantified by liquid scintillation counting. To ensure that the radioactivity in the organic phase only represented [^{14}C]PC, the organic phases were also separated by one-dimensional TLC as described above. Incorporation of [^{14}C]choline into [^{14}C]PC was measured by quantifying the intensity of the PC spot using a Storm PhosphorImager and ImageQuant. The intensity units were later converted to pmoles by interpolation of a standard curve of known amounts of [^{14}C]CDP-DAG.

LC-ESI-MS/MS analysis of phosphatidylcholine

Cultures (500 mL) of *E. coli* BL21(DE3)/pLysS strains expressing LicCA, TDE0021, or both LicCA and TDE0021 were grown to an OD_{620} of 0.6 in LB medium, induced with IPTG (0.2 mM), then grown for 4 hours. Lipids were extracted according to Bligh and Dyer (Bligh and Dyer, 1959). Normal phase LC was performed on an Agilent 1200 Quaternary LC system equipped with an Ascentis Silica HPLC column, 5 μm, 25 cm × 2.1 mm (Sigma-Aldrich, St. Louis, MO). Mobile phase A consisted of $CHCl_3/CH_3OH$/aqueous ammonium hydroxide (800:195:5, v/v); mobile phase B consisted of $CHCl_3/CH_3OH/H_2O$/aqueous ammonium hydroxide (600:340:50:5, v/v); mobile phase C consisted of $CHCl_3/CH_3OH/H_2O$/aqueous ammonium hydroxide (450:450:95:5, v/v). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. With a total flow rate was 300 μL/min, the LC eluent is injected into the ion spray source of a Triple-TOF 5600 quadrupole time-of-flight tandem mass spectrometer (AB SCIEX, Framingham, MA, U.S.A.). Instrumental settings for positive ion ESI and MS/MS analysis of lipid species were as follows: Ion spray voltage (IS) = 5500 V; current gas (CUR) = 20 psi (pressure); gas-1 (GS1) = 20 psi; de-clustering potential (DP) = +55 V; and focusing potential (FP) = +150 V. The MS/MS analysis used nitrogen as the collision gas, with the collision energy (CE) set at 45 eV. Data analysis was performed using the Analyst TF1.5 Software.

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