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Induction of non-bilayer lipid structures by functional signal peptides

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Using ^{31}P NMR and freeze-fracture electron microscopy we investigated the effect of several synthetic signal peptides on lipid structure in model membranes mimicking the lipid composition of the *Escherichia coli* inner membrane. It is demonstrated that the signal peptide of the *E.coli* outer membrane protein PhoE, as well as that of the M13 phage coat protein, strongly promote the formation of non-bilayer lipid structures. This effect appears to be correlated to *in vivo* translocation efficiency, since a less functional analogue of the PhoE signal peptide was found to be less active in destabilizing the bilayer. It is proposed that signal sequences can induce local changes in lipid structure that are involved in protein translocation across the membrane.

Key words: model membranes/non-bilayer lipid structures/^{31}P NMR/protein translocation/signal peptides

Introduction

Proteins that are destined for export out of the cytoplasm of *Escherichia coli* cells are synthesized as precursor proteins with N-terminal extensions or signal sequences, which are essential for translocation of the protein across the inner membrane (for reviews, see Baker *et al.*, 1987; Briggs and Gierasch, 1986; Benson *et al.*, 1985; Gierasch, 1989). Although the 15–25 amino acids long signal sequences of various precursor proteins have no primary structure homology, they all consist of a positively charged N-terminal region, followed by a stretch of ~10–15 hydrophobic amino acids and a more polar region containing the cleavage site (Von Heijne, 1985).

In recent years a number of biophysical studies have been carried out on the properties of signal peptides in order to get insight into the role of these sequences in protein translocation (Batenburg *et al.*, 1988a,b; Bruch *et al.*, 1989; Cornell *et al.*, 1989; Gierasch, 1989; Nagaraj *et al.*, 1987; Reddy and Nagaraj, 1989). However, in spite of extensive research in this area, the mechanism by which proteins translocate across membranes and the exact role of signal sequences therein is still not known. In principle, two types of translocation pathways have been postulated. One hypothesis, originally proposed for the analogous eukaryotic system, is that proteins translocate via proteinaceous pores (Blobel and Dobberstein, 1975; Rapoport, 1986). Such a pore-forming role has been recently proposed for the SecY protein, one of the components of the export machinery of *E.coli* (Watanabe and Blobel, 1989). Another possibility is that membrane lipids are directly involved in translocation of proteins across membranes (Wickner, 1979, 1988). The observation that the M13 phage procoat protein can cross a pure lipid bilayer in the absence of any proteinaceous factors (Geller and Wickner, 1985) strongly supports this possibility. Consistent with this hypothesis is the observation that phosphatidylglycerol (PG) is involved in translocation of the *E.coli* outer membrane protein PhoE (De Vrije *et al.*, 1988).

Evidently, translocation of a water-soluble protein across a lipid bilayer would be energetically unfavourable if the polar side chains were in contact with the hydrophobic lipid acyl chains. However, an attractive translocation pathway could be formed if locally and transiently an alternative, non-bilayer lipid structure were induced (Batenburg *et al.*, 1988b; Nesmeyanova, 1982). Such non-bilayer lipid intermediates have been proposed to play a role in a number of functional abilities of biological membranes and could be regulated, among other factors, by interactions between lipids and proteins or peptides (for reviews, see De Kruijff, 1987; De Kruijff *et al.*, 1985 and references therein). Since signal peptides can interact strongly with lipids (Batenburg *et al.*, 1988b; Cornell *et al.*, 1989; Gierasch, 1989; Nagaraj *et al.*, 1987), the possibility should be considered that signal sequences, in addition to their role in efficient targetting and possible other roles as discussed by Gierasch (1989), can act by locally inducing the formation of non-bilayer lipid structures which may be involved in translocation of proteins across the membrane.

We explored this possibility by studying the effect of several synthetic signal peptides on lipid structure in model membranes mimicking the lipid composition of the *E.coli* inner membrane. Using ^{31}P NMR and freeze-fracture electron microscopy it was investigated how membrane lipid organization is affected upon insertion of the signal peptide of the *E.coli* outer membrane protein PhoE and whether changes in lipid organization correlate with the functional activity of the signal sequence in protein translocation. The specificity of the process was further investigated by studying the effect of various other (signal) peptides as well as signal peptide fragments.

We will demonstrate here that the signal peptide of PhoE is extremely effective in inducing the formation of non-bilayer lipid structures in model membrane systems. This effect appears to be related to *in vitro* and *in vivo* translocation efficiency and is most likely a general property of signal peptides.

Our results support, but do not prove, the hypothesis that non-bilayer lipid structures are involved in protein translocation and suggest that signal sequences could act by inducing a local change in lipid structure that allows the protein to cross the membrane.

	-20 ↓ -1 ↓
PhoE signal peptide	MKKSTLALVVMGIVASASVQA
Fragment Met^{-21}-Ala^{-15}	MKKSTLA
Fragment Met^{-21}-Gly^{-10}	MKKSTLALVVMG
Fragment Ile^{-9}-Ala^{-1}	IVASASVQA
N-formylated signal peptide	f-MKKSTLALVVMGIVASASVQA
[Asp^{-19},Asp^{-20}] signal peptide	MDDSTLALVVMGIVASASVQA
M13 coat signal peptide	MKKSLVLKASVAVATLVPMLSFA

Fig. 1. Primary structure of the signal peptides and PhoE signal peptide fragments used in this study. The arrow at position −1 indicates the cleavage site of the signal sequence.

Results

In this study we investigated the effect of several signal peptides and signal peptide fragments on membrane lipid structure. The peptides (see Figure 1 for their structure) were introduced through the aqueous medium to model membranes composed of dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylglycerol (DOPG) in a 3/1 molar ratio to mimic the lipid composition of the *E.coli* inner membrane (Burnell *et al.*, 1980b). At 30°C these model membranes form stable bilayers, which give rise to a characteristic ^{31}P NMR spectrum (Seelig, 1977; Cullis and De Kruijff, 1979) with a low field shoulder and a high field peak, as shown in Figure 2A.

Addition of the PhoE signal peptide to these model membranes results in dramatic changes in ^{31}P NMR characteristics, that can be observed already at a very low molar ratio of peptide to lipid of 1/3200 (Figure 2B). Superimposed on the 'bilayer' signal a second component is visible, with a smaller overall linewidth, indicating that the motional characteristics of part of the lipids are strongly affected by the presence of the signal peptide.

Increasing the peptide/lipid ratio leads to further spectral changes, as illustrated in Figure 2B–D for peptide/lipid ratios ranging from 1/3200 to 1/200. The relative intensity of the second spectral component increases with the peptide/lipid ratio whereas its linewidth decreases. At the highest molar ratio of 1/200 a sharp 'isotropic' signal is observed which indicates that a change in lipid organization has occurred in which the lipids undergo fast isotropic motion with a correlation time of $<10^{-5}/s^{-1}$ (Burnell *et al.*, 1980a). The occurrence of an isotropic ^{31}P NMR signal as in Figure 2D is commonly observed during a transition from a bilayer organization of lipids to a hexagonal H_{II} phase and in that case is indicative of the formation of type II non-bilayer structures (for review, see Lindblom, 1989), which are structures with a net concave surface curvature at the lipid/water interface. Interestingly, when pure lipid samples are incubated at elevated temperatures a similar change from a bilayer to isotropic ^{31}P NMR signal is observed as upon introduction of the signal peptide (not shown). This behavior has been described in detail in several analogous lipid systems and is typical for a temperature induced transition from a bilayer organization of lipids to a type II cubic structure (Lindblom, 1989). Thus, it is very likely that the isotropic ^{31}P NMR signal induced by the PhoE signal peptide represents a type II non-bilayer lipid organization. This is further supported by the observations that: (i) bilayer stabilization occurred upon incorporation of 5 mol% palmitoyllysophosphatidylcholine (LPC), a typical type I lipid, which on its own organizes in structures with a net convex surface curvature and therefore mitigates against the formation of type II structures (Madden and Cullis, 1982); and that (ii) after spinning the sample for 15 min at 30 000 *g* the isotropic signal was associated with the pellet, excluding the possibility (Burnell *et al.*, 1980a) that this signal originated from the formation of small vesicles.

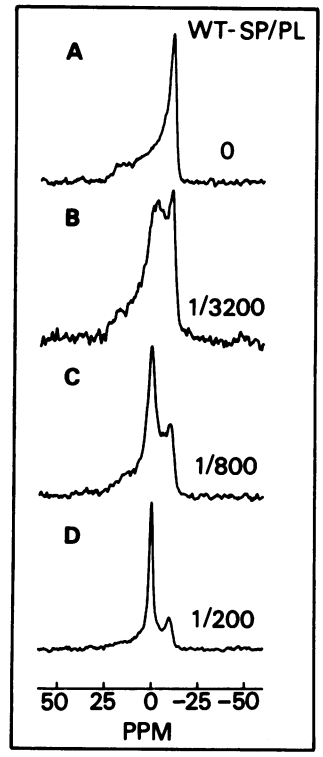

Fig. 2. ^{31}P NMR spectra of DOPE/DOPG (75/25 molar ratio) dispersions upon addition of PhoE signal peptide in various molar ratios of peptide to lipid as indicated in figure.

To further characterize the nature of the structures giving rise to the observed isotropic ^{31}P NMR signal, freeze-fracture electron microscopy was performed. As shown in Figure 3A and in accordance with the ^{31}P NMR results a control sample of pure lipid shows the presence of large vesicles (diameter 2000–20 000 Å). Addition of the PhoE signal peptide (1/200 molar ratio of peptide to lipid) clearly results in a change of morphology. The electron micrograph (Figure 3B) shows the presence of lipidic particles of ~120 Å in diameter as well as larger volcano-like protrusions (diameter ~300 Å), that most likely represent interbilayer fusion points. A very similar change in morphology as upon introduction of the signal peptide was observed in pure lipid samples after incubation at elevated temperatures (not shown). Since such a morphology typically occurs under conditions that induce a transition from a bilayer organization of lipids to a hexagonal H_{II} phase (Verkleij, 1984), the electron microscopy experiments are

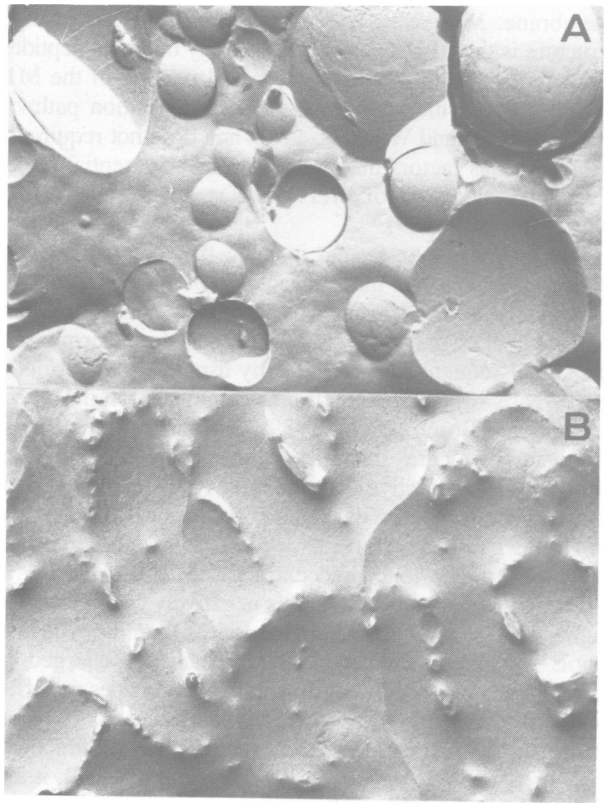

Fig. 3. Electron micrographs of DOPE/DOPG (75/25 molar ratio) dispersions in the absence of peptide (**A**) and upon addition of PhoE signal peptide in a 1/200 molar ratio of peptide to lipid (**B**). Magnification: 37 000×.

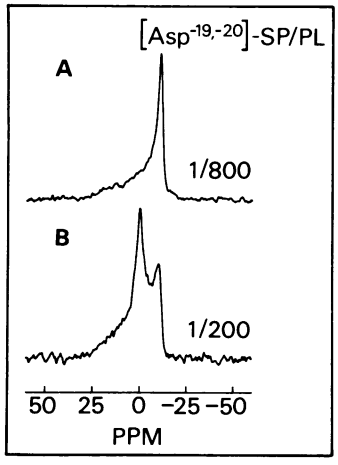

Fig. 4. ^{31}P NMR spectra of DOPE/DOPG (75/25 molar ratio) dispersions upon addition of PhoE [$Asp^{-19,-20}$] signal peptide in molar ratios of peptide to lipid of 1/800 (**A**) and 1/200 (**B**).

in full agreement with the ^{31}P NMR results, both indicating that the PhoE signal peptide induces the formation of type II non-bilayer lipid structures.

To investigate whether this effect of the signal peptide on lipid structure can be correlated to its functional activity, we next studied the effect of replacement of the positively charged lysine residues at the N terminus by aspartic acid, which modification in the signal sequence of prePhoE results in a decrease of the *in vivo* and *in vitro* translocation efficiency (Bosch *et al.*, 1989). Figure 4A shows that when

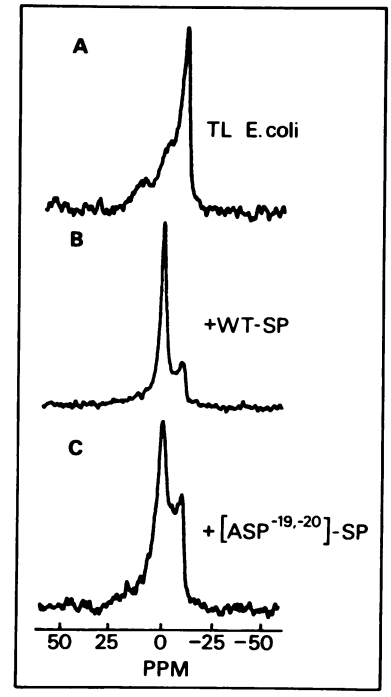

Fig. 5. ^{31}P NMR spectra of model membranes of the total lipid extract of *E.coli* cells in the absence of peptide (**A**) and upon addition of PhoE signal peptide (**B**) or PhoE [$Asp^{-19,-20}$] (**C**) signal peptide in a molar ratio of peptide to lipid of 1/100.

added in a molar ratio of peptide to lipid of 1/800 the PhoE [$Asp^{-19,-20}$] signal peptide is unable to perturb lipid structure under conditions at which the wild-type signal peptide induces a large isotropic signal (Figure 2C). Increasing the peptide/lipid ratio to 1/200 also for the [$Asp^{-19,-20}$] signal peptide results in the formation of an isotropic spectral component (Figure 4B) but with a relative intensity that is considerably less than that induced by the wild-type signal peptide under identical conditions (compare with Figure 2D). A qualitatively similar effect on lipid structure of both peptides was observed in model membranes composed of the total lipid extract of *E.coli* cells (Figure 5). Sucrose-density gradient centrifugation experiments of these samples showed a quantitative association of the wild-type signal peptide as well as of the [$Asp^{-19,-20}$] signal peptide with the model membranes (not shown) demonstrating that the differences in effect on lipid structure are not due to differences in binding affinity. In these *E.coli* lipid model membranes twice the amount of both signal peptides was required to induce approximately the same percentage of isotropic ^{31}P NMR signal as in the DOPE/DOPG mixtures. This lower efficiency of perturbation of the bilayer is most likely due to the more saturated character of the *E.coli* lipids.

The percentage of isotropic signal induced by the various signal peptides in DOPE/DOPG mixtures is quantified in Figure 6A. From this figure it is clear that the formylated form of the PhoE signal peptide (see Figure 1), which is expected to be the form in which the signal sequence functions *in vivo*, has a similar effect on lipid structure as the non-formylated signal peptide. Furthermore it was found that the signal peptide of the M13 coat protein (see Figure 1 for its chemical structure) also was able to induce an isotropic component in the DOPE/DOPG mixtures, indicating that this lipid structure perturbing activity is most likely

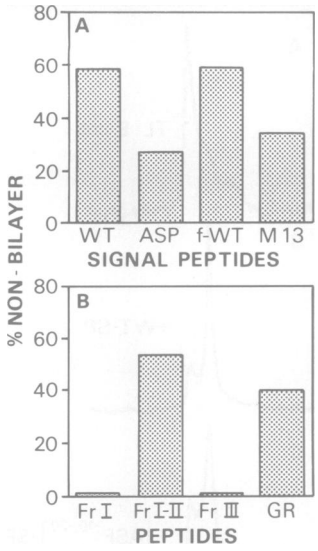

Fig. 6. Quantification of the relative amount of isotropic ^{31}P NMR signal induced in dispersions of DOPE/DOPG (75/25 molar ratio) by various signal peptides (**A**) and other peptides or signal peptide fragments (**B**) in a 1/200 molar ratio of peptide to lipid. **A**: PhoE signal peptide (WT), PhoE [$Asp^{-19,-20}$] signal peptide (ASP), N-formylated PhoE signal peptide (f-WT) and signal peptide of M13 coat protein (M13). **B**: signal peptide fragments Met^{-21}-Ala^{-15} (Fr I), Met^{-21}-Gly^{-10} (Fr I-II) and Ile^{-9}-Ala^{-1} (Fr III) and gramicidin A′ (GR).

a general property of functional signal peptides. Although this peptide appeared to be less effective than the PhoE signal peptide, it should be realized that the extent of the effect of the signal peptide of the M13 coat protein on lipid structure cannot be directly compared on a functional level with that of the PhoE signal peptides, since it is not known whether the *in vivo* and *in vitro* translocation efficiency of the M13 procoat protein is similar to that of prePhoE in the same system.

To investigate which part of the PhoE signal peptide is responsible for its effect on lipid structure, ^{31}P NMR experiments were carried out using the various fragments of the peptide indicated in Figure 1. These experiments showed that neither the N-terminal positively charged fragment Met^{-21}-Ala^{-15}, nor the hydrophobic C-terminal fragment Ile^{-9}-Ala^{-1}, was able to induce the formation of an isotropic signal (Figure 6B). In contrast, the longer N-terminal fragment Met^{-21}-Gly^{-10} was found to induce an isotropic signal with an intensity rather similar to that induced by the intact signal peptide.

Addition of gramicidin A′, a very strong promotor of the formation of type II lipid structures in model membrane systems (Killian and De Kruijff, 1986), also was found to result in the induction of an isotropic signal (Figure 6B), but with an efficiency that is less than that of the PhoE signal peptide (Figure 6A). This observation thus illustrates the strength of the lipid perturbing activity of the signal peptide.

Discussion

The results in this paper demonstrate a completely new property of signal peptides, i.e. their ability to induce the formation of type II non-bilayer lipid structures when introduced through the aqueous phase to model membranes mimicking the lipid composition of the *E.coli* inner membrane. Moreover, it is shown that this effect on lipid structure is most likely a general property of signal peptides, since it also is observed for the signal peptide of the M13 coat protein, which has a different translocation pathway (Ohno-Iwahita and Wickner, 1983) and does not require the proteinaceous factors that are known to be essential for efficient translocation of prePhoE (De Vrije *et al.*, 1988; Kusters *et al.*, 1989).

Furthermore, our results indicate that the type II non-bilayer lipid structure inducing activity of the signal peptides is related to their efficiency in protein translocation, since replacement of the two lysine residues at the N terminus of the signal sequence of prePhoE by aspartic acid results in a decrease of the rate of translocation across the *E.coli* membrane (Bosch *et al.*, 1989) and since similarly we demonstrated here that the PhoE [$Asp^{-19,-20}$] signal peptide is less effective than the wild-type signal peptide in inducing the formation of type II non-bilayer lipid structures in DOPE/DOPG mixtures as well as in model membranes composed of the total lipid extract of *E.coli* cells.

From the observations that this negatively charged mutant signal peptide affects lipid structure less than the wild-type PhoE signal peptide and that the positively charged N-terminal fragment of the PhoE signal peptide does not affect lipid structure at all, it can be concluded that overall hydrophobicity, the most evident requirement for functional signal sequences (Von Heijne, 1985; Gierasch, 1989), is also essential for the bilayer destabilizing activity of the signal peptides, whereas electrostatic interactions appear to determine the efficiency of the process. Of the various fragments of the PhoE signal peptide however, it was shown that fragment Met^{-21}-Gly^{-10}, but not the hydrophobic C-terminal fragment, was able to induce changes in lipid organization, demonstrating that hydrophobicity alone is not sufficient. In this respect it is interesting to note that fragment Met^{-21}-Gly^{-10} but not Ile^{-9}-Ala^{-1} was found to be capable of adopting an α-helical structure in apolar solvents (Batenburg *et al.*, 1988a), a property that has been shown to be essential for functional signal sequences (Briggs and Gierasch, 1984; Emr and Silhavy, 1983).

Summarizing, the results presented in this paper are consistent with our hypothesis as outlined in the Introduction, that signal sequences can act by inducing a local and transient formation of type II non-bilayer lipid structures that allow proteins to translocate across the membrane. However, we would like to emphasize that care should be taken in extrapolating the effects of signal peptides on lipid structure to their importance for *in vivo* protein translocation, since as yet nothing is known about the localization of signal sequences during translocation across membranes, nor is it clear whether a direct interaction occurs between the signal sequence of a precursor protein and membrane lipids.

Materials and methods

Materials

The PhoE signal peptide, its N-formylated derivative and PhoE [$Asp^{-19,-20}$] signal peptide were prepared by solid-phase synthesis by D.Olshevski (University of California, San Diego, CA). M13 coat signal peptide was from the same source and was a kind gift of Dr W.Wickner (University of California, LA). HPLC analysis, performed as described previously (Batenburg *et al.*, 1988a), indicated a >95% purity of the various signal peptides. Fragments Met^{-21}-Ala^{-15}, Met^{-21}-Gly^{-10} and Ile^{-9}-Ala^{-1} of the PhoE signal peptide were synthesized and purified as described by Batenburg *et al.* (1988a). The amino acid composition of the

various peptides was confirmed by amino acid analysis on a Kontron Liquimat III amino acid analyzer. Gramicidin was purchased from Sigma (St. Louis, MO) and used as such.

1,2-Dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1-palmitoyl-*sn*-glycero-3-phosphocholine (LPC) were obtained as described previously (Killian *et al.*, 1986). All lipids were judged pure from HPTLC using chloroform/methanol/water/ammonia (68/28/2/2, by vol.) as eluens. *E.coli* lipids were isolated as described before (Batenburg *et al.*, 1988b) from a culture of *E.coli* SD12 cells, grown at 37°C and harvested at the late logarithmic phase. The amount of lipids was quantified on the basis of the phosphate content of the total lipid extract according to Rouser *et al.* (1975).

All solvents and other reagents were of analytical grade.

Sample preparation

Lipid dispersions were prepared by lyophilizing mixtures of DOPE (15 μmol) and DOPG (5 μmol) or 20 μmol of *E.coli* lipids, from 1 ml benzene, followed by hydration in 1 ml 100 mM NaCl, 10 mM PIPES, 0.2 mM EDTA buffer, pH 7.4. The lipid mixtures were allowed to swell for 1 h at room temperature and then subjected to a minimum of five cycles of freeze-thawing.

All peptides were first dissolved in trifluoroacetic acid in a concentration of 50 mg/ml and then dried under a stream of N_2. Next, 5 mM stock solutions of the peptides were prepared in trifluoroethanol (TFE) which were used as such or which prior to addition to the lipid dispersion were diluted, such that each sample composed of DOPE and DOPG contained 2 vol% of TFE whereas to all *E.coli* lipid samples a total of 4 vol% of TFE was added. Increasing the amount of TFE to 4 vol% in these former samples did not affect the results. The peptide was added slowly, using a Hamilton syringe, and under gentle stirring to the lipid dispersion in a 5 ml polyethylene vial. The sample was then transferred to a 10 mm NMR tube and incubated at 30°C for 2 h. ^{31}P NMR spectra were recorded at 30°C as described below.

^{31}P NMR

^{31}P NMR experiments were carried out as described before (Chupin *et al.*, 1987) on a Bruker MSL 300 spectrometer at 121.4 MHz. Prior to Fourier transformation an exponential multiplication was applied to 2000 accumulated free induction decays, resulting in a 100 Hz linebroadening. Percentages of isotropic and bilayer signals were obtained by subtraction with an estimated maximal error of 5%. All spectra were scaled to the same height of the largest peak. No changes in total intensity were observed upon addition of the peptide.

Freeze-fracture electron microscopy

Immediately after incubation samples were rapidly frozen from room temperature with a Reichert Jung KF 80 plunge-freezing device (Sitte *et al.*, 1987) without the use of cryoprotectants. The freeze-fracture replicas were made in a Balzers 300 apparatus and analysed with a Philips 420 electron microscope.

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