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A CDP-choline pathway for phosphatidylcholine biosynthesis in *Treponema denticola*

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Summary

The genomes of *Treponema denticola* and *Treponema pallidum* contain a gene, *licCA*, which is predicted to encode a fusion protein containing choline kinase and CTP:phosphocholine cytidylyltransferase activities. Because both organisms have been reported to contain phosphatidylcholine, this raises the possibility that they use a CDP-choline pathway for the biosynthesis of phosphatidylcholine. This report shows that phosphatidylcholine is a major phospholipid in *T. denticola*, accounting for 35–40% of total phospholipid. This organism readily incorporated [^{14}C]choline into phosphatidylcholine, indicating the presence of a choline-dependent biosynthetic pathway. The *licCA* gene was cloned, and recombinant LicCA had choline kinase and CTP:phosphocholine cytidylyltransferase activity. The *licCA* gene was disrupted in *T. denticola* by erythromycin cassette mutagenesis, resulting in a viable mutant. This disruption completely blocked incorporation of either [^{14}C]choline or ^{32}Pi into phosphatidylcholine. The rate of production of another phospholipid in *T. denticola*, phosphatidylethanolamine, was elevated considerably in the *licCA* mutant, suggesting that the elevated level of this lipid compensated for the loss of phosphatidylcholine in the membranes. Thus it appears that *T. denticola* does contain a *licCA*-dependent CDP-choline pathway for phosphatidylcholine biosynthesis.

Introduction

Phosphatidylcholine (PC) is a major lipid in eukaryotic membranes, in which it accounts for 40–60% of the phospholipids. Phosphatidylcholine is a major structural component of membrane bilayers and lipoproteins, and participates in several signal transduction pathways. Phosphatidylcholine is also found in a wide variety of bacteria, notably symbionts and pathogens (reviewed in Sohlenkamp *et al*., 2003), where it may constitute only a few per cent of total lipids as in *Pseudomonas aeruginosa* (Albelo and Domenech, 1997) or may be a major lipid component as in *Acetobacter aceti* (Hanada *et al*., 2001).

In eukaryotes, PC is made by either of two pathways: the CDP-choline or Kennedy pathway (overview shown in Fig. 1) consists of three enzymatic steps catalysed by choline kinase, CTP:phosphocholine cytidylyltransferase (CCT), and a CDP-choline:1,2-diacylglycerol choline phosphotransferase. The phospholipid N-methyltransferase pathway consists of the stepwise methylation of phosphatidylethanolamine (PE), with S-adenosylmethionine as methyl donor. It has been long assumed that bacteria do not possess the CDP-choline pathway for PC biosynthesis (Lopez-Lara and Geiger, 2001). To date prokaryotes have been shown to make PC by either the phospholipid N-methyltransferase pathway (Kaneshiro and Law, 1964) or the phosphatidylcholine synthase pathway, a pathway unique to bacteria in which choline reacts with CDP-diacylglycerol to form PC (de Rudder *et al*., 1999). Most bacteria having PC as a membrane lipid probably possess both currently known bacterial pathways for PC biosynthesis though some, including *Borrelia*, *Pseudomonas* and *Burkholderia* spp., possess only one PC biosynthesis pathway (reviewed in Sohlenkamp *et al*., 2003)

Some bacteria do have a CDP-choline pathway for attaching phosphocholine to complex, cell-surface oligosaccharides (Fig. 1). The *lic1* operon in *H. influenzae* and *lic* operon in *S. pneumoniae* have been identified as being involved in this process (Weiser *et al*., 1997; Zhang *et al*., 1999). Gene *licA* was proposed to be a choline kinase (Weiser *et al*., 1997) and this identity has recently been confirmed by cloning, expressing, and characterizing the gene product (H. A. Campbell and C. Kent, unpubl. data). The *licC* gene has been identified as the CCT of this pathway, although its primary structure is not similar to that of the eukaryotic CCTs (Campbell and Kent, 2001; Rock *et al*., 2001). The gene encoding the choline phosphotransferase that donates phosphocholine to the oligosaccharide has not been definitively identified, but it has

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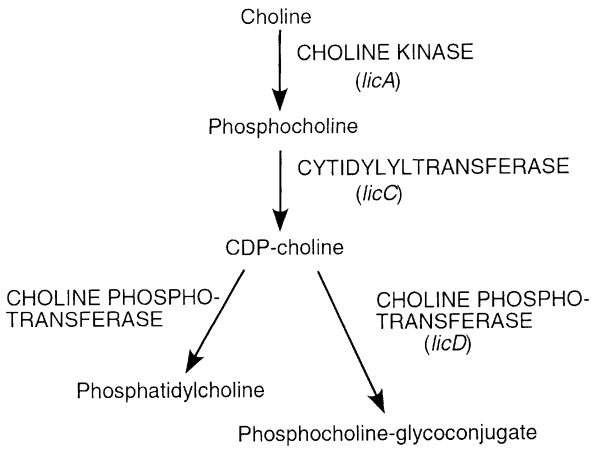

Fig. 1. CDP-choline pathways for biosynthesis of phosphatidylcholine and glycoconjugate-linked phosphocholine. The CDP-choline pathway for phosphatidylcholine biosynthesis is found in animals, plants, yeasts and other eukaryotes. The CDP-choline pathway for addition of phosphocholine to glycoconjugates has been demonstrated in *H. influenzae* and *S. pneumoniae* and is presumably present in other bacteria containing the *lic* genes. In organisms containing the *lic* genes, choline kinase is the product of *licA*, cholinephosphate cytidylyltransferase is the product of *licC*, and the cholinephosphotransferase that transfers phosphocholine to the complex oligosaccharide is the presumed product of *licD*.

been proposed to be *licD* (Zhang *et al*., 1999; Lysenko *et al*., 2000).

Genomic analysis has revealed that several bacteria appear to encode a fusion protein in which a *licC*-encoded CCT is the amino terminal half and a *licA*-encoded choline kinase is the carboxyl terminal half (Fig. 2). We refer to this fusion gene as *licCA*. The *licCA*-containing organisms include *Treponema pallidum*, which causes syphilis, *Fusobacterium nucleatum*, which is involved in periodontal disease, and *Clostridium perfringens*, which causes gangrene. Although little information is available on phospholipid synthesis and composition in fusobacteria and clostridia, it is of interest that the treponemes have been reported to contain PC as a major membrane phospholipid (Livermore and Johnson, 1974; Matthews *et al*., 1979; Barbieri *et al*., 1981; Belisle *et al*., 1994). The presence of the *licCA* fusion gene in treponemes suggests that they may utilize a CDP-choline pathway for biosynthesis of PC. In this report we show that *T. denticola* possesses a CDP-choline pathway for PC biosynthesis, and that a *licCA* gene similar to that of *T. pallidum*, encodes proteins in that pathway. This is the first report of a CDP-choline pathway for PC biosynthesis in bacteria.

Results

Phosphatidylcholine in T. denticola

Several species of *Treponema*, including *T. denticola*, have been reported to contain PC and several other phospholipids including PE and PG (Livermore and Johnson, 1974; Smibert, 1976; Barbieri *et al*., 1981), but the amount of each phospholipid in *T. denticola* has not previously been reported quantitatively. In order to confirm the presence of and measure the amount of PC in *T. denticola*, total lipids were extracted and chromatographed by thin-layer chromatography in several different solvent systems. The major phospholipids, PC, PE and phosphatidylglycerol (PG) were identified by co-chromatography with known standards. Phosphatidylcholine and PE were the most abundant phospholipids, each accounting for about a third of total phospholipids, while PG was about 10% (Fig. 3). Cardiolipin was a minor lipid (not shown).

Although the choline-containing sphingolipid, sphingomyelin, has been reported to be a constituent of *T. pallidum* (Matthews *et al*., 1979), we found no evidence for this lipid in *T. denticola*, either by measuring lipid phosphate or by incorporation of [^{14}C]-choline or ^{32}Pi into a lipid that co-chromatographed with authentic sphingomyelin.

If *T. denticola* were using a CDP-choline pathway for PC biosynthesis, one would expect that this lipid would be specifically labelled after incubating the bacteria with radiolabelled choline. Indeed, *T. denticola* had a robust system for uptake and incorporation of either [^{3}H]choline (not shown) or [^{14}C]choline (Fig. 4) into lipids. Separation by thin-layer chromatography and quantification of the individual lipids from such an experiment revealed that at least 96% (^{3}H) or 99% (^{14}C) of the radioactivity in the total lipid extract was in PC.

Cloning and expression of licCA *and enzymatic activity of the recombinant protein*

The *licCA* gene was identified in preliminary *T. denticola* genome sequence contigs based on similarity to the predicted *licCA* gene of *T. pallidum* (Fraser *et al*., 1998). The *T. denticola licCA* DNA sequence was found to be identical to that shown in the preliminary genomic contigs (data not shown). The original cloned fragment in pSY107 was designed to contain about 1300 base pairs upstream of the translation start site predicted from the *T. pallidum licCA* sequence (^{79}MAAGFGS…, Fig. 2). Recombinant expression of a *T. denticola* construct that initiated at this translation start site, however, resulted in a protein that was insoluble and inactive. Inspection of the sequences upstream of this predicted start site revealed another possible translation initiation site, which added 78 residues to the protein sequence. The *T. pallidum* and *T. denticola* sequences were 45% identical within this segment. Expression of a construct designed to initiate at this new site (^{1}MKRRYF…, Fig. 2) resulted in a protein that was soluble and active. The protein was purified to near homo-

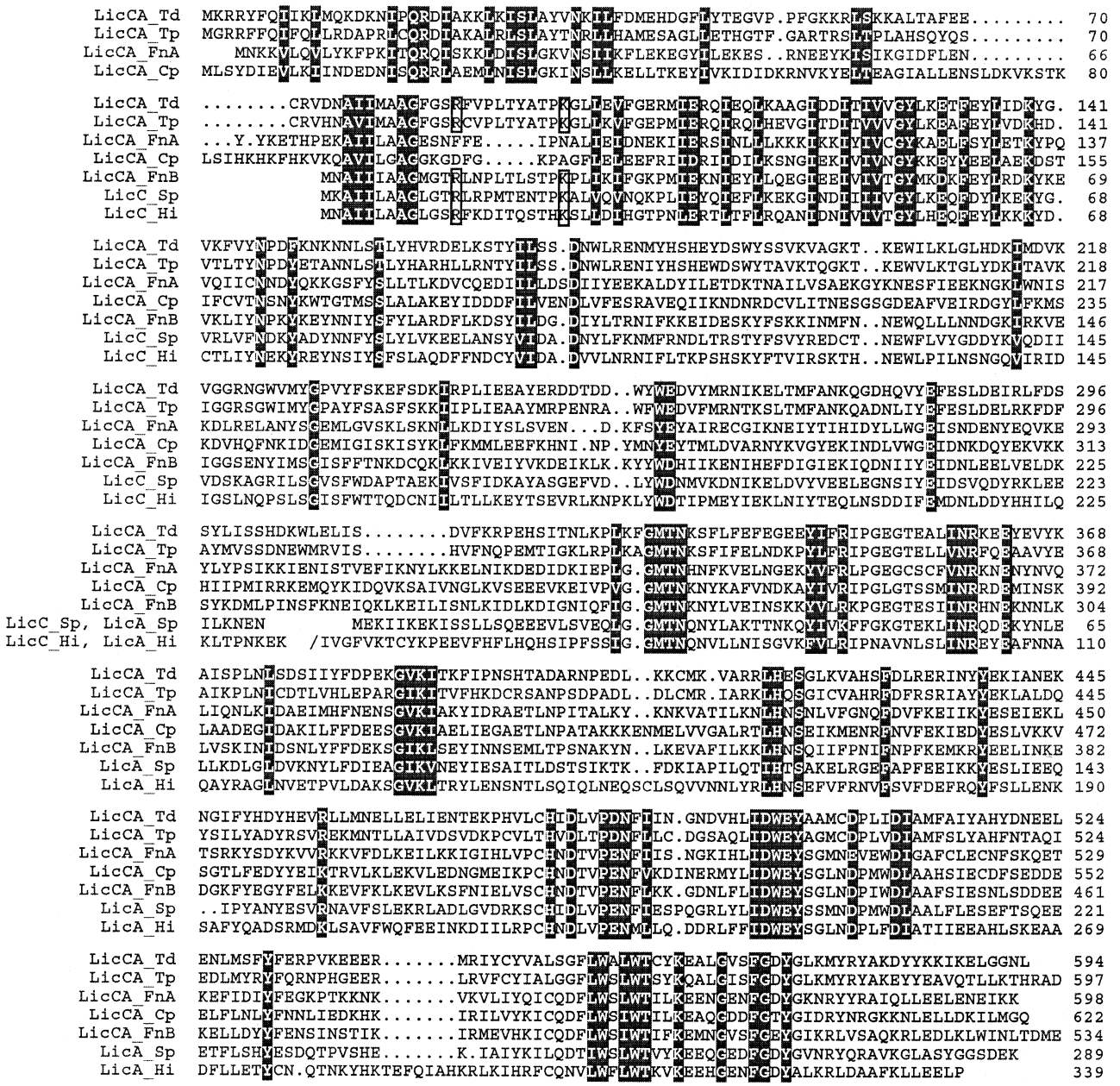

Fig. 2. Alignment of LicC, LicA and LicCA gene products. The alignment was according to the CLUSTALW algorithm in the MEGALIGN program of the Lasergene package. Shaded residues are those that are conserved (identical or Y = F =W, I = L = V, K = R, E = D, and S = T) in all sequences. Boxed residues are the critical Arg and Lys active site residues (see *Discussion*). Sequences, abbreviations and accession numbers are Td, *Treponema denticola* (AY322155); Tp, *Treponema pallidum* (NP_218547); Fn, *Fusobacterium nucleatum* (NP_602486 [FnA] and NP_604134 [FnB]); Cp, *Clostridium perfringens* (NP_561543); Sp, *Streptococcus pneumoniae* (AAK94072 [LicC] and AAK94073 [LicA]); Hi, *Haemophilus influenzae* (NP_439688 [LicC] and P14181 [LicA]). The putative initiator methionine in the *T. pallidum* LicCA is encoded by GTG. The last six residues of the *T. pallidum* LicCA are not shown. *Streptococcus pneumoniae* LicA and *H. influenzae* LicA are aligned with the C-terminal regions of LicCA, such that the conserved Gly-Met-Thr-Asn motif near the N-terminus of the LicA region (330–333 in *T. denticola*) is aligned in all sequences. A series of 4-residue tandem repeats at the amino terminus of *H. influenzae* LicA is not shown, and is indicated by '/'.

geneity (Fig. 5) and was active in assays for both choline kinase and CCT (Table 1).

Because the enzyme activities of the LicCA protein were 50–200 times lower than the activities of the individual enzymes from *S. pneumoniae* (Campbell and Kent, 2001; Rock *et al.*, 2001; H. A. Campbell and C. Kent, unpubl. data), we also constructed expression vectors that would encode the individual enzymes. These were termed C308 for the CCT from the *licC* portion and A317 for the choline kinase from the *licA* portion. Residue 308 was the

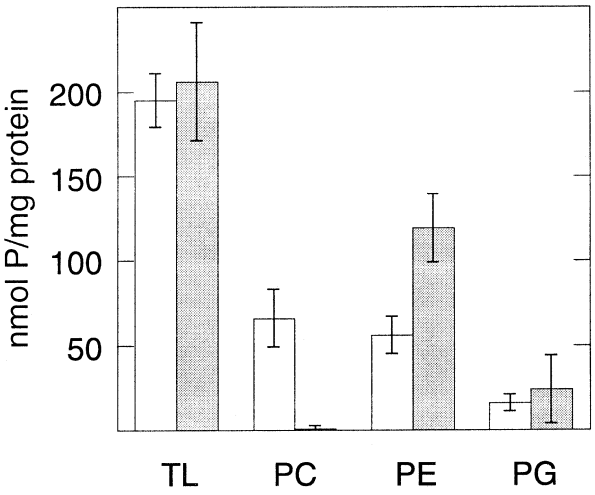

Fig. 3. Phospholipid composition of parent and mutant *T. denticola*. Levels of major phospholipids were determined as indicated in *Experimental procedures*. Data for *T. denticola* 35405 (open bars) and isogenic mutant LBE3 (shaded bars) are the averages and standard deviations from five samples. Phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and total phospholipids (TL).

C-terminus of C308 and residue 317, preceded by an initiator Met, was the N-terminus of A317. These proteins were also purified to near homogeneity (Fig. 5) and assayed for their activities. The A317 choline kinase activity was about fivefold greater than the choline kinase of the fusion protein, but the C308 CCT activity was negligible (Table 1).

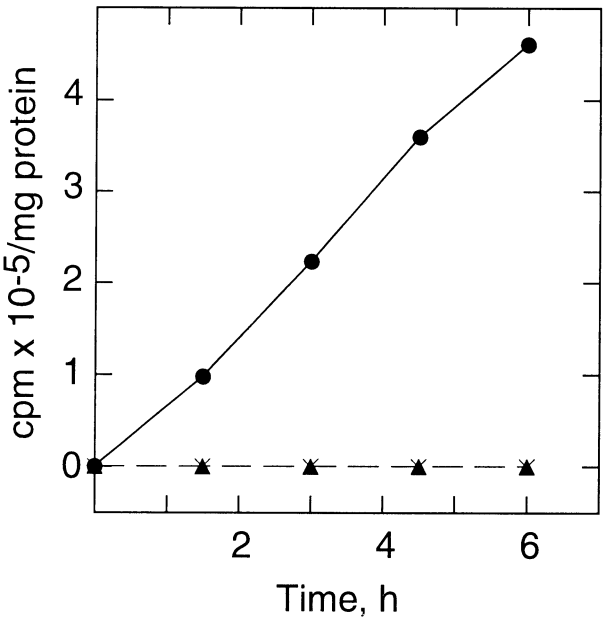

Fig. 4. Incorporation of [^{14}C]choline into lipids in parent and mutant *T. denticola*. Parent (35405, solid circles) and two clonal isolates of mutant (LBE, X′s and solid triangles) cells were grown to log phase, then 2 μCi ml^{-1} [^{14}C]choline were added to the culture medium. Cells were harvested at the indicated times and processed as described in *Experimental procedures*. Total radioactivity in the lipid extract, normalized for the total cellular protein, is reported.

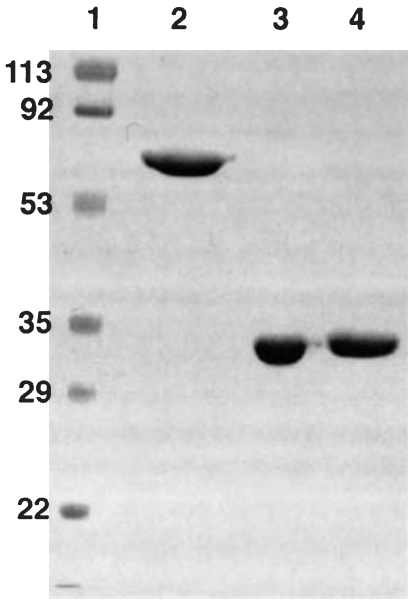

Fig. 5. Analysis of purified recombinant proteins. Purified LicCA (lane 2), C308 (lane 3), and A317 (lane 4) were subjected to SDS-PAGE. Lane 1 contained molecular weight standards.

Allelic replacement mutagenesis of licCA

To determine whether LicCA was essential for phosphatidylcholine biosynthesis in *T. denticola*, *licCA* was disrupted by allelic replacement mutagenesis. The strategy for *licCA* mutagenesis is shown in Fig. 6. Briefly, a 693 bp *BstZ*17I-*Bcl*I fragment including the 5′ end of *licCA* in pSY107 was replaced with a 2.1 kb *ermF/ermB* cassette. The vector sequence was released by restriction enzyme digestion and *T. denticola* was transformed by electroporation with the linearized disrupted *licCA*. Six erythromycin-resistant isolates were recovered, of which two had the desired allelic replacement. Construction of the isogenic mutant, designated *T. denticola* LBE3, was con-

Table 1. Enzymatic activities of LicCA and fragments.

Recombinant protein	Cytidylyltransferase activity (nmol min^{-1} mg protein^{-1})	Choline kinase Activity (nmol min^{-1} mg protein^{-1})
LicCA	813 ± 9	228 ± 6
C308	<1	NA
A317	NA	1100 ± 96

NA: not applicable.
Values are the averages and ranges of determinations from two separate protein preparations.

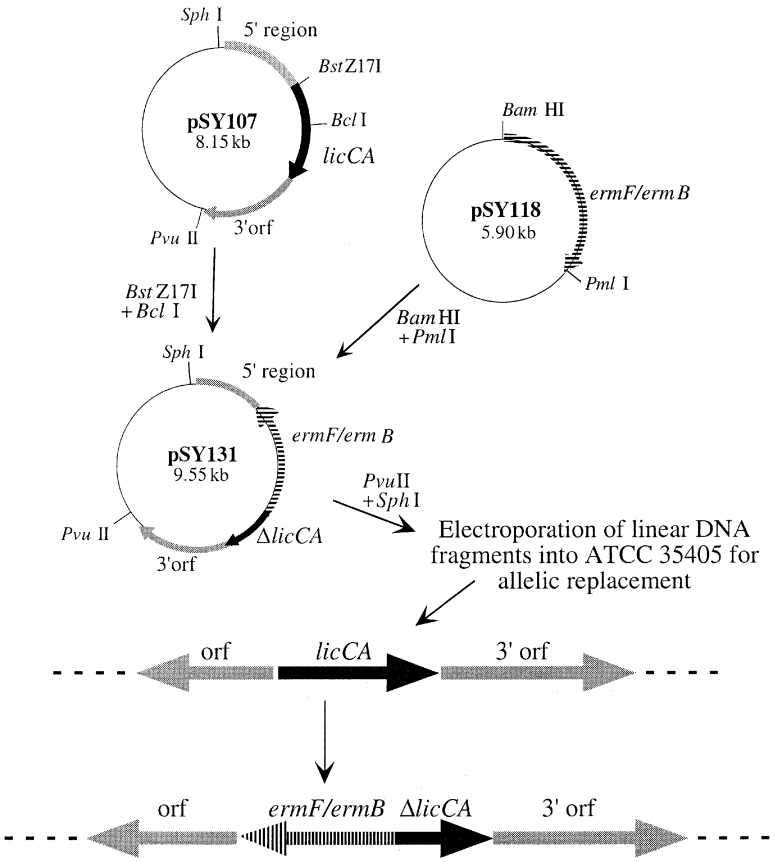

Fig. 6. Strategy for mutagenesis of *licCA*. Plasmid pSY107, which contained the original *licCA* product plus 5′ and 3′ flanking sequences, was digested with *Bcl*I and *Bst*Z17I to remove a 693 bp fragment of *licCA* including the 5′ end of the gene. The 2.1 kb *ermF/ermB* cassette was isolated from *Bam*HI-*Pml*I digested pSY118 and ligated to the previously digested pSY107. In the resulting plasmid, pSY131, the *ermF/ermB* cassette is in opposite transcriptional orientation to the disrupted *licCA* gene. pSY131 was digested to completion with *Sph*I and *Pvu*II to release the vector sequence. *Treponema denticola* 35405 was electroporated with the resulting linear DNA, resulting in allelic replacement of native *licCA*. Arrows show transcriptional orientation of *licCA* (black), *ermF/ermB* (hatched) and predicted open reading frames adjacent to *licCA* in *T. denticola* (grey).

firmed by PCR analysis using oligonucleotide primers CX226 and CX227. As expected, the amplicon from LBE3 was approximately 1.4 kb larger than that of the parent strain, confirming the double crossover homologous recombination event. Quantitative RT-PCR using a primer set within *licCA* but downstream of the insertion site demonstrated that *licCA* mRNA was present in 35405 and was absent in LBE3. Similarly, QRT-PCR using a primer set within the open reading frame directly downstream of *licCA* in the *T. denticola* genome sequence showed that this gene was transcribed in 35405, but was not transcribed in LBE3. The polar effect of *ermF/ermB* insertion in *T. denticola* is consistent with previous mutagenesis studies (Lee *et al.*, 2002). (data not shown).

The parent and mutant strains displayed no obvious phenotypic differences. Growth rates and final densities of broth cultures of the parent, 35405 and mutant LBE3 were not significantly different. Cell morphology and motility under phase-contrast microscopy were similar. Expression of membrane-associated proteins including Msp, PrcA and OppA was not altered in LBE3 compared with 35405. Peptidase activities of *T. denticola* parent and mutant strains as tested by hydrolysis of chromogenic substrates SAAPFNA and BApNA were indistinguishable (data not shown).

Lipid metabolism in the licCA *mutant*

Targeted disruption of the *licCA* gene completely eliminated incorporation of [^{14}C]choline into lipids and ^{32}Pi into phosphatidylcholine (Figs 4 and 7A). In addition, incorporation of [^{14}C]choline into phosphocholine and CDP-choline were also eliminated (Fig. 7B), as would be expected for a *licCA* disruption. In the mutant, levels of soluble [^{14}C]choline were reduced by about 40% compared to the parent strain. This may be due to absence of transcription in LBE3 of the open reading frame downstream of *licCA*, which has homology with predicted carnitine, choline or glycine betaine transporters (data not shown).

Because *T. denticola* was grown in the presence of serum, it was possible that the mutant was using serum lipoproteins as a source for either PC or lysoPC, which then might be acylated to form PC. Separation and quantification of lipid mass, however, revealed that the mutant contained little, if any, PC (Fig. 3).

To determine if the levels and biosynthetic rates of other phospholipids were altered in the mutant, a time course of ^{32}Pi incorporation was carried out (Fig. 8). As expected, essentially no ^{32}Pi was incorporated into PC in the mutant. The rate of incorporation of ^{32}Pi into PG was increased by about 50%, although there was not a signif-

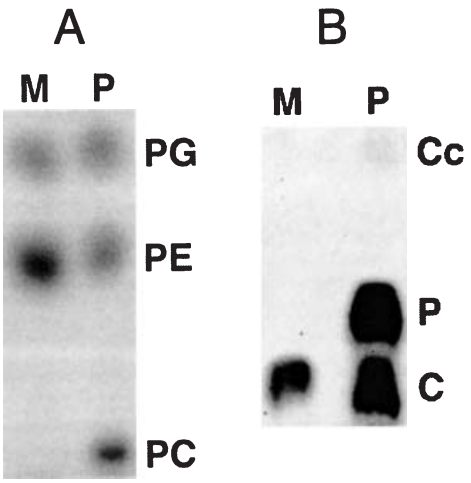

Fig. 7. Chromatography of radiolabelled precursors and lipids.
A. Cells were labelled in 5 μCi ml^{-1} ^{32}Pi for 4 h, then ^{32}P-labelled lipids from *T. denticola* LBE3 (M) and 35405 parent (P) were prepared and separated by TLC in solvent system I and visualized by autoradiography. Phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG).
B. Cells were labelled in 2.5 μCi ml^{-1} [^{14}C]choline for 6 h, then soluble metabolites were prepared and separated by TLC and visualized by autoradiography. Metabolites are choline (C), phosphocholine (P) and CDP-choline (Cc).

icant increase in the mass of PG (Fig. 3). The rate of incorporation of ^{32}Pi into PE in the mutant was increased by 2.7-fold (Fig. 8), and the level of PE mass was increased by twofold (Fig. 3). Thus it appears that the mutant compensates for the lack of PC primarily by increasing PE levels.

Discussion

The results presented here show that *T. denticola* contains phosphatidylcholine as a major phospholipid and has a choline-dependent mechanism for phosphatidylcholine biosynthesis. This mechanism is dependent on an intact *licCA* gene, implying that the two enzymatic functions of this gene, choline kinase and CCT, participate in the *T. denticola* pathway for phosphatidylcholine biosynthesis. Thus it appears that this bacterium uses a CDP-choline pathway for PC biosynthesis, which has not previously been reported for bacteria. Although we cannot definitively rule out the possibility that the LicCA protein only indirectly governs PC biosynthesis in *T. denticola*, it is much more likely that the LicCA choline kinase and CCT activities directly participate in the CDP-choline pathway. Strong evidence for these conclusions comes from the disruption of *licCA*, which prevents conversion of labelled precursors to phosphocholine and PC, and eliminates all detectable PC from this organism. Thus, there is no evidence for PC biosynthesis by either sequential methylation of PE or by a choline-dependent PC synthase activity in the *licCA* mutant, and the preliminary *T. denticola* genome sequence does not appear to contain genes encoding key enzymes required for these pathways.

The *licC* and *licA* genes were originally characterized in *H. influenza* (Weiser *et al*., 1997) and *S. pneumonia* (Zhang *et al*., 1999), where they are contained in an operon as separate, non-contiguous genes. The two genes are fused, however, in several organisms (Fig. 2). The advantage of such a fusion is not clear. It is possible that the proximity of the two active sites affords a more efficient means of catalysis. The low activity of the fusion protein as expressed heterogeneously was surprising, considering the much higher activity of the recombinant *licC* and *licA* gene products from *S. pneumonia* (Campbell and Kent, 2001; Rock *et al*., 2001; H. A. Campbell and C. Kent, unpubl. data) and eukaryotic choline kinases (Porter and Kent, 1990; Kim *et al*., 1998; Gee and Kent, 2003). Possibly the *T. denticola licCA* gene product is post-translationally modified or cleaved *in vivo*, leading to a higher level of activity in the native form. Our attempts to produce 'cleaved' recombinant proteins using molecular tools had mixed results. The A317 fragment had higher choline kinase activity than recombinant LicCA, but the CCT activity of the C308 fragment was much lower than that of recombinant LicCA. Further studies are needed to quantify activity of native LicCA.

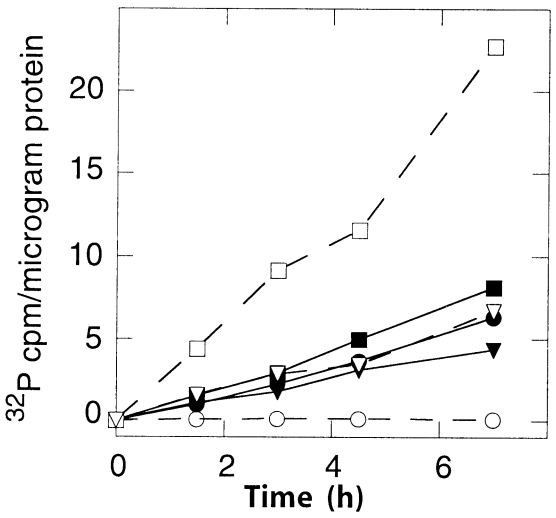

Fig. 8. Incorporation of ^{32}Pi into lipids in parent and mutant *T. denticola*. Parent and LBE3 were incubated with 5 μCi ml–1 ^{32}Pi for the indicated times in hours (h). Total lipids were prepared as described in *Experimental procedures*, and separated by TLC in solvent system I before scraping and counting. Closed symbols and solid lines, parent; open symbols and dashed lines, LBE3. Circles are phosphatidylcholine, triangles are phosphatidylglycerol, and squares are phosphatidylethanolamine.

In addition to the *licCA* genes in *T. denticola* and *T. pallidum*, three other putative *licCA* genes have been identified by genomic sequencing, one from *Clostridium perfringens* and two from *Fusobacterium nucleatum* (Fig. 2). LicCA from *C. perfringens* and one of the LicCA's from *F. nucleatum* are predicted to have the extended amino terminal segment that we found necessary for solubility and activity of recombinant LicCA in *T. denticola*. The other LicCA from *F. nucleatum*, however, does not appear to have this extension. Also of interest is that the two longer genes from *C. perfringens* and *F. nucleatum* are altered in a region known to be important for activity in the related GlmU/RmlA superfamily of nucleotidyltransferases (Brown *et al*., 1999; Blankenfeldt *et al*., 2000) (Fig. 2). The alterations leave these two proteins without the critical residues Arg-86 and Lys-96 (numbering for *T. denticola*). In the crystal structures of these nucleotidyltransferases from *E. coli* and *S. pneumoniae* (Brown *et al*., 1999; Kwak *et al*., 2002), these residues are found in a loop which might be flexible enough to allow other residues in the altered, shorter loop to carry out their function. Alternatively, the proteins with the altered catalytic loop may not have active CCT activity, and there may be an alternative function of this portion of the LicCA protein, such as binding phosphocholine. Additional studies in these organisms will be necessary to determine if this region is part of the translated *C. perfringens* and *F. nucleatum* LicCA proteins and whether they have these potential activities.

A CDP-choline pathway for PC biosynthesis would also necessitate the presence of a gene encoding a choline phosphotransferase, which catalyses the last step in which phosphocholine is transferred from CDP-choline to diacylglycerol (Fig. 1). The TP0671 gene in *T. pallidum* is quite similar to the choline- and ethanolaminephosphotransferases of yeast and humans, with the highest similarity in the CDP-alcohol phosphotransferase motif, known to be part of the active site (Williams and McMaster, 1998). A sequence similar to TP0671 can be found in *T. denticola*; the *Treponema* homologues are 49% identical to each other in the amino terminal half, which contains the active site (not shown). Thus, it is reasonable to propose that both of these treponemes encode a choline phosphotransferase, and would have a complete CDP-choline pathway for PC biosynthesis.

Of the relatively small number of prokaryotes that synthesize PC, most have specific symbiotic or pathogenic associations with eukaryotic hosts. In at least some of these microbes, PC synthesized from host-derived choline appears to be important in either bacterial growth or for specific microbe–host interactions (Lopez-Lara and Geiger, 2001). In *T. denticola*, PC does not appear to be essential under *in vitro* culture conditions, and PC incorporation was not a result of the uptake of exogenous PC from serum in the medium. In the absence of a functional *licCA* gene, this organism appears to compensate for the lack of PC by increasing its content of PE. It is possible that this may be due a bias in nutrient availability in the complex culture medium required for growth of this organism. Some other PC-producing bacteria have the ability to modulate membrane phospholipid expression in response to environmental conditions (Tang and Hollingsworth, 1998; Hanada *et al*., 2001; Russell *et al*., 2002). This phenomenon is distinct from the phase-variable expression of phosphocholine in *H. influenzae*, which is produced from the LicC and LicA activity of this organism (Weiser *et al*., 1997) but is consistent with the hypothesis that production of PC or phosphocholine in various eukaryote-associated bacteria is important for bacterial survival in these environments. Further studies are required to understand the unique membrane physiology of treponemes, as well as the potential role of choline metabolites in the interaction between these organisms and host cells.

Experimental procedures

Chemicals

Unless otherwise noted, chemicals were purchased at the highest available purity from Sigma Chemical Co. (St Louis, MO) or Fisher Scientific (Chicago, IL). [^{14}C-methyl]-choline, [^{3}H-methyl]-choline, [^{14}C-methyl]-phosphocholine, [^{14}C-methyl]-CDP-choline, and ^{32}Pi were from Amersham.

Bacterial strains, plasmids and growth conditions

Treponema denticola ATCC 35405 and isogenic mutants were grown and maintained under anaerobic conditions in NOS broth medium as previously described (Haapasalo *et al*., 1991), with erythromycin (40 μg ml^{-1}) added as appropriate. For radiolabelling experiments, anaerobic pouches (Mitsubishi Gas Chemical Company) were used. For allelic replacement mutagenesis, mutants were selected on NOS/GN plates (Chan *et al*., 1997) containing erythromycin (40 μg ml^{-1}) as described previously (Li *et al*., 1996; Fenno *et al*., 1998). Cultures were examined by phase-contrast microscopy for purity and typical strain morphology before use.

Escherichia coli strains JM109 and JM110 (Yanisch-Perron *et al*., 1985) were used for routine subcloning and plasmid preparations, and *E. coli* BL21(DE3) was used for recombinant expression studies. Plasmid vector pSTBlue-1 (Novagen) was used for cloning of PCR fragments. Plasmid vector pET21b (Novagen) was used for expression studies. The *ermF/ermB* cassette from pVA2198 (Fletcher *et al*., 1995) was PCR-amplified using oligonucleotide primers CX247 and CX249 (Table 1) and ligated into the TA cloning site of pSTBlue-1 to yield pSY118, which was used as the source for *ermF/ermB* in constructing plasmids for allelic replacement mutagenesis. *E. coli* strains were grown in LB broth or agar medium supplemented with ampicillin

(50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), chloramphenicol (34 μg ml^{-1}) or erythromycin (200 μg ml^{-1}), as appropriate.

Labelling and analysis of phospholipids

For radiolabelling experiments, the label was added at 1–5 μCi ml^{-1} to log phase *T. denticola* cells, which were then aliquoted, incubated in anaerobic pouches at 37°C, and harvested at the indicated times. At harvest, cells were centrifuged at 4000 g_{max} for 15 min at 4°C. The medium was removed and the pellet was washed by addition of 1 ml of phosphate-buffered saline followed by gentle resuspension, and the centrifugation was repeated. The final cell pellet was resuspended in 1.0 ml H_2O and aliquots were taken for lipid extraction and protein determination (Lowry *et al*., 1951).

Lipids were extracted by the Bligh-Dyer method (Bligh and Dyer, 1959). The aqueous fraction was evaporated to dryness with air and the aqueous metabolites were separated by thin-layer chromatography (TLC) on silica gel G plates in methanol/0.5% NaCl/NH_4OH, 50:50:1. ^{14}C-Labelled standards were also chromatographed, and standards as well as experimental samples were visualized by autoradiography. Radioactive TLC spots were scraped into scintillation vials, then suspended in 0.5 ml of H_2O and 4.5 ml Ecolite (ICN) scintillation fluid. Levels of radioactivity were determined in a Beckman liquid scintillation counter.

The chloroform phase from the lipid extraction was washed by re-extracting with methanol and saline, then evaporated to dryness under N_2. Lipids were dissolved in chloroform/methanol, 2:1 and the lipid classes separated by TLC in three systems: I, silica gel H plates developed in chloroform/2-propanol/methyl acetate/methanol/0.25% KCl/acetic acid, 25:25:25:10:5:4; II, silica gel H plates developed in chloroform/methanol/NH_4OH, 45:45:4; III, silica gel G plates in chloroform/methanol/acetic acid/H_2O, 50:30:8:4. Non-radioactive standards were visualized by exposure to iodine vapour and radioactive lipids were visualized by autoradiography.

For determination of phospholipid mass, organic phosphorus was assayed as described (Ames, 1967). Silica gel containing lipids was scraped from TLC plates and the lipids were eluted from the silica gel in chloroform/methanol/acetic acid/H_2O, 50:30:8:4 and methanol. The solvents were then evaporated before organic phosphorus determination.

Enzymatic activity assays

Choline kinase (Gee and Kent, 2003) and CCT (Morand and Kent, 1989) were assayed as described. Peptidase activities of *T. denticola* parent and mutant strains were tested by hydrolysis of chromogenic substrates succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPFNA) and N-α-benzoyl-L-arginine-*p*-nitroanilide (BA*p*NA) as described previously (Fenno *et al*., 2001).

Recombinant DNA methods

Unless stated otherwise, standard methods found in Ausubel *et al*. (1995) or Sambrook *et al*. (1989) were followed. DNA fragments were eluted from agarose gels using the Gene Clean II kit (QBiogene, La Jolla, CA). Genomic DNA′s and plasmid DNA′s were isolated using the Wizard Genomic DNA Purification Kit and Wizard Plus SV Minipreps Kit (Promega, Madison, WI) respectively. Oligonucleotide primers (Invitrogen, Carlsbad, CA) were designed using the GeneFisher algorithm (Giegerich *et al*., 1996) or Primer Express software (Perkin-Elmer Applied Biosystems).

Cloning of licCA

The *licCA* region identified in preliminary unannotated contigs of the *T. denticola* genome sequence (http://www.tigrblast.tigr.org/ufmg) was amplified from *T. denticola* genomic DNA with oligonucleotide primers CX226 and CX227 (Table 2). The 4.4 kb PCR product, including approximately 1.3 kb upstream of *licCA* and 1.5 kb downstream of the *licCA* stop codon, was cloned in pSTBlue-1, yielding pSY107. For expression studies, DNA fragments of interest were cloned by PCR with Vent polymerase, with pSY107 as template. Fragments were cloned for optimal expression from the T7 promoter in pET21b. Full-length *licCA* was cloned with forward primer EX10 and reverse primer EX11 (Table 2). The *licC* portion, C308, was cloned as the first 308 residues of the *licCA* gene, with EX10 as forward primer and EX12 as reverse primer. The *licA* portion, A317, was cloned with forward primer EX13, which added an ATG codon in front of codon 317, and was extended to the end of the *licCA* gene with reverse primer EX11. For expression, plasmid-containing cells were grown in LB media to an optical density of 0.8,

Table 2. Oligonucleotide primers used in this study.

Primer	Target[a]	Sequence	
CX226	5′ to *licCA*	(F)	5′ dACC CAT ACC TGC TTC ATT C 3′
CX227	Beyond *licCA* 3′end	(R)	5′ dCTA CCT ATA CCC TCC GTT ATG 3′
CX247	*ermF/ermB* 5′end	(F)	5′ dGGC ATA TGC GAT AGC TTC CGC TAT TG 3′
CX249	*ermF/ermB* 3′end	(R)	5′ dGGC ATA TGA GCT GTC AGT AGT ATA CC 3′
CX329	*licCA*	(F)	5′ dGGA TGC CCG TAA TCC TGA AGA 3′
CX330	*licCA*	(R)	5′ dCGT TCA CGT AGA TCA AAA GAA TGC 3′
CX323	16S rRNA	(F)	5′ dAGG GAT ATG GCA GCG TAG CA 3′
CX324	16S rRNA	(R)	5′ dTTG CGG GAC TTA ACC CAA CA 3′
EX10	licCA	(F)	5′ dCG CGG ATC CAT ATG AAA AGA AGA TAT TTT CAA ATT ATA AAA CTT ATG
EX11	licCA	(R)	5′ dCCG CTC GAG TCA TAG ATT ACC TCC TAG TTC TTT TAT TTT TTT ATA ATA ATC c
EX12	C308	(R)	5′ dCCG CTC GAG TCA CTC AAG CCA TTT ATC ATG GGA GG
EX13	A317	(F)	5′ dcgc GGA TCC CAT ATG CCT GAG CAC AGC ATC

a. Orientation of the primer (F, forward; R, reverse) with respect to gene of interest.

then induced with 1 mM isopropylthiogalactopyranoside. LicCA was induced for 3 h at 37°C, and C308 and A317 were induced for 20 h at 25°C. Cell pellets were stored at −20°C.

Quantitative RT-PCR

Total RNA was extracted from cultures harvested during active growth (optical density at 600 nm of 0.2) using the RNeasy Mini Kit (Qiagen). RNA samples were reversed transcribed from random primers using SuperScript™ First-Strand Synthesis System (Invitrogen). One microlitre of the resulting cDNA was amplified using QuantiTectTM SYBR Green PCR (Qiagen) in a 25 μl reaction, using *licCA* primers CX329 and 330. Amplification of 16SrRNA with primers CX323 and 324 served as an internal control (Table 2). Thermal cycling was performed in an iCycler iQ™ Multi-Color Real Time PCR Detection System (Bio-Rad) at 95°C for 15 min, and followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s.

DNA sequence analysis

DNA sequencing of expression plasmids was performed by the Molecular Biology Core, University of Michigan Medical Center. Analysis of DNA sequence data was performed using SeqEd 1.0 (Applied Biosystems) and DNA Strider 1.3 (Service de Biochimie, Department de Biologie, Institut de Recherche Fondamentale Commissariat a l'Energie Atomique, Saclay, France). The non-redundant SWISS-PROT, PIR, EMBL and GenBank databases were searched for homologous peptide and nucleotide sequences using the BLAST (Altschul *et al*., 1990) network service at the National Center for Biotechnology Information, National Institutes of Health, USA.

Allelic replacement mutagenesis

Isogenic defined mutants were constructed as described previously (Lee *et al*., 2002). Briefly, *T. denticola* was electroporated with linear DNA consisting of the selectable *ermF/ermB* gene cassette (Fletcher *et al*., 1995) cloned between fragments of the target sequence. DNA fragments to be introduced into *T. denticola* were UV-irradiated at 25 mJ/cm as described by Picardeau *et al*. (2001) before electroporation. Following 24 h recovery in NOS broth, mutants were selected by growth in NOS/GN agar containing erythromycin (40 μg ml^{-1}).

Protein production and purification

All procedures were performed at 4°C. Cell pellets from 250 ml cultures were resuspended in 2.0 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 30 mM NaCl, 2 mM dithiothreitol, 0.2 mM EDTA, 2.5 μg ml^{-1} leupeptin, 2 μg ml^{-1} chymostatin, 2 μg ml^{-1} pepstatin, 1 μg ml^{-1} antipain, 10 μg ml^{-1} *p*-aminobenzamidine, 10 μg ml^{-1} benzamidine, and 0.2 mM phenylmethylsulphonylfluoride). The cells were disrupted by three 30 s intervals of sonication. The cell lysate was centrifuged at 330 000 *g* for 25 min to produce a cleared lysate.

The LicCA cleared lysate was passed over a 2-ml CM-Sepharose column (Pharmacia) equilibrated with buffer D (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 1 mM 2-mercaptoethanol) and eluted with 0.5 M KCl in buffer D. The protein was diluted 200-fold with buffer D, then passed over a 1-ml Blue-Sepharose column equilibrated with buffer D and eluted with 1 M KCl in buffer D. The C308 cleared lysate was passed over a 0.5-ml TMAE column (EM Science) were equilibrated with buffer D, The flow-through was collected and saturated ammonium sulphate was added to 60% saturation. The precipitate was removed by centrifugation for 10 min at 18 000 *g*, then the supernatant was brought to 75% saturation and the precipitation repeated. The 75% pellet was dissolved in buffer D and the C308 protein was further purified by gel filtration on Sephacryl-S100. The A317 cleared lysate was passed over a 1-ml Decyl-Sepharose column (Pharmacia) equilibrated with buffer D containing 1 M KCl and eluted in 100 ml buffer D. The eluate was passed over a 0.5-ml TMAE column and eluted with 0.5 M KCl in buffer D. In initial purification of each protein, fractions containing significant protein peaks were assayed by SDS-PAGE and enzymatic activity to identify the active enzyme peak. In subsequent purifications, the corresponding protein peaks were identified without assaying enzyme activity.

Nucleotide sequence accession number

The nucleotide sequence of *T. denticola licCA* has been assigned GenBank accession number AY322155.

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References

Albelo, S.T., and Domenech, C.E. (1997) Carbons from choline present in the phospholipids of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **156:** 271–274.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Ames, B.N. (1967) Assay of inorganic phosphate, total phosphate, and phosphatases. *Methods Enz* **10:** 115–118.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds). (1995) *Current Protocols in Molecular Biology*. New York: Wiley-Interscience.

Barbieri, J.T., Austin, F.E., and Cox, C.D. (1981) Distribution of glucose incorporated into macromolecular material by *Treponema pallidum*. *Infect Immun* **31:** 1071–1077.

Belisle, J.T., Brandt, M.E., Radolf, J.D., and Norgard, M.V. (1994) Fatty acids of *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins. *J Bacteriol* **176:** 2151–2157.

Blankenfeldt, W., Asuncion, M., Lam, J.S., and Naismith, J.H. (2000) The structural basis of the catalytic mechanism and regulation of glucose-1-phosphate thymidylyltransferase (RmlA). *EMBO J* **19:** 6652–6663.

Bligh, E.G., and Dyer, W.J. (1959) A rapid method of total lipd extraction and purification. *Can J Biochem Physiol* **37:** 911–917.

Brown, K., Pompeo, F., Dixon, S., Mengin-Lecreulx, D., Cambillau, C., and Bourne, Y. (1999) Crystal structure of the bifunctional N-acetylglucosamine 1-phosphate uridyltransferase from *Escherichia coli*: a paradigm for the related pyrophosphorylase superfamily. *EMBO J* **18:** 4096–4107.

Campbell, H.A., and Kent, C. (2001) The CTP: phosphocholine cytidylyltransferase encoded by the *licC* gene of *Streptococcus pneumoniae*: cloning, expression, purification, and characterization (1). *Biochim Biophys Acta* **1534:** 85–95.

Chan, E.C.S., DeCiccio, A., McLaughlin, R., Klitorinos, A., and Siboo, R. (1997) An inexpensive solid medium for obtaining colony-forming units of oral spirochetes. *Oral Microbiol Immunol* **12:** 372–376.

Fenno, J.C., Wong, G.W., Hannam, P.M., and McBride, B.C. (1998) Mutagenesis of outer membrane virulence determinants of the oral spirochete *Treponema denticola*. *FEMS Microbiol Lett* **163:** 209–215.

Fenno, J.C., Lee, S.Y., Bayer, C.H., and Ning, Y. (2001) The *opdB* locus encodes the trypsin-like peptidase activity of *Treponema denticola*. *Infect Immun* **69:** 6193–6200.

Fletcher, H.M., Schenkein, H.A., Morgan, R.M., Bailey, K.A., Berry, C.R., and Macrina, F.L. (1995) Virulence of a *Porphyromonas gingivalis* W83 mutant defective in the *prtH* gene. *Infection Immunity* **63:** 1521–1528.

Fraser, C.M., Norris, S.J., Weinstock, G.M., White, O., Sutton, G.G., Dodson, R., *et al.* (1998) Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. *Science* **281:** 375–388.

Gee, P., and Kent, C. (2003) Multiple isoforms of choline kinase from *Caenorhabditis elegans*: cloning, expression, purification, and characterization. *Biochim Biophys Acta* **1648:** 33–42.

Giegerich, R., Meyer, F., and Schleiermacher, C. (1996) GeneFisher – software support for the detection of postulated genes. *Proc Int Conf Intell Syst Mol Biol* **4:** 68–77.

Haapasalo, M., Singh, U., McBride, B.C., and Uitto, V.-J. (1991) Sulfhydryl-dependent attachment of *Treponema denticola* to laminin and other proteins. *Infect Immun* **59:** 4230–4237.

Hanada, T., Kashima, Y., Kosugi, A., Koizumi, Y., Yanagida, F., and Udaka, S. (2001) A gene encoding phosphatidylethanolamine N-methyltransferase from *Acetobacter aceti* and some properties of its disruptant. *Biosci Biotechnol Biochem* **65:** 2741–2748.

Kaneshiro, T., and Law, J.H. (1964) Phosphatidylcholine synthesis in *Agrobacterium tumefaciens* I. Purification and properties of a phosphatidylethanolamine N-methyltransferase. *J Biol Chem* **239:** 1705–1713.

Kim, K.H., Voelker, D.R., Flocco, M.T., and Carman, G.M. (1998) Expression, purification, and characterization of choline kinase, product of the CKI gene from *Saccharomyces cerevisiae*. *J Biol Chem* **273:** 6844–6852.

Kwak, B.Y., Zhang, Y.M., Yun, M., Heath, R.J., Rock, C.O., Jackowski, S., and Park, H.W. (2002) Structure and mechanism of CTP: phosphocholine cytidylyltransferase (LicC) from *Streptococcus pneumoniae*. *J Biol Chem* **277:** 4343–4350.

Lee, S.Y., Bian, X.L., Wong, G.W., Hannam, P.M., McBride, B.C., and Fenno, J.C. (2002) Cleavage of *Treponema denticola* PrcA polypeptide to yield protease complex-associated proteins Prca1 and Prca2 is dependent on PrtP. *J Bacteriol* **184:** 3864–3870.

Li, H., Ruby, J., Charon, N., and Kuramitsu, H. (1996) Gene inactivation in the oral spirochete *Treponema denticola*: construction of an *flgE* mutant. *J Bacteriol* **178:** 3664–3667.

Livermore, B.P., and Johnson, R.C. (1974) Lipids of the Spirochaetales: comparison of the lipids of several members of the genera *Spirochaeta*, *Treponema*, and *Leptospira*. *J Bacteriol* **120:** 1268–1273.

Lopez-Lara, I.M., and Geiger, O. (2001) Novel pathway for phosphatidylcholine biosynthesis in bacteria associated with eukaryotes. *J Biotechnol* **91:** 211–221.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **193:** 265–275.

Lysenko, E., Richards, J.C., Cox, A.D., Stewart, A., Martin, A., Kapoor, M., and Weiser, J.N. (2000) The position of phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* affects binding and sensitivity to C-reactive protein-mediated killing. *Mol Microbiol* **35:** 234–245.

Matthews, H.M., Yang, T.K., and Jenkin, H.M. (1979) Unique lipid composition of *Treponema pallidum* (Nichols virulent strain). *Infect Immun* **24:** 713–719.

Morand, J.N., and Kent, C. (1989) Localization of the membrane-associated CTP: phosphocholine cytidylyltransferase in Chinese hamster ovary cells with an altered membrane composition. *J Biol Chem* **264:** 13785–13792.

Picardeau, M., Brenot, A., and Saint Girons, I. (2001) First evidence for gene replacement in *Leptospira* spp. Inactivation of *L. biflexa flaB* results in non-motile mutants deficient in endoflagella. *Mol Microbiol* **40:** 189–199.

Porter, T.J., and Kent, C. (1990) Purification and characterization of choline/ethanolamine kinase from rat liver. *J Biol Chem* **265:** 414–422.

Rock, C.O., Heath, R.J., Park, H.W., and Jackowski, S. (2001) The *licC* gene of *Streptococcus pneumoniae* encodes a CTP: phosphocholine cytidylyltransferase. *J Bacteriol* **183:** 4927–4931.

de Rudder, K.E., Sohlenkamp, C., and Geiger, O. (1999) Plant-exuded choline is used for rhizobial membrane lipid biosynthesis by phosphatidylcholine synthase. *J Biol Chem* **274:** 20011–20016.

Russell, N.J., Coleman, J.K., Howard, T.D., Johnston, E., and Cogdell, R.J. (2002) *Rhodopseudomonas acidophila* strain 10050 contains photosynthetic LH2 antenna complexes that are not enriched with phosphatidylglycerol, and the phospholipids have a fatty acyl composition that is unusual for purple non-sulfur bacteria. *Biochim Biophys Acta* **1556:** 247–253.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Smibert, R.M. (1976) Cultivation, composition, and physiol-

ogy of avirulent treponemes. In *The Biology of Parasitic Spirochetes*. Johnson, R.C. (ed). New York: Academic Press, pp. 49–56.

Sohlenkamp, C., Lopez-Lara, I.M., and Geiger, O. (2003) Biosynthesis of phosphatidylcholine in bacteria. *Prog Lipid Res* **42:** 115–162.

Tang, Y., and Hollingsworth, R.I. (1998) Regulation of lipid synthesis in *Bradyrhizobium japonicum*: low oxygen concentrations trigger phosphatidylinositol biosynthesis. *Appl Environ Microbiol* **64:** 1963–1966.

Weiser, J.N., Shchepetov, M., and Chong, S.T. (1997) Decoration of lipopolysaccharide with phosphorylcholine: a phase- variable characteristic of *Haemophilus influenzae*. *Infect Immun* **65:** 943–950.

Williams, J.G., and McMaster, C.R. (1998) Scanning alanine mutagenesis of the CDP-alcohol phosphotransferase motif of *Saccharomyces cerevisiae* cholinephosphotransferase. *J Biol Chem* **273:** 13482–13487.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33:** 103–119.

Zhang, J.R., Idanpaan-Heikkila, I., Fischer, W., and Tuomanen, E.I. (1999) Pneumococcal *licD2* gene is involved in phosphorylcholine metabolism. *Mol Microbiol* **31:** 1477–1488.