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}\text{C}]\text{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}\text{C}]\text{choline}\ or \[^{32}\text{P}]\text{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* (Albedo and Domenech, 1997) or may be a major lipid component as in *Acetobacter acetii* (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 *lic* 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...
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 gas 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), 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 [14C]-choline or 32P 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 [3H]-choline (not shown) or [14C]-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% ([3H]) or 99% ([14C]) 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 (5'GAGGAAGTG...). 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 (5'GAGGAAGTG...), resulted in a protein that was soluble and active. The protein was purified to near homo-
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 geneity (Fig. 5) and was active in assays for both choline kinase and CCT (Table 1).

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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).

**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 *Bst*Z17I-*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.**

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>Cytidylyltransferase activity (nmol min⁻¹ mg protein⁻¹)</th>
<th>Choline kinase Activity (nmol min⁻¹ mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LicCA</td>
<td>813 ± 9</td>
<td>228 ± 6</td>
</tr>
<tr>
<td>C308</td>
<td>&lt;1</td>
<td>NA</td>
</tr>
<tr>
<td>A317</td>
<td>NA</td>
<td>1100 ± 96</td>
</tr>
</tbody>
</table>

NA: not applicable.

Values are the averages and ranges of determinations from two separate protein preparations.

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 [14C]choline into lipids and 32Pi into phosphatidylycerine (Figs 4 and 7A). In addition, incorporation of [14C]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 [14C]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 32Pi incorporation was carried out (Fig. 8). As expected, essentially no 32Pi was incorporated into PC in the mutant. The rate of incorporation of 32Pi into PG was increased by about 50%, although there was not a signif-
significant increase in the mass of PG (Fig. 3). The rate of incorporation of 32Pi 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. 7.](image) Chromatography of radiolabelled precursors and lipids. A. Cells were labelled in 5 μCi ml⁻¹ ³²P for 4 h, then ³²P-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 [¹⁴C]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).

![Fig. 8.](image) Incorporation of ³²P into lipids in parent and mutant *T. denticola*. Parent and LBE3 were incubated with 5 μCi ml⁻¹ ³²P 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). [14C-methyl]-choline, [3H-methyl]-choline, [14C-methyl]-phosphocholine, [14C-methyl]-CDP-choline, and 32Pi 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 gmax 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 H2O 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/NH4OH, 50:50:1. 14C-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 H2O 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 N2. 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/acetate acid, 25:25:25:10:5:4; II, silica gel H plates developed in chloroform/methanol/NH4OH, 45:45:4; III, silica gel G plates in chloroform/methanol/acetate acid/H2O, 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 chromatography (TLC) on silica gel G plates in systems: I, silica gel H plates developed in chloroform/methanol, 2:1 and the lipid classes separated by TLC in three systems: II, silica gel H plates developed in chloroform/methanol/NH4OH, 45:45:4; III, silica gel G plates in chloroform/methanol/acetate acid/H2O, 50:30:8:4. Non-radioactive 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 H2O and 4.5 ml Ecolite (ICN) scintillation fluid. Levels of radioactivity were determined in a Beckman liquid scintillation counter.

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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/acetate acid/H2O, 50:30:8:4 and methanol. The solvents were then evaporated before organic phosphorus determination.

Table 2. Oligonucleotide primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target*</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CX226</td>
<td>5’ to licCA</td>
<td>5’ dACC CAT ACC TGC TTC ATT C 3’</td>
</tr>
<tr>
<td>CX227</td>
<td>Beyond licCA 3’ end</td>
<td>5’ dCTA CCT ATA CCC TCC GTT ATG 3’</td>
</tr>
<tr>
<td>CX247</td>
<td>ermF/ermB 5’ end</td>
<td>5’ dGGA TGC CCG TAA TCC TGA AGA 3’</td>
</tr>
<tr>
<td>CX249</td>
<td>ermF/ermB 3’ end</td>
<td>5’ dGGA TGA TGA GCT GTG AGT ATA CC 3’</td>
</tr>
<tr>
<td>CX329</td>
<td>licCA</td>
<td>5’ dGGC ATA TGC GAC TCG TCG TAT G 3’</td>
</tr>
<tr>
<td>CX330</td>
<td>licCA</td>
<td>5’ dGGG TTA TCC TCA TGA TCG TAT G 3’</td>
</tr>
<tr>
<td>CX332</td>
<td>16S RNA</td>
<td>5’ dCTG CCG GAT ATG AAG GAA TGC GAT G 3’</td>
</tr>
<tr>
<td>CX324</td>
<td>16S rRNA</td>
<td>5’ dGGC GAT ATG AGC TGA TCA TGA 3’</td>
</tr>
<tr>
<td>EX10</td>
<td>licCA</td>
<td>5’ dGAA TCC TGA TGA TGA TCG TAT G 3’</td>
</tr>
<tr>
<td>EX11</td>
<td>licCA</td>
<td>5’ dGGA TCC TGA TGA TGA TCG TAT G 3’</td>
</tr>
<tr>
<td>EX12</td>
<td>C308</td>
<td>5’ dGAA TCC TGA TGA TGA TCG TAT G 3’</td>
</tr>
<tr>
<td>EX13</td>
<td>A317</td>
<td>5’ dGAA TCC TGA TGA TGA TCG TAT G 3’</td>
</tr>
</tbody>
</table>

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

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 (BApNA) 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/ulmg) 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 licCA 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,
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 microtitre of the resulting cDNA was amplified using QuantiTect™ SYBR Green PCR (Qiagen) in a 25 μl reaction, using licCA primers CX329 and 330. Amplification of 18SrRNA 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


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