Dynamic localization of membrane proteins in *Bacillus subtilis*

A. S. Johnson,† S. van Horck and P. J. Lewis

School of Environmental and Life Sciences, Biological Sciences, University of Newcastle, Callaghan, NSW 2308, Australia

The subcellular localization of membrane proteins in *Bacillus subtilis* was examined by using fluorescent protein fusions. ATP synthase and succinate dehydrogenase were found to localize within discrete domains on the membrane rather than being homogeneously distributed around the cell periphery as expected. Dual labelling of cells indicated partial colocalization of ATP synthase and succinate dehydrogenase. Further analysis using an ectopically expressed phage protein gave the same localization patterns as ATP synthase and succinate dehydrogenase, implying that membrane proteins are restricted to domains within the membrane. 3D reconstruction of images of the localization of ATP synthase showed that domains were not regular and there was no bias for localization to cell poles or any other positions. Further analysis revealed that this localization was highly dynamic, but random, implying that integral membrane proteins are free to diffuse two-dimensionally around the cytoplasmic membrane.

INTRODUCTION

Biological membranes are not homogeneous structures, but consist of domains enriched for specific lipids and proteins often referred to as lipid rafts (Edidin, 2003). These rafts appear to be highly dynamic structures, in terms of both lipid/protein content and size (Edidin, 2003). Although lipid rafts and membrane heterogeneity have been intensively studied in eukaryotic systems, relatively little work has been carried out with microbial systems, probably in part because of their small size. In recent years, however, convincing evidence has been presented indicating the presence of lipid domains in microbial cytoplasmic membranes (Fishov & Woldringh, 1999; Mileykovskaya & Dowhan, 2000; Kawai *et al.*, 2004). In particular, the use of specific fluorescent probes has indicated that the phospholipid cardiolipin localizes preferentially to the cell poles and division sites (mid-cell regions) of both Gram-positive and Gram-negative bacteria (Mileykovskaya & Dowhan, 2000; Kawai *et al.*, 2004). Further experiments using 2-pyrene derivatives of the phospholipids phosphatidylethanolamine (PE) and phosphatidyleglycerol (PG) indicated that the two lipids segregate into distinct domains in both Gram-positive and Gram-negative cell membranes, and that the PE domains were enriched for protein content (Vanounou *et al.*, 2003). Despite the similarity of results obtained with Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) model systems, the two organisms have quite different proportions of common phospholipids in their cytoplasmic membranes. In *B. subtilis* PE accounts for approximately 50% of phospholipid, PG 15%, the lysine ester of PG 2-4%, cardiolipin (CL) 0-8%, and mono- and triglucosyldiacylglycerols 30% (de Mendoza *et al.*, 2002), whereas in *E. coli* PE accounts for about 70–80% of phospholipid, PG 15–25% and CL 5–10% (Kadner, 1996).

Do integral membrane proteins also segregate into domains within the cytoplasmic membrane? The presence of chemoreceptors at cell poles is well established (Maddock & Shapiro, 1993) and this may reflect a preference of these proteins for acidic lipids such as cardiolipin. Penicillin-binding proteins involved in cell wall synthesis have also been shown to localize in several patterns, including to division septa and discrete foci along the long axis of the cell, reflecting the role of these proteins in specific stages of cell wall synthesis (Scheffers *et al.*, 2004). However, analysis of the localization of the *E. coli* Sec protein secretion machinery, BglF sugar sensor and *B. subtilis* phage φ29 DNA replication protein p16.7 indicates that these proteins are homogeneously distributed around the cytoplasmic membrane, with no reported preference for cell poles or other observable domains (Brandon *et al.*, 2003; Lopian *et al.*, 2003; Meijer *et al.*, 2001). Certainly, the images presented in these papers indicate that the localization pattern of these proteins creates a clear outline of the cytoplasmic membrane.

We have examined the localization of ATP synthase and succinate dehydrogenase, which carries out steps in both...
the tricarboxylic acid cycle and the electron-transport chain (as complex II), and re-examined the localization of p16.7 using a series of high-resolution fluorescence microscopy techniques with fluorescent protein fusions in live B. subtilis cells. We found that all of the proteins localized around the cytoplasmic membrane heterogeneously, and appeared to be free to move randomly throughout the membrane. We propose that such localization to domains is a general feature of integral membrane proteins.

METHODS

Strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. All cloning was performed using E. coli DH5α (Gibco-BRL). Unless otherwise stated B. subtilis strains were cultured in CH medium (Sharpe et al., 1994) supplemented with the appropriate antibiotics and inducers: chloramphenicol, 50 μg ml⁻¹; spectinomycin, 50 μg ml⁻¹; xylose 0-5 % (w/v); IPTG 0-5 mM. The functionality of atpA– and sdhA–gfp fusions was checked on succinate minimal media plates (SMM; Santana et al., 1994) supplemented with the appropriate antibiotics and inducers, and grown at 37 °C.

Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Source/construction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. subtilis</strong></td>
<td>trpC2</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>168</td>
<td>trpC2 chr::pNG24 (atpA–gfp Ppxyl–cat)</td>
<td>168 transformed with pNG24</td>
</tr>
<tr>
<td>BS23</td>
<td>trpC2 chr::pNG25 (atpA–cfp Ppxyl–cat)</td>
<td>168 transformed with pNG25</td>
</tr>
<tr>
<td>BS24</td>
<td>trpC2 chr::pNG107 (sdhA–gfp Ppxyl–sdhA cat)</td>
<td>168 transformed with pNG107</td>
</tr>
<tr>
<td>BS112</td>
<td>trpC2 chr::pNG117 (sdhA–yfp lacI_pspc–sdhA cat)</td>
<td>168 transformed with pNG117</td>
</tr>
<tr>
<td>BS121</td>
<td>trpC2 chr::pNG25 [atpA–cfp Ppxyl–atpA (catR spcR)]</td>
<td>BS24 transformed with pCm:Sp; inserts by double crossover into cat gene of pNG25 and converts to spcR</td>
</tr>
<tr>
<td>BS131</td>
<td>trpC2 chr::pNG25 [atpA–cfp Ppxyl–atpA (catR spcR)]</td>
<td>BS131 transformed with BS112 chromosomal DNA</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td>bla (cat–spc–cat)</td>
<td>Bacillus Genetic Stock Center</td>
</tr>
<tr>
<td>pSG1186</td>
<td>bla cat cfp</td>
<td>Feucht &amp; Lewis (2001)</td>
</tr>
<tr>
<td>pSG1187</td>
<td>bla cat yfp</td>
<td>Feucht &amp; Lewis (2001)</td>
</tr>
<tr>
<td>pNG24</td>
<td>bla cat Ppxyl–atpA–gfp</td>
<td>Man–EcoRI digest of atpA inserted into EcoRI-cut pSG1164</td>
</tr>
<tr>
<td>pNG25</td>
<td>bla cat Ppxyl–atpA–cfp</td>
<td>EcoRI–SpeI digest of pSG1186 inserted into the large fragment of similarly cut pNG24</td>
</tr>
<tr>
<td>pNG107</td>
<td>bla cat Ppxyl–sdhA–gfp</td>
<td>Acc651–Xhol digest of 3’ terminal portion of sdhA inserted into similarly cut pSG1164</td>
</tr>
<tr>
<td>pNG115</td>
<td>bla cat Ppxyl–sdhA–yfp</td>
<td>Xhol–SpeI digest of pSG1187 inserted into the large fragment of similarly cut pNG107</td>
</tr>
<tr>
<td>pNG117</td>
<td>bla cat lacI Ppxyl–sdhA–yfp</td>
<td>Acc651–SpeI sdhA–yfp fragment from pNG115 inserted into large fragment of similarly cut pSG1170</td>
</tr>
</tbody>
</table>
set 31044V2, YFP with set 41029, and FM4-64 was visualized using set 61000v2SBX with a 61560 TRITC exciter (all sets from Chroma Technology). Cells were mounted onto 1-2% agarose pads as described by Glaser et al. (1997) or within Gene Frames (Advanced Biotechnologies) as described by Lewis et al. (2000) for microscopy. Image acquisition was performed using MetaMorph version 5.0 (UIC); backgrounds were subtracted, and out-of-focus light removed, using the Nearest Neighbours deconvolution drop-in. Linescans were performed in MetaMorph and data transferred to Microsoft Excel for further analysis. Final figures were prepared for publication using Adobe Photoshop version 7.0.

**3D imaging and deconvolution.** Image stacks at 20 nm incremental steps were collected using a Pifoc PI P-721.10 microscope focus drive (Physik Instrumente) controlled via MetaMorph. Background subtractions and image stack alignments were carried out in MetaMorph and image stack deconvolution in AutoDeblur version 6.0 (AutoQuant Imaging). 3D reconstruction was performed in MetaMorph.

**Time lapse.** For time-lapse microscopy, exponentially growing cells were placed onto 1-2% agarose pads in Gene Frames and a suitable field of cells was located by phase-contrast microscopy. Cells were then imaged using the ACQUIRE TIMELAPSE drop-in from MetaMorph with a sampling interval of 1 min. Stacks were aligned and images processed as detailed above prior to analysis.

**Fluorescence recovery after photobleaching (FRAP).** FRAP was carried out using a Zeiss LSM 510 confocal microscope fitted with an Orca ER CCD camera (Hamamatsu) using a 100 × PlanApo NA1.3 objective to view exponentially growing cells mounted on an inverted agarose pad. GFP fluorescence was viewed using the 488 nm laser line at 10% full power. A region of interest was bleached using 40 ms pulses at 40% power over a 2 s exposure period. One-second exposures were obtained at the intervals specified in the text during photobleaching recovery. Twelve-bit images were analysed in MetaMorph.

### RESULTS

**atpA– and sdhA–gfp fusions are functional**

It is important to ascertain whether a fluorescent protein fusion is functional, partially functional or non-functional when making conclusions about observed localization patterns. This is particularly important when considering the localization pattern of fusions involved in the formation of multi-protein complexes such as ATP synthase and succinate dehydrogenase. Both atpA and sdhA, to which fusions were made, lie within operons, encoding ATP synthase and succinate dehydrogenase, respectively, and so on single crossover of fusions into the chromosome it was important to ensure the expression of genes downstream of the insertion through use of either xylose- or IPTG-inducible promoters (Lewis & Marston, 1999). The growth of strains BS23 (atpA–gfp), BS112 (sdhA–gfp), BS121 (sdhA–yfp) and BS133 (atpA–cfp and sdhA–yfp) (Table 1) was tested on SMM agar plates. With succinate as the only carbon source, these strains should be dependent on a functional electron-transport chain and ATP synthase via ATP synthase. Growth of the strains on SMM supplemented with either xylose or IPTG is shown in Fig. 1(A, B). Xylose was required for induction of expression of genes downstream of the fluorescent protein fusions in strains BS23, BS112 and BS133, whereas IPTG was required in strain BS121. In the presence of xylose, extensive growth of strains BS23, BS112 and BS133 was observed (Fig. 1A), indicating expression of the fusion, and the genes downstream in both the atp and sdh operons led to formation of functional protein complexes. These results were confirmed by growing strain BS23 in liquid SMM (Fig. 1C). In the presence of xylose, the fusion strain grew with very similar kinetics to the control strain (168; t_d approximately 85 min; not shown), whereas very little growth was observed in the absence of xylose. In addition, Western blots of whole-cell extracts together with cytoplasmic and membrane fractions showed that there was no detectable degradation of the fusions, which localized exclusively to the membrane fraction (data not shown). Virtually no growth of BS121 containing an IPTG-inducible promoter downstream of an sdhA–yfp insertion was observed (Fig. 1A), indicating the dependence of these strains on the formation of functional enzyme complexes for growth. Conversely, when these strains were grown on SMM supplemented with IPTG

![Image of Fig. 1](http://mic.sgmjournals.org)
instead of xylose, growth was only observed for strain BS121 (Fig. 1B).

Together, these results indicate that functional fusions to both AtpA and SdhA were constructed. It was possible that xylose could be used as an alternative carbon source in these strains and so permit growth by bypassing the requirement for functional electron transport and ATP synthase. However, the lack of growth of strain BS121 on SMM supplemented with xylose, and growth of the same strain on SMM supplemented with the non-metabolizable galactose analogue IPTG, indicate that this does not occur.

**Heterogeneous localization of integral membrane proteins**

The vital membrane stain FM4-64 has been used for many years as an indicator of the cell boundaries and cell membrane in cell biology and shows the membrane to be a smooth homogeneous structure enveloping the cytoplasm (e.g. Pogliano et al., 1999; Lewis et al., 2000). When exponentially growing cells of strain 168 (Table 1) were stained with FM4-64 a homogeneous band outlining the cells was observed, and this was confirmed when a linescan through the top border of the cells was performed (Fig. 2A). The peaks in the trace correspond to the division septa that contain two membranes and so appear more heavily stained. When exponentially growing cells of strains BS24 (atpA–gfp) and BS112 (sdhA–gfp) were visualized using the same image processing techniques a much more heterogeneous staining pattern was observed (Fig. 2B, C). To test the prevalence of this phenomenon, the localization of a completely unrelated integral membrane protein, p16.7 from the *B. subtilis* phage ϕ29, was tested. In the absence of other phage proteins, p16.7 is simply a small integral membrane protein with no known role in *B. subtilis* physiology. The localization of a p16.7–GFP fusion has been previously reported and shown to be confined to the cytoplasmic membrane in a pattern very similar to FM4-64 (Meijer et al., 2001). However, we found that the p16.7–GFP localization pattern was indistinguishable from that of either SdhA–GFP or AtpA–GFP (Fig. 2D), suggesting that this punctate distribution pattern was most likely a general feature for the localization of integral membrane proteins. An explanation for the apparently different localization pattern for p16.7 found by us and by Meijer et al. (2001) is given in the Discussion.

Although the above results indicate that membrane proteins preferentially localize to submembranous domains, they do not tell us whether this localization is to the same or different domains. The rather punctate pattern of distribution was intriguing, and it was possible that electron transport (succinate dehydrogenase) and ATP synthesis were closely juxtaposed within the cell due to their connected roles in energy generation. There is some circumstantial evidence for close juxtapositioning of these enzymes, as it is possible to isolate respiratory chain supercomplexes containing various combinations of components of the electron-transport chain and ATP synthase (Schägger, 2002). In order to address this issue, dual-labelled cells were constructed containing atpA–cfp and sdhA–yfp fusions (strain BS133; Table 1). In this strain the fusions were inserted by single crossover into the chromosomal locus, similar to their respective single-labelled strains, with expression of genes downstream of both *atpA* and *sdhA* dependent on the presence of xylose (see Fig. 1A, B). For reasons we cannot explain, this appeared to be the only combination of fusions that gave reproducibly bright signals, despite numerous attempts to construct strains in which one fusion was expressed ectopically from the *amyE* locus or when one of the fusions was placed under the control of the IPTG-inducible *P*pscA promoter, which we know produces functional fusions (Fig. 1A, B) and gives bright signals in single-labelled strains (not shown). Nevertheless, chromosomal PCR and fluorescence microscopy checks indicated that strain BS133 contained the correct constructs in the right chromosomal context, and growth on SMM supplemented with xylose indicated that the strain produced functional fluorescent protein fusions (Fig. 1A).

Fluorescence micrographs of strain BS133 grown in CH medium supplemented with 0.5% (w/v) xylose at 37°C are shown in Fig. 3. Fluorescence crossover experiments

![Fig. 2](image-url)
confirmed that there was no detectable crossover of CFP signal into the YFP channel and vice versa (not shown). The fluorescence patterns of both CFP-labelled ATP synthase and YFP-labelled succinate dehydrogenase appear very similar to that observed for the single-labelled strains (compare Fig. 2B and C with Fig. 3A and B). The ATP synthase signal was pseudocoloured red, while the succinate dehydrogenase was pseudocoloured green in the overlay shown in Fig. 3(C). Usually, when red and green signals of approximately equal intensity coincide in overlays, a yellow colour is observed. While yellow can be seen in the overlay, there are clearly regions of either green or red signal, implying that there was relatively little colocalization of the signals. However, careful examination of the linescan shown in Fig. 3(D) indicates that there were substantial regions of signal overlap, but that there was considerable variation in the intensity of red or green signal at some regions of overlap that may have given rise to the appearance of a red or green region in the overlay in Fig. 3(C) (see regions arrowed in Fig. 3D). However, the linescan also shows that the signals are not perfectly coincident, with considerable regions of signal heterogeneity. Analysis of multiple fields of cells indicated a mean level of 62% signal overlap with respect to the number of AtpA-CFP peaks.

Overall, we feel these results are consistent with the localization of both proteins to submembranous regions, with both enzymes present in similar, at least partially overlapping, domains.

**Integral membrane proteins localize in large amorphous patches**

A more detailed analysis of the localization of integral membrane proteins was carried out using image stack deconvolution and 3D reconstruction. Results are presented for the AtpA–GFP fusion, but SdhA and p16.7 fusions have similar localization patterns.

A 3D reconstruction of ATP synthase localization in a pair of cells in a series of orientations is shown in Fig. 4(A) and as an animation in supplementary movie 1 with the online version of this paper at http://mic.sgmjournals.org. The 3D rendition clearly shows heterogeneously distributed ATP synthase over the membranes. There is no clearly ordered organization of the enzyme; rather it appears to be distributed in a series of discrete foci and larger heterogeneous patches. A more detailed analysis of the image stacks was used to confirm this observation. Three image stacks were used to confirm this observation.

---

**Fig. 3.** Colocalization of AtpA–CFP and SdhA–YFP. (A) AtpA–CFP fluorescence; (B) SdhA–YFP fluorescence; (C) an overlay of the two signals. AtpA–CFP has been pseudocoloured red and SdhA–YFP pseudocoloured green. A linescan around the pair of rightmost cells indicated with the arrow using the same colour assignment is shown in (D); the arrows above the linescan indicate regions of signal colocalization.
slices taken from positions indicated in the top right panel corners are shown in Fig. 4(B). A slice from towards the top of the cell is shown in red, from the middle in green, and towards the bottom in blue. These three slices were overlaid (Fig. 4B bottom right panel) and a linescan made around the margin of the overlaid image; the arrow indicates the start point of the scan. Colour coding is the same as in (B). In (B) and (C), ‘a’ and ‘b’ refer to a diffuse domain and discrete focus, discussed in more detail in the text.

Fig. 4. 3D localization of ATP synthase. (A) A contrast-inverted 3D rendering of ATP synthase localization. The numbers indicate the degrees of rotation of the image. (B) Three processed image slices taken from near the top (red), middle (green) and bottom (blue) of the cell. An overlay of the three slices is shown in the fourth panel. (C) A linescan around the margin of the overlaid image; the arrow indicates the start point of the scan. Colour coding is the same as in (B). In (B) and (C), ‘a’ and ‘b’ refer to a diffuse domain and discrete focus, discussed in more detail in the text.

of recently described actin-like cytoskeletal filaments (see Discussion; Jones et al., 2001; Kruse et al., 2003).

Protein mobility within the membrane

Given the heterogeneous localization of proteins in the membrane, we wanted to know whether the observed localization represented protein restricted to specific domains within the cell membrane, or whether proteins were free to diffuse around the membrane. Time-lapse movies of the AtpA–GFP fusion strain were obtained at 1 min intervals over 30 min. A full movie sequence is provided in supplementary movie 2 with the online version of this paper (http://mic.sgm.journals.org), and a more detailed analysis of a 4 min period of the time-course is shown in Fig. 5. It is clear from both the movie and Fig. 5 that the localization of AtpA–GFP is highly dynamic within the membrane. Images taken at 0, 2 and 4 min into the time-lapse are shown in red, green and blue, respectively in Fig. 5(A) along with an overlay of the three images. The lack of coincidence of the signals in the overlay panel presents a graphic illustration of the movement of AtpA–GFP over the imaging period. A linescan around the cell, starting at the point indicated by the arrow, is shown in Fig. 5(B). The movement of a small focus of AtpA–GFP, marked ‘a’, ‘b’ and ‘c’ in the red, green and blue panels, respectively, was mapped for further analysis (Fig. 5A, B). This particular focus appears to start near the cell pole (a), diffuse slightly and move away from the pole (b) then move back towards the pole (c), suggesting that movement was due to random diffusion through the membrane. However, interpretation of these images is complicated, with unequivocal assignment of foci in each panel difficult. It may be that the focus mapped in ‘b’ and ‘c’ starts at ‘a’ rather than ‘a’ (Fig. 5A, B). If this is the case, then the time-lapse images suggest that movement is unidirectional within the membrane. However, it is not possible to draw such conclusions from this experiment as it is unlikely that all the images were obtained in exactly the same focal plane. As indicated by the 3D reconstruction images in Fig. 4, a small change in focal plane could result in focusing on a very different distribution pattern for AtpA–GFP. In order to resolve the issue of whether movement within the membrane was random or directed, we performed fluorescence recovery after photobleaching (FRAP) experiments using a confocal microscope system. Following bleaching of a portion of a cell, the recovery of fluorescence could be monitored to determine if it is uni- or bi-directional. Unidirectional recovery would imply that Atp–GFP moved around the observed plane of the cell in one direction, whereas bi-directional recovery implies random movement of Atp–GFP.

The results of a FRAP experiment are shown in Fig. 6. Images of cells before, immediately following, 3 and 6 min after photobleaching are shown in Fig. 6(A). Linescans from the top right cell in Fig. 6(A) are shown in Fig. 6(B), with the arrow indicating the start of the linescan. Linescans were made down the left-hand (L) and
right-hand (R) sides of the cell and are shown in Fig. 6(B). The colour scheme used in Fig. 6(A) is retained in Fig. 6(B) for comparative purposes with the region that was bleached lying to the right of the dashed line in the linescans. The cells indicated by ‘a’ in Fig. 6(A) were totally bleached, and the lack of fluorescence recovery in these cells indicates that there was no detectable de novo synthesis of AtpA–GFP over the course of this experiment.

Following photobleaching, the intensity of fluorescence in the bottom half of the analysed cell dropped considerably (compare the red and green panels in Fig. 6A and the red and green linescans in Fig. 6B). At 6 min following photobleaching, fluorescence recovery throughout the cell appeared to be largely complete (cyan panel and linescan in Fig. 6A and B). The image taken 3 min following photobleaching represents an intermediate stage between bleaching and recovery (blue panel and linescan Fig. 6A and B). Comparison of the linescans through the left- and right-hand side of the cell indicated that the rate of fluorescence recovery was approximately the same on both sides (Fig. 6B), which was observed in all FRAP experiments. This result suggests that AtpA–GFP movement within the membrane is most likely due to random diffusion.

**DISCUSSION**

We have shown in this study that microbial membrane proteins appear to be restricted to domains rather than being homogeneously distributed within the cytoplasmic membrane. This appears to be a general property, which was analysed with GFP-tagged subunits of ATP synthase (AtpA) and succinate dehydrogenase (SdhA) as well as the *B. subtilis* phage φ29 protein p16.7. All the proteins appeared to have a similar heterogeneous pattern of localization that was strikingly different from the distribution of total membrane when observed with the membrane stain FM4-64. The localization pattern for p16.7 has previously been reported and described as being similar to that of FM4-64 (Meijer *et al.*, 2001). Indeed, in this work we used the same strain, 110WA, to observe p16.7 localization. However, we do not feel that our results contradict those previously reported; rather, our analysis and interpretation has been more focused on the signal heterogeneity. In addition, our images were processed to remove out-of-focus light (see Methods) whereas Meijer *et al.* (2001) used unprocessed images. Both sets of results indicate that p16.7 is distributed within the cytoplasmic membrane, and it is possible to observe signal heterogeneity in the insert box of Fig. 5 in Meijer *et al.* (2001), indicating that strain 110WA gives similar results in our hands to those of Meijer *et al.* (2001).

Dual-labelling experiments with AtpA–CFP and SdhA–YFP fusions suggested that membrane proteins localize to approximately similar submembranous domains, as observed by the partial colocalization of AtpA–CFP and ShdA–YFP signals (Fig. 3). However, the degree of colocalization of these signals also indicated that it is very
unlikely that ATP synthase and succinate dehydrogenase
aggregate together in specific ‘energy-generating’ domains,
but rather that membrane proteins segregate to domains
that are approximately similar. Heterogeneities observed
between the two signals could be due to differences in
phospholipid preferences for the membrane complexes
and/or the levels of expression of the genes. ATP synthase
fusions were extremely bright in comparison to the other
fusions, possibly indicating a higher level of expression
of this operon, and it has been reported that the level of
expression of the atp operon is extremely high (Santana
et al., 1994).

3D reconstruction of ATP synthase distribution indicated
that protein domains varied between small concentrated
foci to rather large diffuse domains that spread over a
significant proportion of the cell (Fig. 4 and supplementary
movie 1). These imaging experiments were performed in
live, non-fixed cells and so it is possible that the domains
were moving during the image acquisition process. This
did probably occur, although we feel that movement was
not large during the acquisition process. At 200 ms expo-
sures, 60 images could be acquired in little over 12 s. As
seen in Figs 5 and 6, movement was monitored over a period
of minutes, not seconds. Time-lapse movies taken with
short (5 s) gaps showed little observable movement of
protein domains over a 10–15 s period (see supplementary
Fig. S1 at http://mic.sgm.journals.org). Therefore, we feel
the results presented in Fig. 4 and supplementary movie
1 do provide a reasonable snapshot of ATP synthase and
other integral membrane protein distribution around the
membrane.

We also analysed the dynamic distribution of ATP syn-
thase in more detail, and time-lapse microscopy experi-
ments showed that the irregular protein domains were
highly mobile within the membrane (Fig. 5). It was not
difficult to conclude from these experiments whether move-
ment within the membrane was unidirectional (specific) or
bidirectional (random). Further experiments (Fig. 6) moni-
toring the recovery of fluorescence around the membrane
in cells where half the membrane was bleached indicated
that movement of the protein domains was most likely by
random diffusion.

In eukaryotic cells, lipid rafts are known to have contacts
with the actin cytoskeleton via actin-binding proteins such
as annexins (Edidin, 2003), and many proteins are known

Fig. 6. FRAP of the AtpA–GFP fusion. (A) A group of cells analysed in a FRAP experiment. Cells prior to bleaching are
shown in red, immediately after bleaching in green, 3 min post-bleach in blue, and 6 min post-bleach in cyan. (B) Linescans of
the cell indicated by an arrow in (A) down the left and right hand margins, indicated by L and R, respectively in (A). The colour
coding is the same as in (A). The letter ‘a’ indicates a pair of cells that were totally photobleached and failed to redevelop
fluorescence during the experiment (see text for more details). The dotted line in the linescans in (B) indicates the approximate
rightmost limit of photobleaching.
to interact with actin filaments, including enzymes involved in glycolysis (Minaschek et al., 1992). It has recently become clear that bacteria also contain filamentous structures composed of proteins that belong to the actin superfamily, and that these cytoskeletal filaments form regular helical structures that are probably closely juxtaposed with the cytoplasmic membrane (Jones et al., 2001; Kruse et al., 2003). If there were discrete ‘energy-generating domains’ within the cell, it is possible that glycolysis, the tricarboxylic acid cycle, electron transport and ATP synthase could be closely juxtaposed. However, we found no evidence for the proteins examined in this study localizing in a pattern consistent with a connection with cytoskeletal filaments. In addition, we found our protein patches to be highly mobile within the membrane, with substantial movements observed over a period of a few minutes. FRAP of the Mbl cytoskeleton in B. subtilis occurred over a period of tens of minutes (Carballido-López & Errington, 2003), indicating that the dynamics of protein movement in membranes and cytoskeletons occurs on different timescales. Most likely, integral membrane proteins are simply inserted into the membrane directly via translation from ribosomes with little or no further connection with the cytoplasm. We do know that ribosomes preferentially localize towards the cell poles (Lewis et al., 2000), and membrane protein localization does not reflect a similar polar bias. However, once inserted into the membrane, it is clear that most proteins (or those that do not have a specific function dependent on their subcellular localization) are free to diffuse within the membrane and so would have a localization pattern unrelated to their site of synthesis.

In conclusion, integral membrane protein localization is probably dependent on the lipid distribution within the membrane, and is highly dynamic and random. Given the apparent preference of these proteins for submembranous domains, it will be interesting to determine whether formation of these protein-rich domains is correlated with lipid raft distribution.

ACKNOWLEDGEMENTS

This work was supported by the Australian Research Council through grants A00105184 and DP0449482 and University of Newcastle RMC grants to P.L. P. L. acknowledges the help of Scott Merrington and Eileen McLaughlin with confocal imaging and FRAP experiments.

REFERENCES


