

# Phospholipid synthesis in *Borrelia burgdorferi*: BB0249 and BB0721 encode functional phosphatidylcholine synthase and phosphatidylglycerolphosphate synthase proteins

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Phospholipids are an important component of bacterial membranes. *Borrelia burgdorferi* differs from many other bacteria in that it contains only two major membrane phospholipids: phosphatidylglycerol (PG) and phosphatidylcholine (PC). *B. burgdorferi* appears to lack enzymes required for synthesis of PC through the well-described methylation pathway. However, *B. burgdorferi* does contain a gene (BB0249) with significant identity to a recently described phosphatidylcholine synthase gene (*pcs*) of *Sinorhizobium meliloti*. *B. burgdorferi* also contains a gene (BB0721) with significant identity to the gene (*pgs*) encoding phosphatidylglycerolphosphate synthase, an enzyme in the synthetic pathway of PG. Activity of BB0249 was confirmed by cloning the gene into *Escherichia coli*, which does not produce PC. Transformation with a plasmid carrying BB0249 resulted in production of PC by *E. coli*, but only in the presence of exogenously supplied choline, as would be predicted for a *Pcs*. Because loss of *Pgs* activity is lethal to *E. coli*, activity of BB0721 was confirmed by the ability of BB0721 to complement an *E. coli* *Pgs*<sup>−</sup> mutant. A plasmid containing BB0721 was transformed into a *Pgs*<sup>−</sup> mutant of *E. coli* containing a copy of the native gene on a temperature-regulated plasmid. The temperature-regulated plasmid was exchanged for a plasmid containing BB0721 and it was shown that BB0721 was able to replace the lost *Pgs* function and restore bacterial growth. This study has established the existence and function of two critical enzymes in the synthesis of PC and PG in *B. burgdorferi*. Understanding of the biosynthetic pathways of PC and PG in *B. burgdorferi* is the first step in delineating the role of these phospholipids in the pathogenesis of Lyme disease.

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## INTRODUCTION

Phospholipids are an important component of both prokaryotic and eukaryotic membranes. Most bacterial membranes contain the anionic phospholipids phosphatidylglycerol (PG) and cardiolipin (CL) and the zwitterionic phosphatidylethanolamine (PE) as the major membrane phospholipids. Many other phospholipids, including but not limited to phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylcholine (PC) can be found in smaller amounts or in specific bacteria (Sohlenkamp *et al.*, 2003). The different phospholipids have been shown to participate in many important cellular functions. Foremost among their functions is the ability to act as a permeability barrier for

cells and for organelles by forming phospholipid bilayers. The phospholipid bilayers act as a matrix supporting functions such as protein targeting, DNA replication, solute transport and signal transduction. The individual phospholipids appear to differ in their ability to facilitate certain functions; for example PE exhibits chaperone-like functions in protein folding, whereas PG assists in translocation of proteins across membranes and initiation of DNA replication (Bogdanov & Dowhan, 1995, 1998, 1999; Bogdanov *et al.*, 1996, 1999; Crooke, 2001; de Vrije *et al.*, 1988; Dowhan, 1997).

*Borrelia burgdorferi*, the causative agent of Lyme disease, appears to differ from many other bacteria, including related spirochaetes such as *Treponema pallidum*, in its phospholipid content. *B. burgdorferi* appears to contain only PG and PC as its major membrane phospholipids (Belisle *et al.*, 1994). PC is the major membrane phospholipid found in eukaryotic cells, but it had previously been believed that PC was found in only a very small number of bacteria with highly specialized function – e.g. photosynthetic bacteria

**Abbreviations:** CL, cardiolipin; DAG, diacylglycerol; dH<sub>2</sub>O, distilled water; G3P, glycerol 3-phosphate; PC, phosphatidylcholine; *Pcs*, phosphatidylcholine synthase; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; Pgp, phosphatidylglycerol phosphate; *Pgs*, phosphatidylglycerolphosphate synthase; *Pmt*, phosphatidylmethyltransferase; PS, phosphatidylserine.

containing extensive internal membrane structures or symbiotic bacteria living in close contact with eukaryotic cells (Sohlenkamp *et al.*, 2003). The majority of bacteria synthesize PC using a methylation pathway involving phosphatidylmethyltransferases (Pmt) (Lopez-Lara & Geiger, 2001). In this pathway, CDP-diacylglycerol (CDP-DAG) is firstly condensed with serine by phosphatidylserine synthase to form PS. PS is further converted by phosphatidylserine decarboxylase (Psd) into PE, which is then converted to PC through methylation by PmtA. Previous searches of the *B. burgdorferi* genome have not revealed any ORFs with significant identity to other bacterial Pmts, and *B. burgdorferi* does not appear to contain either PS or PE as intermediaries for PC synthesis.

Geiger and his colleagues have recently described a novel pathway by which some bacteria may generate PC (de Rudder *et al.*, 2000; Lopez-Lara & Geiger, 2001; Sohlenkamp *et al.*, 2000, 2003). In this pathway, which was first described for *Sinorhizobium meliloti*, a phosphatidylcholine synthase (Pcs) directly condenses exogenously acquired choline with CDP-DAG to form PC. Since the description of the *pcs* pathway in *S. meliloti*, paralogues of the gene have been found in a number of other bacteria including *Rhizobium leguminosarum*, *Mesorhizobium loti*, *Brucella melitensis*, *Pseudomonas aeruginosa*, *Legionella pneumophila* and *Borrelia burgdorferi* (Sohlenkamp *et al.*, 2003).

We were interested in understanding phospholipid biosynthesis by *B. burgdorferi*. In addition to a potential gene encoding Pcs, the *B. burgdorferi* genome also contains a potential paralogue of the gene encoding phosphatidylglycerol-phosphate synthase (Pgs), which is the key enzyme in the formation of PG from CDP-DAG, and CDP-DAG synthase (CdsA), which is the key enzyme in the formation of the CDP-DAG that is utilized as substrate by both Pgs and Pcs. Here, we present our studies into the phospholipid composition of *B. burgdorferi* and the activity of the potential *B. burgdorferi* orthologues of Pcs and Pgs.

## METHODS

**Bacterial strains and cultivation.** The borrelial species *B. burgdorferi* (strain B31), *B. coriaceae*, *B. anserina*, *B. hermsii*, *B. parkeri*, *B. turicatae*, *B. garinii* and *B. afzelii* were maintained in BSK H (Sigma) at 37 °C. *E. coli* BL21 CodePlus (DE<sub>3</sub>)-RIL (Stratagene) was cultivated in LB supplemented with 50 µg chloramphenicol ml<sup>-1</sup> at 37 °C. *E. coli* HD38/pHD102, a kind gift of Dr William Dowhan, was grown in LB medium supplemented with 50 µg chloramphenicol ml<sup>-1</sup> and 50 µg tetracycline ml<sup>-1</sup> at 30 °C. *E. coli* Top10 (Invitrogen) was incubated in LB medium at 37 °C.

**Plasmid construction.** Total DNA of *B. burgdorferi* N40 was prepared as previously described (Hinnebusch & Barbour, 1992). The putative *B. burgdorferi* *pcs* (BB0249) and *pgsA* (BB0721) genes were amplified by PCR from *B. burgdorferi* template DNA using the following primer pairs: PcsF (5'-AGCTATACATATGAAAAATATCAATTTGATT-3'), PcsR (5'-TGTGTGCTCGAGTCTATTTCTTGT-TTTATAAG-3'), PgsBF (5'-GAACAGGATCCATGAATAATTTAATCAAGGTCA-3') and PgsSR (5'-ATATTGTGCGACTTATTTATTTT-CATATTTTTTTTAC-3'). The underlined sequences represent the

restriction sites for *Nde*I, *Xho*I, *Bam*HI or *Sal*I. PCR was performed using the following cycling parameters: denaturing at 95 °C for 5 min; followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min; and a final extension phase of 72 °C for 7 min. PCR products (705 bp for BB0249 and 627 bp for BB0721) were restricted, purified from low-melt agarose gels (QiaquickGel Extraction kit, Qiagen), and then ligated into the expression vectors pET21b (Novagen) at *Nde*I and *Xho*I sites and p<sup>tac</sup>85 (Marsh, 1986) at *Bam*HI and *Sal*I sites to form the recombinant pET125 and p<sup>tac</sup>56 respectively. Recombinant plasmids were amplified in *E. coli* Top10 host cells. Positive transformants were initially screened by growing bacterial cells on LB plates supplemented with 100 µg ampicillin ml<sup>-1</sup> for both recombinant pET125 and p<sup>tac</sup>56. Cloning of the appropriate sequences was established by DNA sequencing (Tufts University Core Sequencing Facility).

**Transformation and expression.** pET125 was transformed into chemically competent *E. coli* BL21 CodePlus (DE<sub>3</sub>)-RIL (Stratagene) according to the manufacturer's instructions. BL21/pET125 transformants were screened by growing bacterial cells on LB plates supplemented with 100 µg ampicillin ml<sup>-1</sup> and 50 µg chloramphenicol ml<sup>-1</sup> at 37 °C. p<sup>tac</sup>56 was electroporated into *E. coli* HD38/pHD102 cells prepared according to standard procedures (Sambrook & Russell, 2001). HD38/p<sup>tac</sup>56 transformants were screened by growing bacterial cells on LB plates containing 100 µg ampicillin ml<sup>-1</sup> at 37 °C. Loss of the pHD102 plasmid and replacement with p<sup>tac</sup>56 was confirmed by PCR specific for each plasmid, by restriction digest analysis and by the inability of the HD38/p<sup>tac</sup>56 colonies to grow in the presence of chloramphenicol.

Gene expression from BL21/pET125 transformants was induced by adding IPTG to a final concentration of 0.5 mM. Bacterial cells were incubated at 37 °C unless otherwise stated, and harvested by centrifugation at 2000 g for 20 min at 4 °C.

**Extraction of phospholipids.** For phospholipid preparations, bacterial cells were grown to mid-exponential phase, and harvested by centrifugation at 2000 g for 15 min. Cell pellets were washed once with TBS (50 mM Tris/HCl pH 8.0, 100 mM NaCl) and then twice with distilled water (dH<sub>2</sub>O). Phospholipids were extracted from cell pellets as described by Ames (1968). Briefly, cell pellets were suspended in dH<sub>2</sub>O (0.8 ml dH<sub>2</sub>O per g wet cells), and methanol and chloroform were added to a final ratio of 2:1:0.8 (methanol/chloroform/H<sub>2</sub>O; by vol.). The mixture was rotated at 4 °C for 1 h, after which an additional 1 vol. chloroform and then 1 vol. of dH<sub>2</sub>O were added. The mixture was centrifuged at 2000 g at 4 °C for 30 min. The chloroform phase was transferred to a fresh tube and then vacuum dried. The dried powder was redissolved in chloroform and stored at -20 °C.

**Thin-layer chromatography (TLC).** HPLC silica Gel 60 plates (Whatman) were pre-washed by solvent migration with chloroform/methanol (1:1, v/v) and air-dried in a fume hood. Samples were applied to the plate (approx. 30 µg) and TLC was performed in solvent containing butyl acetate/n-propanol/chloroform/methanol/0.25 % KCl (25:25:28:10:7, by vol.) (Heape *et al.*, 1985). After TLC was completed, the plates were baked in an oven at 100 °C for 5 min, and then sprayed with primuline [1 mg primuline powder (Sigma) in 100 ml acetone/dH<sub>2</sub>O (80:20, v/v)]. The fluorescent spots of phospholipids were viewed and photographed under UV light. Choline was stained specifically using Dragendorff reagent containing 20 ml solution A (0.5 g bismuth nitrate [Bi(NO<sub>3</sub>)<sub>3</sub>·5H<sub>2</sub>O] in 20 % acetic acid), 5 ml 40 % KI and 70 ml dH<sub>2</sub>O.

Phospholipid was recovered from TLC plates by scraping the desired spots from the glass plates. The collected silica was heated to 180 °C after adding 0.2 ml dH<sub>2</sub>O and 0.4 ml perchloric acid. The mixture was allowed to cool and then centrifuged at low speed. The supernatants

were used to determine phosphorus content. Amounts of phospholipid were estimated on the basis of phosphorus content according to the method of Chalvardjian & Rudnicki (1970).

## RESULTS

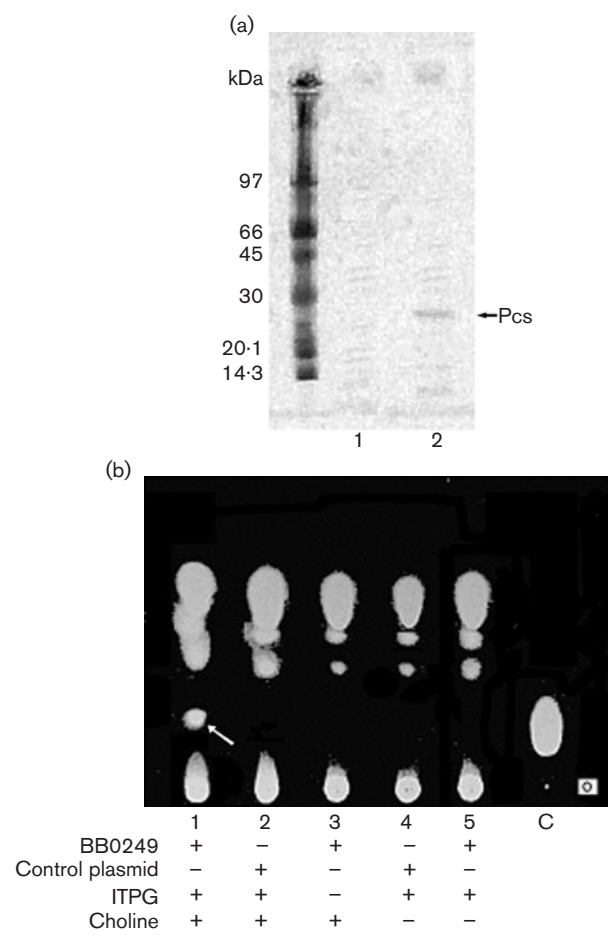
### Phospholipid composition of *Borrelia* species

Belisle *et al.* (1994) have previously established that the *B. burgdorferi* membrane is composed of primarily two phospholipids, PC and PG. We analysed the membrane phospholipids of *B. burgdorferi* using a non-radiolabelled method and compared our results to the published data, which were obtained using radiolabelled substrates. Phospholipids were extracted according to early reported protocols (Ames, 1968) and TLC of the extracts was performed. Staining of the TLC plates confirmed that PC and PG are the major phospholipids in the *B. burgdorferi* membrane (data not shown) and suggested that this method was a reliable method for examining *B. burgdorferi* membrane phospholipids.

We then used this method to determine the phospholipid compositions of seven other *Borrelia* species: two subspecies of *B. burgdorferi sensu lato* (*B. garinii* and *B. afzelii*), three relapsing fever spirochaetes (*B. hermsii*, *B. parkeri* and *B. turicatae*), the bovine pathogen *B. coriaceae*, and the avian pathogen *B. anserina*. All spirochaetes were grown in BSK H at 37°C to the late-exponential phase and membrane phospholipids were extracted and analysed as described above. All of the tested *Borrelia* species appeared to have very similar phospholipid compositions (data not shown). PC with an  $R_F$  value of  $0.13 \pm 0.03$  constituted the majority (68–74%) of the total phospholipids, whereas PG with an  $R_F$  value of  $0.75 \pm 0.08$  made up a smaller component (26–32%) (Fig. 1b). None of the membrane extracts from the borrelian species contained PE. TLC of the lipid extractions revealed an unidentified lipid in all of the tested borrelian strains that ran near the solvent migration front ( $R_F$   $0.95 \pm 0.02$ ). However, tests for phosphate on extractions of these lipids were negative, suggesting that these were not phospholipids.

### *B. burgdorferi* BB0249 functions as a phosphatidylcholine synthase

PC found in other bacterial membranes can be generated either through a pathway involving methylation of PE to PC by Pmt or via a recently described Pcs pathway that utilizes externally derived choline. A search of the *B. burgdorferi* genome did not reveal any genes with significant similarity to bacterial PmtA genes, which code for the enzyme responsible for methylation of PE to generate PC. However, *B. burgdorferi* does have an ORF (BB0249) that showed strong homology to the *pcs* gene of *S. meliloti* (50% similarity and 30% identity). Hydropathy plots for *S. meliloti* and *B. burgdorferi* Pcs are very similar (mean hydropathicity 0.86 and 0.72 respectively). The divergence between the proteins is located mainly at the N-terminus, where the



**Fig. 1.** Transformation of BB0249 into *E. coli*. (a) Expression of BB0249: Coomassie-stained SDS-PAGE gel of cellular lysates from *E. coli* expressing BB0249. The molecular mass of standard proteins is marked on the left. Lane 1, IPTG-induced *E. coli* transformed with pET21b vector alone; lane 2, IPTG-induced *E. coli* transformed with pET125, which carries the insert encoding BB0249. (b) TLC analysis of the total phospholipids extracted from *E. coli* expressing BB0249. *E. coli* containing either pET125 or the control plasmid pET21b was grown in the presence or absence of choline and IPTG as indicated. Cell extracts of total phospholipids were analysed by one-dimensional TLC in solvent containing butyl acetate/n-propanol/chloroform/methanol/0.25% KCl (25:25:28:10:7, by vol.). O, origin; C, standard PC from Sigma. PC is indicated by the white arrow.

predicted *B. burgdorferi* protein is more hydrophobic than that of *S. meliloti*. This difference appears to be due to the loss of seven residues at the N-terminus, four of which are polar amino acids.

To confirm the function of BB0249 as a Pcs, we cloned BB0249 from *B. burgdorferi* (strain B31) into the expression vector pET21b to form the recombinant plasmid pET125. pET125 was transformed into *E. coli* BL21 CodePlus (DE<sub>3</sub>)-RIL. This strain was chosen as the host strain because it

contains extra copies of the *argU*, *ileY* and *leuW* tRNA genes to correct for codon bias when expressing AT-rich borrelial genes. Coomassie staining of cellular lysates of transformed *E. coli* run on SDS-PAGE (Fig. 1a) showed a new protein band at the predicted migration position for the putative Pcs ( $\sim 27$  kDa).

It has previously been shown that *E. coli* does not synthesize PC (Ames, 1968; Sohlenkamp *et al.*, 2003). However, *E. coli* does synthesize CDP-DAG, which it utilizes to form PG; CDP-DAG can also be utilized by Pcs to form PC. We compared the membrane phospholipid content of *E. coli* transformed by pET125 with *E. coli* transformed with vector alone. As predicted, *E. coli* that did not express the putative *B. burgdorferi* Pcs did not contain PC. The membranes of *E. coli* transformed with pET125 contained a new phospholipid with a migration on TLC that matched that of PC. This phospholipid was only present in transformed *E. coli* when choline was supplied in the culture medium (Fig. 1b). Without supplementation of additional choline in the medium, PC was undetectable even in *E. coli* transformed by pET125. Control *E. coli* did not produce PC, regardless of the addition of choline to the medium.

### ***B. burgdorferi* BB0721 functions as a phosphatidylglycerolphosphate synthase**

PG is the other major phospholipid found in borrelial membranes. In other bacteria, biosynthesis of PG is catalysed by two enzymes, phosphatidylglycerolphosphate synthase (Pgs) and phosphatidylglycerol phosphatase (Pgp). In this pathway, conversion of CDP-DAG and glycerol 3-phosphate (G3P) to form phosphatidylglycerolphosphate (PGP) by Pgs is the rate-limiting step. By CLUSTAL W multiple sequence alignment, the predicted protein for BB0721 has slight homology to Pgs of *Bacillus subtilis*, *Treponema pallidum*, *Mycoplasma genitalium* and *Mycoplasma pneumoniae* (16 % similarity and 8 % identity). However, BB0721 possesses a conserved CDP-alcohol phosphatidyltransferase motif, DG(X)<sub>2</sub>AR(X)<sub>8</sub>G(X)<sub>3</sub>D(X)<sub>3</sub>D (Sohlenkamp *et al.*, 2003; Williams & McMaster, 1998), at amino acid positions 57–80, which is characteristic for other Pgs enzymes. Thus, despite the low similarity, it is possible that BB0721 may function as a Pgs converting CDP-DAG and G3P to generate PGP.

To ascertain whether BB0721 functions as a Pgs, we cloned and inserted BB0721 into the expression vector p<sup>tac</sup>85 at *Bam*HI and *Sal*I restriction sites to form the recombinant plasmid p<sup>tac</sup>56. This plasmid was transformed into *E. coli* HD38/pHD102. *E. coli* HD38 is a PgsA<sup>−</sup> strain that is incapable of synthesizing PG and CL. Because loss of PgsA is a lethal mutation, viability of HD38 is dependent upon the presence of a functional copy of the *pgsA* gene carried by the plasmid pHD102. Replication of pHD102 is temperature sensitive, with replication decreasing at temperatures above 30 °C. Cell growth ceases at temperatures restrictive for plasmid replication and the level of PG drops below 10 % of wild-type levels (Heacock & Dowhan, 1987). HD38/p<sup>tac</sup>56

transformants were successfully selected after growth on plates containing 100 µg ampicillin ml<sup>−1</sup> and 50 µg kanamycin ml<sup>−1</sup>. Confirmation of the acquisition of p<sup>tac</sup>56 was confirmed by the ability of transformed strains to grow in the presence of ampicillin but not in the presence of chloramphenicol, by restriction fragment analysis of purified plasmid preparations, and by PCR for each plasmid. These results indicate that the isolated HD38 transformants contained only the p<sup>tac</sup>56 plasmid.

The ability of BB0721 to replace the function of *E. coli* PgsA in the PgsA<sup>−</sup> mutants was determined by comparing growth curves for HD38/p<sup>tac</sup>56, HD38/pHD102 and wild-type *E. coli*. Bacteria were grown overnight at 37 °C and then inoculated into 50 ml fresh LB medium supplemented with the appropriate antibiotics and allowed to incubate at 43 °C. As previously reported, HD38/pHD102 grew slowly for the first 4 h after the shift to 43 °C (representing utilization of previously expressed PgsA) and then essentially ceased replication. In contrast, HD38/p<sup>tac</sup>56 continued to grow steadily at 43 °C, although the growth rate did not match that of wild-type *E. coli* (Top 10). After 8 h of growth at 43 °C, the cell density of HD38/p<sup>tac</sup>56 approached  $7 \times 10^8$  cells ml<sup>−1</sup>, which was 4.3-fold higher than that ( $1.6 \times 10^8$  cells ml<sup>−1</sup>) for HD38/pHD102 (Fig. 2a). The ability of the cells to continue to replicate, which does not occur in the absence of a functional PgsA, suggests that BB0721 inserted into p<sup>tac</sup>56 is able to compensate for the deficiency of the *pgsA* gene in HD38 cells and allow for synthesis of PG. Although HD38/p<sup>tac</sup>56 cells grown at 43 °C did not grow at the same rate as wild-type *E. coli*, HD38/p<sup>tac</sup>56 showed growth equivalent to wild-type *E. coli* when grown at either 30 °C or 37 °C.

To further confirm the Pgs activity of BB0721, we analysed the phospholipid compositions of both HD38/p<sup>tac</sup>56 and HD38/pHD102 growing at 30 °C and 43 °C (Fig. 2b). When bacterial cells were grown at 30 °C, no significant differences in the amount of PG and CL were found between HD38/p<sup>tac</sup>56 and HD38/pHD102. The acidic phospholipids PG and CL constituted approximately 25 % of total phospholipids in both cases. However, when the bacterial strains were grown at 43 °C for 9 h, the amount of PG and CL in HD38/pHD102 dropped to less than 5 % of total phospholipids; in contrast, the percentage of PG and CL in the total phospholipids of HD38/p<sup>tac</sup>56 remained similar to that of cells grown at 30 °C (22 %), consistent with the temperature-independent system of expressing BB0721 in the p<sup>tac</sup> vector.

## **DISCUSSION**

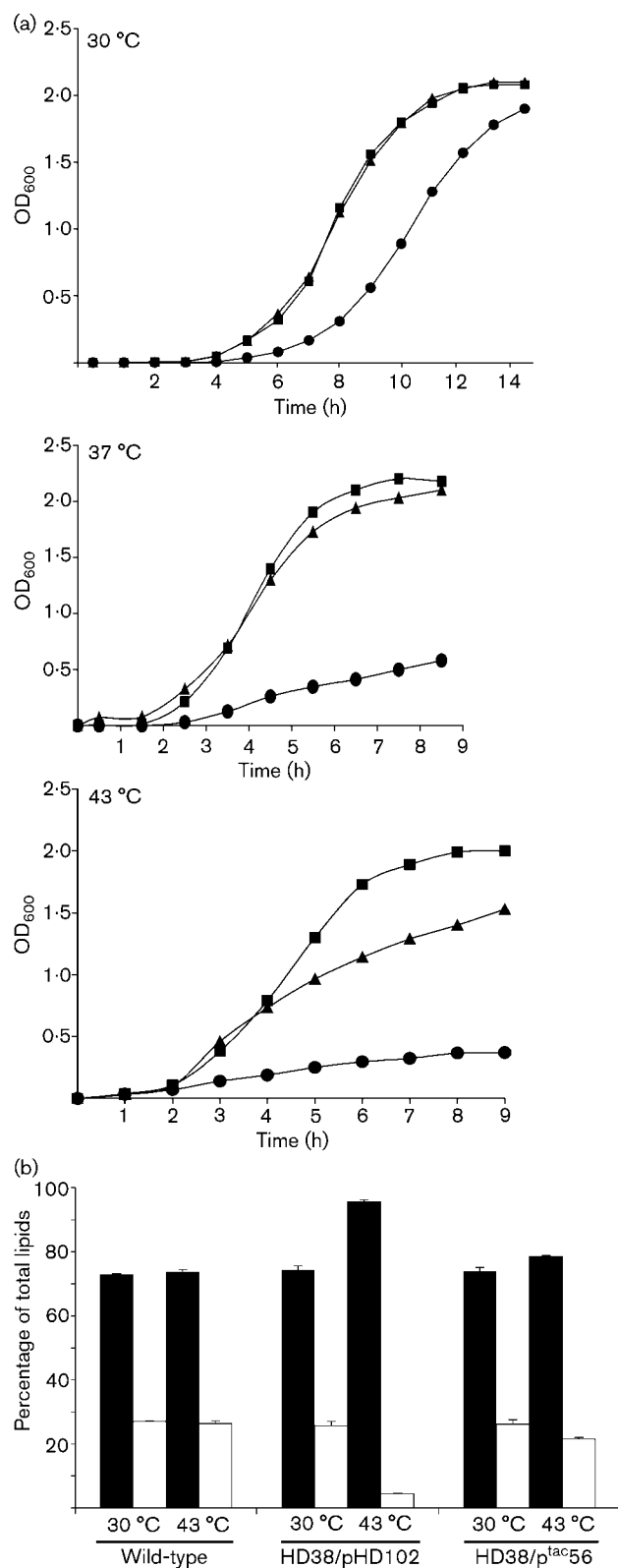
In this study, we attempted to establish the function of enzymes of *B. burgdorferi* that are involved in the synthesis of its major membrane phospholipids PC and PG. We confirmed the function of *B. burgdorferi* coding regions BB0249 and BB0721, which had been identified as genes that could potentially code for proteins with Pcs and PgsA

functions respectively. Introduction of *BB0249* into *E. coli* resulted in the production of PC in non-PC-producing *E. coli* that was dependent upon choline supplementation in

the medium. This clearly demonstrates that *BB0249* encodes a Pcs capable of condensing choline directly with CDP-DAG to yield PC. Based on our analysis of the *B. burgdorferi* genome, we believe that it is likely that the Pcs pathway is the sole mechanism for PC synthesis in *B. burgdorferi*. *B. burgdorferi* lacks both a *pmtA* homologue in its genome and PE, the substrate of Pmt, in its phospholipids, making it highly unlikely that it would be able to synthesize PC through the more common methylation pathway utilized by most other PC-synthesizing bacteria.

To determine the function of the protein encoded by *BB0721*, we expressed the gene (on a temperature-sensitive plasmid) in a *PgsA*<sup>−</sup> *E. coli* mutant. *BB0721* was able to compensate for the deficiency of the chromosomal copy of *pgsA* by restoring both PG production and bacterial growth. Although *BB0721* restored growth in *E. coli* *PgsA*-deficient bacteria to a level equivalent to that of wild-type *E. coli* at 30 °C, growth of *BB0721*-containing *E. coli* at 43 °C did not reach wild-type levels. This may be due to instability or decreased enzymic activity of the borrelial *PgsA* protein at the higher temperature.

The existence of functional Pcs and Pgs in *B. burgdorferi* suggests that PC and PG are generated in a pathway(s) utilizing CDP-DAG. *B. burgdorferi* CdsA (CDP-DAG synthase; BB0119), which converts phosphatidic acid to CDP-DAG, had been putatively identified during the original analysis of the *B. burgdorferi* genome. Cds transfers a CMP moiety from CTP to phosphatidic acid to form an intermediate precursor CDP-DAG. Then Pcs condenses choline directly with CDP-DAG to form PC and releases CMP. Meanwhile, Pgs catalyses the formation of PGP via the transfer of G3P to CDP-DAG and releasing CMP. PGP can be further converted into PG by Pgp. We have not identified a *pgp* gene locus in the *B. burgdorferi* genome; however, our search was limited because only a small number of different Pgp enzymes have been identified to date and critical motifs have not yet been determined. Thus, we cannot rule out the possibility of a *B. burgdorferi* Pgp that is not identified by current amino acid search engines.



**Fig. 2.** Effect of transformation of an *E. coli* *PgsA*<sup>−</sup> mutant with *BB0721*. (a) Growth curves of HD38/p<sup>tac</sup>56 (*B. burgdorferi* *BB0721*) and HD38/pHD102 (*E. coli* *pgsA*) at 30, 37 and 43 °C. Each point represents the mean of duplicate samples. Each experiment was repeated three times with similar results; a representative graph is shown. ●, HD38/pHD102; ■, wild-type *E. coli*; ▲, HD38/p<sup>tac</sup>56. (b) Analysis of the phospholipid content of HD38/p<sup>tac</sup>56 at 30 °C and 43 °C. The percentage of PE and anionic phospholipids (PG and CL) in total phospholipids of HD38/p<sup>tac</sup>56, HD38/pHD102 and wild-type strains was examined by extraction of phospholipids from TLC plates. The phospholipids were quantified using the method of Chalvardjian & Rudnicki (1970). Each bar represents the mean of three separate experiments. Error bars represent standard error.

The importance of maintaining a membrane structure with PC and PG as the primary phospholipids remains to be determined. Individual lipid composition may play a role in the stability and integrity of membrane lipids (Raetz & Dowhan, 1990). Both PC and PG are bilayer-forming phospholipids. How *B. burgdorferi* compensates for the lack of non-bilayer-forming phospholipids such as PE which are critical to cell viability in bacteria such as *E. coli* remains unknown. It is possible that this role may be compensated for by borrelial glycolipids, but further work will be needed to establish this.

Bacteria have been shown to adjust their membrane phospholipid composition in response to environmental changes such as oxygen tension (Tang & Hollingsworth, 1998). *B. burgdorferi* undergoes a complex lifecycle involving both ticks and mammals, where many variables, including oxygen tension, temperature and nutrient availability, vary tremendously. Phospholipid composition of *B. burgdorferi* in its tick and mammalian hosts is not currently known. Changes in phospholipid composition have the potential to affect the activity of cytoplasmic and periplasmic proteins (Dowhan, 1997) which may play a role in host adaptation. The lack of PE and the inability to synthesize PC from PE by methylation means that *B. burgdorferi* is entirely dependent upon acquisition of exogenous choline from its hosts to synthesize PC. Free choline is readily available in eukaryotic fluids, often as a by-product of signalling pathways that activate phospholipases. Availability of choline may play a role in replication and recognition of a eukaryotic host by bacteria. It is also tempting to speculate that, by mimicking the high PC phospholipid content of eukaryotic cells and utilizing choline derived from these cells, PC in *B. burgdorferi* membranes may play a role in host immune evasion and/or in the development of autoimmune responses to the organism. Infection with *Brucella* sp. has resulted in the development of anti-PC antibodies within eukaryotic hosts (Casao *et al.*, 1998). Establishment of the pathways by which *B. burgdorferi* synthesizes PC and PG is the first step towards a clearer understanding of the role of these phospholipids. The relative paucity of different phospholipids in *B. burgdorferi*, and the existence of only a single pathway for synthesizing PC in this organism, presents opportunities for better understanding of the role of PC, which has not been as extensively studied because it is absent in the model Gram-negative and Gram-positive organisms *E. coli* and *Bacillus subtilis*. Recent advances in the tools available for genetic manipulation of *B. burgdorferi* may eventually allow us to better dissect the roles of these phospholipids in disease pathogenesis.

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