Role of anionic phospholipids in the adaptation of *Bacillus subtilis* to high salinity

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The importance of the content of anionic phospholipids [cardiolipin (CL) and phosphatidylglycerol (PG)] in the osmotic adaptation and in the membrane structure of *Bacillus subtilis* cultures was investigated. Insertion mutations in the three putative cardiolipin synthase genes (*ywiE*, *ywnE* and *ywjE*) were obtained. Only the *ywnE* mutation resulted in a complete deficiency in cardiolipin and thus corresponds to a true *clsA* gene. The osmotolerance of a *clsA* mutant was impaired: although at NaCl concentrations lower than 1·2 M the growth curves were similar to those of its wild-type control, at 1·5 M NaCl (LBN medium) the lag period increased and the maximal optical density reached was lower. The membrane of the *clsA* mutant strain showed an increased PG content, at both exponential and stationary phase, but no trace of CL in either LB or LBN medium. As well as the deficiency in CL synthesis, the *clsA* mutant showed other differences in lipid and fatty acids content compared to the wild-type, suggesting a cross-regulation in membrane lipid pathways, crucial for the maintenance of membrane functionality and integrity. The biophysical characteristics of membranes and large unilamellar vesicles from the wild-type and *clsA* mutant strains were studied by Laurdan's steady-state fluorescence spectroscopy. At physiological temperature, the *clsA* mutant showed a decreased lateral lipid packing in the protein-free vesicles and isolated membranes compared with the wild-type strain. Interestingly, the lateral lipid packing of the membranes of both the wild-type and *clsA* mutant strains increased when they were grown in LBN. In a conditional IPTG-controlled *pgsA* mutant, unable to synthesize PG and CL in the absence of IPTG, the osmoresistance of the cultures correlated with their content of anionic phospholipids. The transcriptional activity of the *clsA* and *pgsA* genes was similar and increased twofold upon entry to stationary phase or under osmotic upshift. Overall, these results support the involvement of the anionic phospholipids in the growth of *B. subtilis* in media containing elevated NaCl concentrations.

INTRODUCTION

During growth in hyperosmotic media containing elevated concentrations of inorganic or organic solutes, bacteria develop several strategies, including changes in the regulation of metabolic pathways and activation of transporters, to overcome both the salt and the osmotic stresses (Wood, 1999; Poolman *et al.*, 2002). Upon modification of the external osmolality of the media the cells change their volume and, among other properties, their membrane tension. Moreover, it has been suggested that a change in the membrane potential and ion gradients might also occur (Poolman *et al.*, 2004). The osmotic response is characterized by two different phases. Among the first responses to the new environmental conditions, the transport of large amounts of K^{+} from the extracellular medium via turgor-responsive transport systems has been described both in enteric Gram-negative bacteria such as *Escherichia coli* and

Salmonella typhimurium and in the Gram-positive *Bacillus subtilis* (McLaggan *et al.*, 1994; Whatmore & Reed, 1990). Along with this response, in enteric bacteria glutamate synthesis is activated in order to provide the counterions for the increased positive charges in the cytoplasm (Yan *et al.*, 1996), while in *B. subtilis* synthesis of proline preferentially occurs (Whatmore *et al.*, 1990). As a second response, both Gram-negative and Gram-positive bacteria accumulate in their cytoplasm large amounts of zwitterionic organic molecules such as glycine betaine, carnitine, choline, proline or ectoine, which are highly soluble molecules, commonly named osmoprotectants (Kempf & Bremer, 1998; Wood, 1999). These compounds are accumulated either through *de novo* synthesis or by active transport from the environment, depending on the medium composition (Whatmore *et al.*, 1990). Osmoprotectants like glycine betaine and proline increase the cytoplasmic volume and the free water content of the cells at high salinity; therefore their accumulation allows cell proliferation under adverse environmental conditions (Record *et al.*, 1998). In the case of *B. subtilis*, a family of transporters (OpuA–E) is involved in the transport of these compounds (Kappes *et al.*, 1996; von Blohn *et al.*, 1997).

Until recently studies concerning the adaptation of cells against osmotic stress have been essentially oriented to genetic and physiological regulation aspects (Csonka & Hanson, 1991; von Blohn *et al.*, 1997; Glassker *et al.*, 1998; Kappes *et al.*, 1996; Price *et al.*, 2001). Recently, a transcriptome analysis of the adaptation of *B. subtilis* cells to high salinity showed that at least 125 genes are induced, thus revealing the complexity of this response (Steil *et al.*, 2003).

The behaviour of bacterial envelopes in the presence of elevated NaCl concentrations in the environment has not been sufficiently explored, in spite of the important traffic of compounds that takes place between the cytoplasm and the external medium. It is to be expected that the stability and permeability of the cell membranes will play a fundamental role in the adaptation to different kinds of stresses and that these properties would be closely related to lipid and fatty acid composition. The cell membrane is the first barrier to an increased ionic strength, and the sensing mechanism would take place in it (Poolman *et al.*, 2002, 2004). In addition, several sigma factors required for extracytoplasmic functions (membrane and wall synthesis) have been shown to respond to stress effectors (Cao *et al.*, 2002). In particular, Horsburgh & Moir (1999) showed that in *B. subtilis*, the *sigM* gene product is required for normal cell growth under salt stress. Moreover, the SigM protein is involved in maintaining the membrane and cell wall integrity in response to different stresses in exponential growth phase and is activated by these stresses (Thackray & Moir, 2003).

Although *B. subtilis* cells are able to resist environmental osmotic changes, in our laboratory we have demonstrated that the osmotic response in this bacterium is mainly a stationary-phase-regulated event that shares common regulators with the sporulation process, DegU-P being the

positive regulator (Ruzal & Sánchez-Rivas, 1994, 1998). We have also found that during growth in hypersaline medium *B. subtilis* cells develop an asymmetric septum similar to that developed by cells in sporulation stage II (Ruzal *et al.*, 1998). However, in these cells the sporulation process is inhibited (Ruzal & Sánchez-Rivas, 1994).

We have been studying for several years the biochemical and biophysical characteristics of the cell envelopes of cells grown under osmotic stress both in *B. subtilis* and in *Lactobacillus casei* (López *et al.*, 1998, 2001, 2002; Piuri *et al.*, 2003; Machado *et al.*, 2004; Piuri *et al.*, 2005). In these studies, the analysis of membrane lipids has revealed that there is a significant increase in the anionic lipid fraction such as phosphatidylglycerol (PG) and especially cardiolipin (CL) when *B. subtilis* cells are grown in LB medium containing 1·5 M NaCl. We have also described an important modification in the fatty acid composition due to this stress (the proportion of straight-chain saturated fatty acids increases while that branched-chain saturated fatty acids decreases). These variations are accompanied by a higher membrane microviscosity (López *et al.*, 2001, 2002). Similar variations in the CL content have also been described in protoplasts of *B. subtilis* due to a polyethylene glycol stress (Sánchez-Rivas & Bohin, 1983), in *Rhodobacter sphaeroides* (Catucci *et al.*, 2004) and in *Mesorhizobium loti* grown under acidic stress (Correa *et al.*, 1999), thus suggesting that an increased CL content might be a general cellular response due to membrane adjustments.

The *in silico* examination of the *B. subtilis* genome sequence has revealed the presence of three putative CL synthases (*ywnE*, *ywiE* and *ywjE*). In addition, a transcriptional microarray analysis of stress responses has indicated that the *ywiE* gene is induced during ethanol or NaCl stress, its promoter being sigma B-dependent, as for most of the genes responding to general stress effectors (Price *et al.*, 2001; Petersohn *et al.*, 2001). Moreover, recently Kawai *et al.* (2004) showed that in the *B. subtilis* Marburg strain, *ywnE* is the gene that plays the main role in CL synthesis and therefore renamed it as *clsA*.

Since our previous experiments showed that there are important changes in the *B. subtilis* membrane composition due to elevated NaCl concentrations in the medium, and in particular in the CL content, we decided to investigate the role of the three putative CL synthase genes in our strain context (YB886 derivative of strain 168 cured of prophages: Yasbin *et al.*, 1980). In addition, a conditional mutant (*pgsA*) for synthesis of PG, the precursor of CL, was also studied. Physiological studies of hyperosmotic adaptation and membrane biochemical behaviour of these mutant strains are reported here.

METHODS

Strains, plasmids and primers. These are listed in Table 1.

Bacterial growth, osmoresistance and sporulation. *B. subtilis* strains were grown with vigorous agitation at 37 °C, in Luria–Bertani

Table 1. Bacterial strains, plasmids and primers

	Characteristics	Source/reference
Bacterial strains		
B. subtilis		
YB886	*trpC2 metB5 SPβ (S) xin-1*	Yasbin *et al.* (1980)
CSL1	YB886, *ywiE*::pMutin-4	This study
CSL2	YB886, *ywnE*::pMutin-4*	This study
CSL3	YB886, *ywjE*::pMutin-4	This study
CSL21	YB886, *amy*::pCL31	This study
PGSA1	YB886, *pgsA*::pMutin-4	This study
SDB110	168, *pgsA*::pMutin-4	K. Matsumoto‡
E. coli		
TG1	*supE hsdΔ5 thi Δ(lac–proAB)*	Laboratory stock
	F′(*traD36 proAB⁺ lacI^q lacZΔM15*)	
Plasmids		
p-Mutin 4	Amp^R Erm^R	Vagner *et al.* (1998)
p-GEM-T	Cloning vector	Promega
pEC20	pMutin-4::*ywnE**	P. Stragier‡
pEC6	Integrative at *amy* (Amp^R Erm^R)	Martin-Verstraete *et al.* (1992)
pCL31	pEC6::(P*ywnE-lacZ*)*	This study
pCL29	pMutin-4::*ywiE*	This study
pywjE	pMutin-4::*ywjE*	F. Hullo‡
Primers	**Sequence†**	
C1	5′-ATA*AAGCTT*AAAGGGAAAGCTGTTTAAG-3′	This study
C2	5′-ATA*GGATCC*TCTCTGCTGATATATTC-3′	This study
C5	5′-GGTTTTAAA*GAATTC*TGCCCG-3′	This study
C6	5′-TTT*GGATCC*TAATTCATCGCC-3′	This study
C7	5′-ATTCTGCTTGATATCTTCATG-3′	This study
PGS	5′-AAATCACACTAGCTAGAATCGC-3′	This study
LZ	5′-AGTACAGCGCGGCTGAAATC-3′	This study

*The *ywnE* sequence corresponds to the *clsA* gene according to Kawai *et al.* (2004) and the present work.
†The underlined sequences indicate restriction sites.
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(LB, tryptone 20 g l⁻¹, yeast extract 10 g l⁻¹, NaCl 10 g l⁻¹) medium (control) or LB medium supplemented with 1·5 M NaCl final concentration (LBN) unless otherwise stated in the text. Solid media were obtained by adding 1·5 % (w/v) agar. The antibiotics used were erythromycin (Erm; 1 μg ml⁻¹) and chloramphenicol (5 μg ml⁻¹).

Growth curves were obtained from cultures grown overnight in aerated LB medium at 37 °C. These cultures were diluted (1/50) in fresh LB medium or LB medium containing different concentrations of NaCl as indicated in the figures. Cellular growth was monitored by measuring OD_{600}.

For the osmoresistance assays on solid media, exponential or early stationary phase cultures grown in LB medium were diluted and plated onto LB (control) and LBN plates and incubated for 2–3 days at 37 °C. Osmoresistance is expressed as the percentage of c.f.u. on LBN relative to the control.

The sporulation efficiency was determined from cultures grown in Schaeffer's sporulation medium (Schaeffer *et al.*, 1965) incubated at 37 °C for 48 h. Serial dilutions in 0·85 % NaCl were heat-treated at

70 °C for 10 min and plated on LB for c.f.u. determination. As control, non-heated cells were also diluted and plated and the percentage sporulation efficiency was calculated as (c.f.u. heat-treated/c.f.u. control) × 100.

Construction of *ywiE*, *ywnE*, *ywjE* and *pgsA* mutants in *B. subtilis* YB886. The *ywiE*, *ywnE* and *ywjE* mutant strains were constructed by using the suicide plasmid pMutin-4 (Vagner *et al.*, 1998) harbouring the promoterless *lacZ* gene.

Chromosomal DNA and plasmid isolations were performed according to Bron (1990). The strain mutated in the *ywiE* gene was constructed as follows. DNA from strain YB886 was used as template for PCR amplification using primers C1 and C2 (Table 1) and containing additional *Eco*RI or *Bam*HI sites. This fragment was ligated to pMutin-4 previously digested with the same enzymes. The resulting plasmid, pCL29, was used to transform *B. subtilis* YB886 competent cells prepared as indicated by Kunst & Rapoport (1995). Since the amplicon contains an internal segment of the *ywiE* gene, Erm^R colonies raised by homologous recombination led to a knockout mutation. The recombinants were checked by Southern blotting (data not shown) and one containing the expected construction, named CSL1, was further studied.

The strain with a mutation in the *ywnE* gene was obtained by using plasmid pEC20 (Table 1), which contains an internal fragment of the *ywnE* gene cloned into pMutin-4. The mutant strain in the *ywjE* gene was obtained using the plasmid pywjE (Table 1). These plasmids were used to transform *B. subtilis* YB886 competent cells and the transformants were selected on plates containing Erm as described above. The Erm^R colonies were analysed by PCR using primers C5 and LZ (Table 1) for the *ywnE* mutant strain and primers C7 and LZ (Table 1) for the *ywjE* mutant strain. Colonies identified with the corresponding mutation were named CSL2 (*ywnE*) and CSL3 (*ywjE*).

Strain CSL21 was constructed as follows. A fragment containing part of the putative *ywnE* promoter (nucleotides -143 to $+460$ relative to the ATG) was amplified by standard PCR using primers C5 and C6 (Table 1) and cloned into pGEM-T. The resulting plasmid was subsequently digested using *Eco*RI and *Bam*HI and the insert was purified and subcloned into the pEC6 suicide vector previously digested with the same enzymes. The resulting plasmid was named pCL31 (Table 1). Using this strategy the *ywnE* promoter was fused to the *lacZ* gene, generating a transcriptional fusion flanked by regions with homology to the *amy* locus in *B. subtilis*. pCL31 was then introduced by transformation into *B. subtilis* competent cells. Since this plasmid cannot replicate in this species, recombinants in which homologous recombination within the *amy* locus had occurred were selected on plates containing chloramphenicol and screened by their failure to degrade starch. One of them was chosen, named CSL21, and analysed.

The *pgsA* mutation was transferred from strain SDB110 into the wild-type strain YB886 by transformation using chromosomal DNA from strain SDB110. In this construction the *pgsA* gene is under the control of the *Pspac* promoter, while the *lacZ* gene is under the control of the *pgsA* promoter. Selection of recombinants was performed on plates supplemented with erythromycin and 0·5 M IPTG. Several colonies were analysed by PCR using primers PGS and LZ (Table 1), allowing the selection of the mutant PGSA1.

β-Galactosidase determination. β-Galactosidase activity was determined as described by Miller (1992) but samples were permeabilized by treatment with lysozyme (10 mg ml^{-1}) and sodium deoxycholate (0·01 %, w/v) at 37 °C for 30 min before the assay.

Lipid extraction and purification. Cultures of the different strains grown under specific conditions as described in each figure were harvested by centrifugation and the pellets used for lipid extraction. Total lipids were extracted according to the original method of Rivas & Luzzati (1969) with some modifications as described by López *et al.* (1998). Polar lipids were separated from neutral lipids by preparative TLC run in hexane/ethyl ether/acetic acid (80 : 20 : 1, by vol.). The fractions corresponding to the polar lipids were scraped off and extracted with chloroform/methanol (2 : 1, v/v). The resulting suspension was shaken and filtered according to Kates (1986). This procedure was repeated twice. Polar lipids were analysed by HPTLC (Merck Art. 5074), in chloroform/methanol/acetic acid (65 : 25 : 6, by vol.). Phospholipids and glycolipids were detected with iodine vapour and identified by comparison with authentic purified standards and by treatment with specific stains. Zones from the plates corresponding to each phospholipid fraction were scraped off according to Kates (1986) and quantified. Lipid phosphorus was determined according to Bartlett (1959), and total carbohydrates were quantified as indicated by Hanson & Phillips (1981).

Fatty acid analysis. Total lipid extract was suspended in 2 ml of a fresh solution of KOH 10 % (w/v) in ethanol and digested at 80 °C for 45 min. Distilled water (1 ml) was added and the aqueous solution extracted three times by shaking with 2 ml hexane. The upper organic phase was discarded and the aqueous layer was acidified with 0·5 ml concentrated HCl and extracted twice with 2 ml hexane.

Free fatty acids were dried under a nitrogen stream and submitted to methyl esterification with 2 ml 2 % H$_2$SO$_4$ in methanol at 60 °C for 120 min under a nitrogen atmosphere. Then 2 ml chloroform and 0·67 ml distilled water were added. The aqueous phase was discarded and the organic phase was washed twice with distilled water. After washing, the organic phase was evaporated under a nitrogen stream, resuspended in hexane, and fatty acid methyl esters analysed by gas chromatography using a GLC-FID HP6890 capillary GC (Hewlett Packard) with an Omegawax Capillary column (30 m × 250 μm, 25 μm nominal) (Supelco 11090-02A). As a standard a mixture of bacterial fatty acid methyl esters was used (Bacterial Acid Methyl Esters Mix 4780-U, Supelco).

Isolation of large unilamellar vesicles (LUVs) and membranes. Multilamellar lipid vesicles were prepared by mixing in glass tubes appropriate amounts of stock solutions of total lipids at 1 mM in chloroform/methanol (2 : 1, v/v) and the fluorescent probe. The solvent was evaporated by nitrogen flux and the dried samples were suspended in buffer (sucrose 0·25 M, EDTA 1 mM, pH 7·0), and vortexed vigorously for 5 min. These multilamellar vesicles were extruded through a polycarbonate filter (100 nm pore diameter) mounted in a miniextruder (Avestin) fitted with two 0·5 ml Hamilton syringes. To obtain the LUVs, the samples were subjected to 13 passes through two filters in tandem. The membranes were isolated according to Lysenko *et al.* (1997). A membrane suspension (1 mM) in 0·25 M sucrose and 1 mM EDTA pH 7·0 was mixed with the fluorescent probe and incubated at room temperature for 30 min.

Fluorescence spectroscopy. 2-Dimethylamino-6-lauroylnaphthalene (Laurdan) was purchased from Molecular Probes. The stock solution was prepared in methanol and the final Laurdan probe/lipid phosphorus ratio was 1/400. To calculate the generalized polarization (GP), different Laurdan emission spectra were acquired by using a fixed excitation wavelength of 330 and 410 nm. The Laurdan excitation spectra were also obtained at two different wavelengths (435 and 490 nm) and the four different series of resulting spectra were used to calculate the GP according to Parasassi *et al.* (1994). For each sample ten excitation and emission spectra were acquired and corrected for the background by subtracting the signal of unlabelled samples. The parameter calculated was $GP_{exc} = (I_{435} - I_{490})/(I_{435} + I_{490})$, where I_{435} and I_{490} are the intensities at each excitation wavelength of 330 and 410 nm, using the fixed emission wavelengths of 435 and 490 nm, respectively. The hydration level was calculated as the GP_{exc} value obtained at 350 nm (Hirsch-Lerner & Barenholz, 1999).

RESULTS

Polar lipids of the wild-type, *ywiE*, *ywnE* and *ywjE* mutant strains

The polar lipid composition of the wild-type YB886, CSL1 (*ywiE*), CSL2 (*ywnE*) and CSL3 (*ywjE*) strains grown in LB medium was analysed by HPTLC. The lipid profile and percentage of the main polar lipids obtained for the CSL1 and CSL3 strains was similar to that obtained with the wild-type strain, indicating that there was no change in the content of the major polar lipids in these mutant strains (data not shown). However, during the exponential growth phase the strain harbouring the interrupted *ywnE* gene (CSL2) showed a complete absence of CL and a major increase in PG content compared to its wild-type control (50 % and 25 % of total phospholipids respectively) (Fig. 1a). Concerning the other two phospholipids present
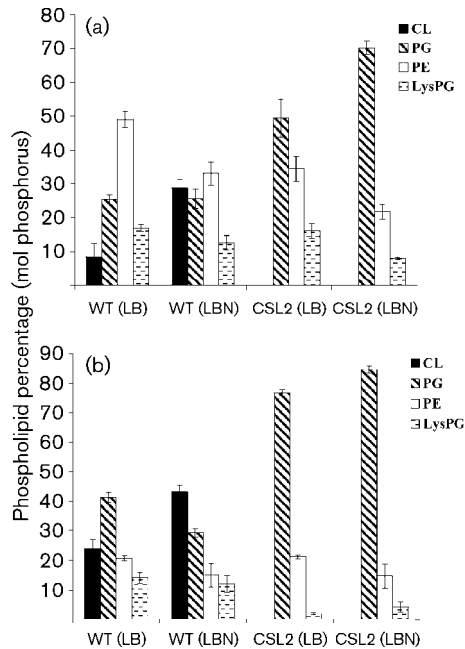
Fig. 1. Phospholipid composition of *B. subtilis* wild-type YB886 and CSL2 mutant strains in (a) exponential phase and (b) stationary phase. The strains were grown in LB and LBN and phospholipids were determined as indicated in Methods. The values represent the mean ± SD of three independent experiments.

in this *B. subtilis* strain, the content of phosphatidylethanolamine (PE) of the CSL2 mutant strain showed a 1·4-fold decrease compared to the wild-type; however, no changes were observed for lysylphosphatidylglycerol (LysPG).

In order to verify if the changes in phospholipid composition described above were specific to the growth phase, we performed the same type of analysis for the wild-type and CSL2 mutant strains grown to stationary phase (Fig. 1b). Interestingly, the results obtained for the wild-type strain indicated that there was an increase of CL in its membrane when the cells reached stationary phase, rising from 8 % to 24 %, suggesting that the presence of CL in the *B. subtilis* membrane is not only dependent on the composition of the media, as we previously described (López *et al.*, 1998), but also on the growth phase (Fig. 1b). In the case of the CSL2 mutant strain, we confirmed that independently of the growth phase (exponential or stationary), this mutant is unable to synthesize CL. Interestingly, we also observed an increased PG content in the CSL2 mutant cells when they reached the stationary phase, rising from 50 % to 80 %, while the content of the other two phospholipids, PE and LysPG, decreased (Fig. 1a, b).

These results clearly show that the interruption of the *ywnE* gene resulted in a complete failure to synthesize the anionic phospholipid CL in the *B. subtilis* YB886 strain context at both stationary and exponential phase. Since this gene encodes the principal CL synthase in this bacterium, from

now on, in accordance with Kawai *et al.* (2004), we will call it *clsA*.

Growth at high NaCl concentration and sporulation efficiency

Since we have previously demonstrated that there is a major increase in the CL content in the *B. subtilis* cells during osmotic stress (López *et al.*, 1998), we wondered whether the absence of this phospholipid would have any effect on the osmotic adaptation. Thus, we obtained growth curves for the CSL2 mutant strain in LB medium supplemented with different concentrations of NaCl ranging from 0·375 to 1·5 M. As shown in Fig. 2, at 0·75 M and 1·0 M NaCl, no differences were observed between the wild-type and CSL2 strains in the lag period, growth rate or maximal OD_{600} reached. In the presence of 1·25 M NaCl, the lag period of the CSL2 mutant strain increased to 8 h, and with 1·5 M NaCl it increased to 12 h. After this extended lag period in the presence of 1·5 M NaCl, the CSL2 strain started to grow, reaching final OD_{600} values 50 % lower than the wild-type strain (data not shown). This result clearly indicates that the absence of CL in the *B. subtilis* membranes, although important for the adaptation to high salinity, is not essential for growth under this condition.

To further investigate the osmoresistance response of the CSL2 strain, cultures were submitted to the plating assay already used in previous studies (Ruzal & Sánchez-Rivas, 1994). Since these studies have shown that the physiological state of the cells is important to withstand the osmotic stress, we analysed the osmotic response of exponential and early stationary phase cultures grown in LB medium. Although we observed that on LB plates containing 1·5 M NaCl the colonies of the CSL2 mutant strain appeared later than those of the wild-type strain, after 48 h incubation at 37 °C their size was similar. These results suggest that the delayed growth of the CSL2 colonies on LBN was due to an extended lag phase, as observed in the growth curves described in Fig. 2. The results obtained in the plate assay (Table 2) indicated that both strains showed the same osmoresistance under the two physiological conditions tested. In addition, as was previously reported, stationary-phase cultures were better able to cope with the ionic strength than exponential-phase cultures, for both the wild-type and the CSL2 mutant.

The sporulation efficiency of the wild-type and CSL2 mutant strains was assayed in Schaeffer's sporulation medium as indicated in Methods. The values obtained for the CSL2 mutant strain were similar to those of the wild-type (85 % and 75 %, respectively), indicating that the *clsA* mutation did not affect the sporulation process.

Phospholipid characterization of the CSL2 cardiolipin null mutant grown in 1·5 M NaCl

Our results showed that the CSL2 strain, which cannot synthesize CL, was able to grow even at elevated NaCl concentrations (1·5 M NaCl) after an extended lag period of
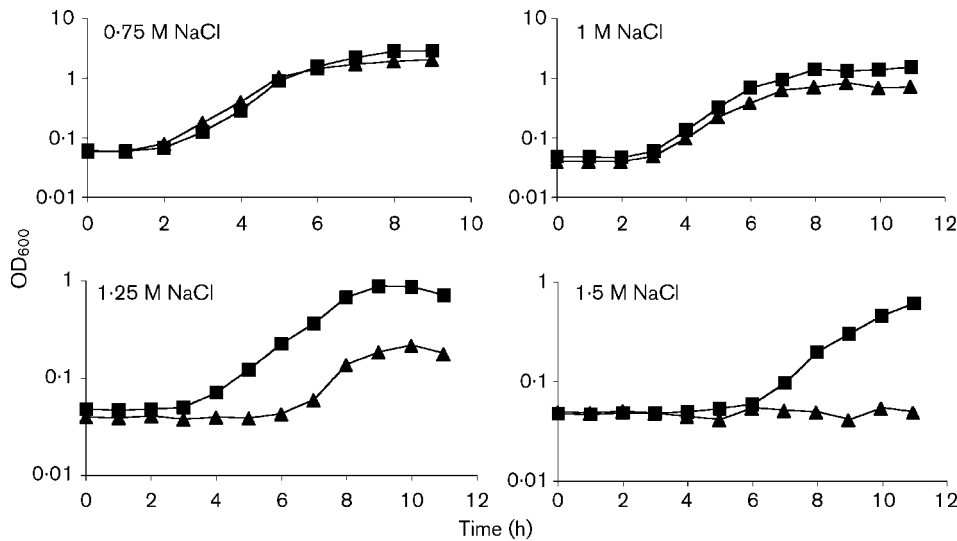
Fig. 2. Growth curves of *B. subtilis* wild-type YB886 (■) and mutant CSL2 (▲) in LB medium supplemented with different NaCl concentrations. Overnight cultures in LB broth were diluted (1 : 50) into fresh LB broth supplemented with 0·75, 1·0, 1·25 and 1·5 M NaCl, and incubated in aerated conditions at 37 °C.

12 h (Fig. 2). However, recently Kawai *et al.* (2004) reported that in *B. subtilis* Marburg a triple mutant of the *cls* homologous genes contains a significant amount of CL during the sporulation phase. In the same report, these authors claimed that in their strain background, this phospholipid could be synthesized by the product of the phosphatidylserine synthase gene (*pss*). Consequently, at least two plausible hypotheses would account for our results: (i) the anionic phospholipid CL is only important for the adaptation of the cells to the new environmental condition but not for their growth (as observed in Fig. 2), or (ii) during the growth of strain CSL2 under high ionic strength conditions the other homologous genes became functional and CL is synthesized. In order to challenge these hypotheses, the phospholipid composition of strain CSL2 grown in LBN was analysed and

compared with that obtained in LB. As shown in Fig. 1(a, b), strain CSL2 did not show any detectable CL in either of the different media or growth phases (exponential and stationary), leading us to confirm that no other gene or pathway would supply CL in this *B. subtilis* strain. In addition, we observed that the PG content in strain CSL2 grown in 1·5 M NaCl increased both in exponential and in stationary phase, when compared with the same strain grown in normal LB medium (Fig. 1a, b).

Fatty acid and diglucosyldiacylglycerol composition of the CLS2 cardiolipin null mutant

To determine whether the mutation in the *clsA* gene affected other membrane lipid components able to replace the CL deficiency, both fatty acids and diglucosyldiacylglycerol (DGDG) were quantified. It is worth mentioning that DGDG is the only glycolipid present in membranes of *B. subtilis* YB886 as described previously (López *et al.*, 1998). Our results indicated that the CSL2 mutant strain grown in LBN showed a 1·6-fold increase in the glycolipid content when compared to the same strain grown in LB medium. These results are in agreement with the changes in DGDG content previously described for the wild-type strain grown under the same conditions (data not shown and López *et al.*, 1998).

Regarding the fatty acid composition, the branched-chain fatty acids are the major components that esterify the membrane lipids in both the wild-type and CSL2 strains (Fig. 3; Kaneda, 1977; López *et al.*, 1998). The fatty acid profile of the wild-type and mutant strains grown in LB showed a significant difference in the straight-chain saturated fatty acids and in the branched-chain iso series; while in LBN we observed a significant difference in the
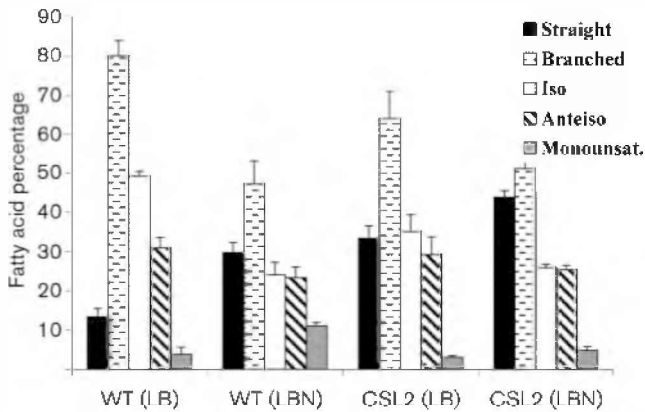
Table 2. Osmoresistance determinations on LB plates supplemented with different NaCl concentrations

Strains were cultured in aerated LB medium, and at exponential or early stationary phase, samples were diluted and plated on LB or on LB with 1 M or 1·5 M NaCl. After 2 days incubation at 37 °C, c.f.u. were counted and osmoresistance calculated as indicated in Methods. WT, wild-type YB886.

Strain (growth phase)	Percentage osmoresistance in:	
	LB + 1 M NaCl	LB + 1·5 M NaCl
WT (exponential)	5·0	1·5
WT (early stationary)	94	80
CSL2 (exponential)	8·0	1·0
CSL2 (early stationary)	99	84

Fig. 3. Fatty acid composition of the *B. subtilis* wild-type YB886 and CSL2 mutant strains grown to stationary phase in LB and LBN. The fatty acids were analysed as indicated in Methods. The values represent the mean ± SD of three independent experiments. Straight, straight-chain saturated fatty acids; Branched, branched-chain saturated fatty acids; Iso, branched-chain saturated fatty acids iso series; Anteiso, branched-chain saturated fatty acids anteiso series; Monounsat, monounsaturated fatty acids. The fatty acid composition of the wild-type versus the mutant strain in each medium was compared using the Student *t*-test.

monounsaturated fatty acids and, interestingly, also in the straight-chain saturated ones. Altogether, these results suggested that these changes are specifically related to the absence of CL in the membranes of the CSL2 mutant strain.

It is clear from Fig. 3 that the fatty acid composition (specifically straight-chain and branched-chain iso series) of the wild-type strain changes drastically upon increasing the NaCl concentration. In contrast, little variation in the content of these fatty acids is observed in the CSL2 mutant when the two growth conditions are compared (LB vs LBN). In this context, it is important to note that the straight-chain and branched-chain saturated fatty acid content in the CSL2 strain grown in LB is similar to that of the wild-type strain in LBN (Fig. 3). These results might explain the small variations observed in the content of these fatty acids when the mutant was grown in the two media (compare CSL2 LB vs WT LBN and CSL2 LB vs CSL2 LBN).

These results clearly indicate that in the CL null mutant strain, not only phospholipids but also other membrane components were modified due to the absence of this anionic lipid, suggesting that these modifications could influence physical characteristics of the membrane such as fluidity, generally involved in adaptation to stresses.

Interface properties and lateral lipid packing of the membranes and LUVs of the CSL2 cardiolipin null mutant

The fluorescent characteristics of the Laurdan probe were used to study the interface properties of LUVs and isolated

membranes of the wild-type and CSL2 strains. Laurdan is a fluorescent probe that shows no preferential phase partitioning between ordered and disordered lipid phases and is considered to have uniform lateral and transbilayer distribution (Bagatolli & Gratton, 2001). Moreover, it has low affinity for proteins in the membrane; therefore this probe is a good reporter molecule to sense molecular relaxation dynamics of solvent dipoles in the membrane as a whole (Parasassi & Gratton, 1995).

The observed wavelength-dependence pattern of the generalized polarization (GP) of LUVs made from total lipids extracted from both strains (studied at 10 °C, 25 °C, 37 °C and 45 °C) changed gradually: the emission (GP_{em}) toward a negative slope and the excitation (GP_{exc}) toward a positive slope, indicating that the lipids were in a liquid ordered state (data not shown and López *et al.*, 2002). In addition, no phase transitions or phase coexistence were detected in both either type of LUVs. The GP_{exc} measured at 350 nm was taken as indicative of the hydration level of the lipid bilayer at the glycerol moiety of the phospholipids (Hirsch-Lerner & Barenholz, 1999). The absolute GP_{exc} values obtained for the LUVs of the CSL2 strain grown in LBN were higher than those obtained for the rest of the samples. Since higher GP_{exc} values indicate a lower degree of dipolar relaxation due to either a lower hydration or water mobility at the polar/non-polar interface, our results suggest an increased lipid packing in this sample (Fig. 4a). At
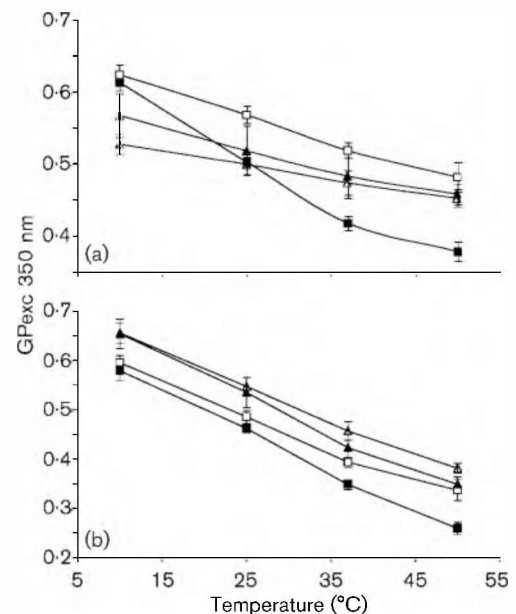

Fig. 4. Generalized polarization (GP_{exc}) of LUVs and membranes. GP_{exc} values were calculated at 350 nm and at the different temperatures with samples from LUVs (a) and isolated membranes (b) obtained from the wild-type (triangles) and CSL2 (squares) cultures in LB (filled symbols) and LBN (open symbols). The values represent the mean ± SD of four independent experiments.

physiological temperature (37 °C), the hydration rate will follow the order CSL2 > WT ~ WT + NaCl > CSL2 + NaCl, LUVs of CSL2 in LB being those presenting the highest interface hydration rate and the lowest lateral lipid packing. Therefore, the changes described in this paper regarding the composition of the membrane (polar lipids and fatty acids) in the CL null mutant undoubtedly lead to changes in the biophysical properties of the LUVs. Moreover, the ΔGP_{exc} of the samples at the different temperatures (data not shown) indicated that the CSL2 LUVs showed more variation of the GP_{exc} values within the temperature range studied, these results being related not only to the lipid composition shown in Fig. 1(b) but also to the fatty acid content of the sample shown in Fig. 3.

In order to determine the influence of the membrane proteins on the biophysical properties of the membranes, the same parameters were studied in isolated membranes of both strains. The wavelength dependence pattern of the samples observed within the 25–45 °C temperature range was characteristic of a liquid ordered state, showing a blue band at ~430 nm as was observed for the LUVs (data not shown). As in the case of the LUVs, no phase transitions or coexistence were detected in the membrane preparations. However, the red excitation band observed at 380 nm for the isolated membranes was higher when compared with that of the LUVs (data not shown). This could be due to changes in the lipid packing and/or the polarity of the environment in which the probe is embedded. At the temperatures studied, the absolute GP_{exc} values obtained for the isolated membranes of strain CSL2 grown in LBN were higher when compared with the same strain grown in LB, suggesting that in the former membranes there is a lower dipolar relaxation of the probe and/or higher lateral lipid packing (Fig. 4b).

The ΔGP_{exc} calculated as a function of the temperature indicated that the membranes of CSL2 grown in LB showed the highest dependence of this parameter within the temperature range studied (data not shown). However, similar values were obtained for the wild-type and strain CSL2 grown in LBN. These results verified that membrane proteins keep the membrane more sensitive to temperature changes than protein-free-lipid vesicles do. The sharp slopes shown in Fig. 4(b) indicate that in natural membranes, Laurdan revealed a greater GP_{exc} dependence on temperature than that obtained in either type of lipid vesicles. Similar results have been described by D'Antuono et al. (2000) in membranes and vesicles obtained from rat renal papillae.

Construction and biochemical characterization of a phosphatidylglycerol mutant strain

As shown in Fig. 1(a, b), the PG content in membranes of strain CSL2 grown in LBN increased, suggesting that this phospholipid might replace CL during the osmotic adaptation. To verify this hypothesis, the PGSA1 mutant, in which the pgsA gene is under the control of an inducible

Pspac promoter, was studied. This strain requires the presence of the inducer (IPTG) for its long-term viability (data not shown), indicating the importance of anionic phospholipids in the membranes of B. subtilis. This behaviour has also been reported by Matsumoto et al. (1999). Also, after 8 h incubation in the absence of IPTG, the cultures started to grow, indicating that the pgsA mutation could be bypassed even in the presence of the LacI repressor. This result led us to choose early stationary phase cultures for the determination of the polar lipid composition and osmoresistance of the mutant strain induced with different IPTG concentrations. As shown in Table 3, the gradually increased content of the anionic phospholipids (PG and CL) was dependent on the IPTG concentration. Regarding the other two major zwitterionic phospholipids present in this strain, PE showed a slight increase at low IPTG concentrations (0·05–0·1 mM) and then its value remained constant, whereas LysPG decreased with increasing IPTG concentrations (Table 3). The phospholipid composition of early stationary phase cultures from the wild-type strain was included as control in the experiments reported in Table 3. The presence of PG and CL in membranes of the PGSA1 mutant grown without IPTG could be due to an inducer-independent transcription of the pgsA gene and not to an increased lifetime of these phospholipids. DGDG accumulated in the membranes of strain PGSA1 grown in the absence of IPTG, but after addition of the inducer the content of this glycolipid decreased (data not shown), indicating a shift in the metabolism of lipid biosynthesis towards the synthesis of anionic phospholipids. The accumulation of DGDG observed in this mutant strain might be due to a change in the regulation of intracellular pools of cytidine 5'-diphosphate diacylglycerol (CDP-DAG), causing an increase in the synthesis of diacylglycerol, which is used as

Table 3. Phospholipid composition of B. subtilis strain PGSA1

Overnight cultures of strain PGSA1 were grown in LB containing 0·375 M IPTG and diluted in LB containing the different IPTG concentrations as indicated; the wild-type strain (WT, strain YB886) was grown in LB. At the early stationary phase the cultures were collected and phospholipids quantified as indicated in Methods. Phospholipid contents are reported as percentage of total phospholipids (mol phosphorus). The values represent the mean ± SD from three independent experiments.

IPTG (mM)	Phospholipid			
	LysPG	PE	PG	CL
0	31 ± 6·0	53 ± 3·8	11 ± 3·0	3 ± 0·9
0·05	25 ± 4·6	60 ± 4·5	12 ± 0·7	3 ± 1·8
0·1	20 ± 1·6	54 ± 1·1	21 ± 3·6	5 ± 2·0
0·375	8 ± 1·4	52 ± 2·7	33 ± 3·9	7 ± 0·8
WT	15 ± 2·2	36 ± 1·9	34 ± 2·7	15 ± 3·8

a substrate for DGDG biosynthesis. However, this assumption should be confirmed. The fatty acid analysis of this strain in the presence of different IPTG concentrations did not reveal any differences when compared with the wild-type (data not shown).

The osmoresistance assays performed with early stationary phase cultures clearly showed a direct correlation between the ability of PGSA1 strain to grow in the presence of 1·5 M NaCl and the induction of the *pgsA* gene: at the maximum *pgsA* expression (0·375 mM IPTG) the osmoresistance of the PGSA1 mutant was 59·6 % and the CL + PG content was 40 %. In similar assays, the wild-type strain showed 80 % osmoresistance, while the anionic phospholipid content was 49 % (Table 4).

Transcriptional analysis of the *clsA* (*ywnE*) and *pgsA* genes

As stated above, the presence of anionic phospholipids is important for the osmotic stress response; therefore we analysed the transcriptional characteristics of the *clsA* and *pgsA* genes by using transcriptional *lacZ* fusions. The transcriptional analysis of the *clsA* gene was performed in both the mutant and wild-type context (strains CSL2 and CLS21) but no significant differences were observed, indicating that CL did not affect the regulation of the *clsA* gene, which is involved in CL synthesis (data not shown). The transcription of the *clsA* gene increased as cells reached the stationary phase, this induction being more significant (twofold increase) when the cultures were grown in the presence of 0·75 M NaCl. These results agree with the biochemical data in which we observed an accumulation of CL in membranes of the wild-type strain not only upon entry into stationary phase but also during growth in LBN medium (Fig. 1a, b).

Table 4. Correlation between anionic phospholipid content in membrane and osmoresistance of PGSA1 and wild-type cultures

Strain PGSA1 was grown in LB medium in the presence of the indicated IPTG concentrations; the wild-type (WT, strain YB886) was grown in LB. Samples were taken at the early stationary phase and used to determine both phospholipid content [percentage of CL + PG (mol phosphorus)] and osmoresistance. The values represent the mean ± SD of three independent experiments.

IPTG (mM)	PG + CL	Osmoresistance (%)*
0	14 ± 3·9	0·16
0·05	15 ± 2·5	1·5
0·1	26 ± 5·6	ND
0·375	40 ± 4·7	59·6
WT	49 ± 6·5	80·0

*Osmoresistance was measured as indicated in Table 2 but all PGSA1 cultures were plated in medium containing 0·375 M IPTG. ND, Not determined.

The transcriptional analysis of the *pgsA* promoter also indicated a slight induction of this gene at the early stationary phase (data not shown). Moreover, upon addition of increasing NaCl concentrations a twofold induction comparable to the *clsA* promoter activity was also observed.

DISCUSSION

In the YB886 genetic background, neither the *ywiE* nor the *ywjE* mutant strains (CSL1 and CSL3) showed a deficiency in CL synthesis. In contrast, the interruption of the *ywnE* gene (strain CSL2) resulted in a complete deficiency in CL synthesis. This mutant strain was completely unable to synthesize CL in the different media and growth phases assayed, thus supporting the proposal that *ywnE* is a true *clsA* gene in *B. subtilis* YB886. It is important to mention that the phospholipid and glycolipid composition of *B. subtilis* strains YB886 (our wild-type context) and Marburg 168 (Kawai *et al.*, 2004) are different; the PG content in YB886 was higher than that in Marburg 168. Furthermore, while in YB886 the *clsA* mutation did not produce a significant change in sporulation efficiency compared to the wild-type, the same mutation in the Marburg strain affected the sporulation process (50 % reduction). Since we cannot rule out the possibility that the phospholipid assay that Kawai *et al.* (2004) used in their experiments could have been more sensitive than our assay, the overall differences discussed above are more likely to be due to differences in the genetic backgrounds of the strains used.

Regarding the ability to respond to an osmotic stress, the CSL2 mutant did not show an absolute defective phenotype. However, the extended lag observed in the presence of 1·5 M NaCl, and the reduced growth, highlight the importance of the membrane CL content in the adaptation of *B. subtilis* to high salt concentrations. Since we observed an increased PG content in the CSL2 strain it is possible that in certain specific reactions that are dependent on the polar head group of the phospholipids, PG might substitute for the lack of CL, as has been described for *Saccharomyces cerevisiae* (Jiang *et al.*, 2000) and *E. coli* (Matsumoto, 2001). Moreover, since changes in the lipid headgroup region of lipid bilayers could lead to modifications in the structure of transmembrane α-helices of membrane proteins and as a result, changes in the packing of these helices, it is important to maintain the presence of charged lipids in the bilayer. This conclusion is also supported by the behaviour of the PGSA1 mutant and by the direct correlation between the anionic phospholipid (CL + PG) content and the osmoresponse observed in our work (Table 4). In addition, the failure of the PGSA1 strain to survive in the absence of anionic lipids corroborates its importance for the integrity of the *B. subtilis* membrane. In fact, most of the genes related to envelope structure in *B. subtilis*, have been shown to be indispensable for viability (Kobayashi *et al.*, 2003). The transcriptional activity of both *pgsA* and *cslA* indicated that these genes were similarly regulated: the intensity of their

promoter was similar and both showed a slight increase (twofold) at stationary phase or under osmotic upshift.

In this work we also demonstrated that the absence of CL in the *B. subtilis* membrane leads to additional modifications of other membrane components such as fatty acids and glycolipids, confirming that there is a tight cross-regulation between the different membrane components. Although a significant difference concerning the fatty acid composition of the CSL2 strain was observed when compared with the wild-type, we also showed that the overall changes produced in these membrane components as a consequence of an elevated NaCl concentration are similar in both strains, suggesting that these modifications might be a general mechanism of membrane adaptation to a high ionic strength.

Several lines of evidence suggest that the biophysical state of the membranes plays a key role in the regulation of some transporters, such as the mechanosensitive channels in *S. cerevisiae* (Gustin *et al.*, 1988) and in *E. coli* (Moe & Blount, 2005). Since the fluidity and functional properties of biological membranes are achieved by the full collaboration of proteins and lipids, we characterized the phase behaviour and interface properties of lipid vesicles and isolated membranes by studying the Laurdan's dipolar relaxation properties. In both the wild-type and the CSL2 strains, the lipid vesicles and membranes were in a liquid-ordered state and no phase coexistence or phase transitions were observed. However, our results clearly indicate that the lateral lipid packing of the vesicles and membranes varied due to the absence of CL. Another interesting feature is that the lateral lipid packing of the membranes of both strains increased when they were grown in LBN. This change could be related to the modifications described in this paper regarding the composition of their membranes (polar lipids and fatty acids). Since it is likely that different proteins require different amounts of water, an osmotic stress would potentially affect the activity of these proteins through changes in the hydration rate of the lipid surface and the membrane proteins as well (Poolman *et al.*, 2002). Therefore, it is possible that the changes observed in the lipid hydration of the isolated membranes of the CSL2 mutant strain would affect the functional properties of specific membrane proteins, such as those involved in the osmotic response. The results in this paper allow us, for the first time, to emphasize the importance of anionic phospholipids in the *B. subtilis* membranes in withstanding high ionic strength.

One possible explanation for the major variations in lipids occurring during stress or growth phase is that they would influence the membrane structure, thus affecting the activity of enzymes involved in different processes such as sporulation (Dartois *et al.*, 1997), osmosensing (Poolman *et al.*, 2004) and modifications of protein function (Jensen & Mouritsen, 2004). van der Heide *et al.* (2001), while studying the ionic regulation of the OpuA transporter of *Lactococcus lactis* in proteoliposomes containing different

phospholipid composition, showed that only the charge present on the lipid headgroups influences the osmotic activation profile of this transporter. Likewise, Poolman *et al.* (2004) demonstrated that the activation threshold of the OpuA transporter, which is an indicator of osmosensor activity, depends on the fraction of anionic lipids present in the samples. These authors argued that this feature would indicate a general requirement for negatively charged lipids in several bacterial transporters even if they are not osmoregulated. In addition, in *E. coli* it has been demonstrated that the autophosphorylation of the sensor kinase KdpD, which regulates the expression of the *kdpFABC* operon, is also dependent on negatively charged phospholipids (Stalkamp *et al.*, 1999).

The presence of negatively charged lipids in the lipid bilayer of the *B. subtilis* cells grown in the presence of elevated NaCl concentrations would increase the negative charge of the surface of the membrane and therefore increase the concentration of positively charged ions or molecules close to the surface (Lee, 2004). This change in phospholipid content would be important for the osmotic adaptation of the cells, since the first response observed in both Gram-negative and Gram-positive bacteria is the uptake of K^+ into the cytoplasm (McLaggan *et al.*, 1994; Whatmore & Reed, 1990). Moreover, as was previously discussed, several transporters and regulatory proteins involved in the osmotic response in different bacteria are not only dependent on the fraction of anionic lipid but also on the physical state of the membranes. However, we cannot discount the possibility that this incorporation of negatively charged lipids would also change the affinities of membrane proteins for charged substrates as was suggested by Lee (2004).

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