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**Clustering and dynamics of cytochrome *bd*-I complexes in the *Escherichia coli*
plasma membrane *in vivo***

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SUMMARY

The cytochrome *bd*-I complex of *Escherichia coli* is a respiratory terminal oxidase and an integral component of the cytoplasmic membrane. As with other respiratory components, the organisation and dynamics of this complex in living membranes is unknown. We set out to visualise the distribution and dynamics of this complex *in vivo*. By exchanging *cydB* for *cydB-gfp* on the *E. coli* chromosome, we produced a strain (YTL01) that expresses functional GFP-tagged cytochrome *bd*-I terminal oxidase complexes under wild-type genetic control. We imaged live YTL01 cells using video-rate epifluorescence and Total Internal Reflection Fluorescence (TIRF) microscopy in combination with Fluorescence Recovery after Photobleaching (FRAP) and saw mobile spots of GFP fluorescence in plasma membranes. Numbers of GFP molecules per spot were quantified by step-wise photobleaching giving a broad distribution with a mean of ~76, indicating that cytochrome *bd*-I is concentrated in mobile patches in the *E. coli* plasma membrane. We hypothesise that respiration occurs in mobile membrane patches which we call “respirazones”.

INTRODUCTION

Oxidative phosphorylation (OXPHOS) is a multi-step membrane process, which is the result of the concerted activities of multiple enzymes. These enzymes have been best characterized in mammalian mitochondria, where they are found in the inner mitochondrial membrane. In prokaryotes, the OXPHOS membrane is usually the plasma membrane. Research on this subject continues to focus mainly on the structure and function of the individual OXPHOS complexes (for review see Rich, 2003) and regulation of expression at the transcriptional level in prokaryotes (for review see Unden, 1997). Relatively little is known about the supramolecular organisation and interactions of OXPHOS complexes in intact bioenergetic membranes. Two contrasting models of organisation have been proposed. For a review of the debate, see Lenaz and Genova (2007). The Random Diffusion model proposes that there is no large-scale organisation of complexes: they are randomly dispersed and functionally connected mainly by lateral diffusion of small redox carriers. "Solid-state" models however suggest that electron transport happens within stable supercomplexes of the respiratory proteins. In agreement with this idea, OXPHOS supercomplexes or "respirasomes" have been isolated from membranes of mammalian, plant and yeast mitochondria and the bacterium *Paracoccus denitrificans* by blue-native polyacrylamide gel electrophoresis (BN-PAGE) (Schägger, 2002). Structures of these supercomplexes have been resolved by single particle cryo-electron microscopy (Dudkina *et al.*, 2006; Schäfer *et al.*, 2006).

More generally, the traditional Fluid Mosaic Model of biological membranes (Singer and Nicholson, 1972), is being supplanted by the "Partitioned" or "Compartmentalized Fluid" model (Kusumi *et al.*, 2005; Engelmann, 2005; Marguet *et al.*, 2006). This new membrane paradigm sees non-random distributions of membrane proteins as the norm rather than the exception.

An attractive approach to studying the organisation and dynamics of membrane proteins *in vivo* is to use gene fusions to tag proteins with Green Fluorescent Protein (GFP) or variants. Fluorescence microscopy can then be used to observe the behaviour of the protein in the intact membrane. Such an approach has been applied to OXPHOS complexes by Johnson *et al.* (2004).

These authors showed that OXPHOS complexes (ATP synthase and succinate dehydrogenase) are heterogeneously distributed in mobile patches in the *Bacillus subtilis* plasma membrane. However, the genes were expressed using heterologous promoters, raising the possibility of artefacts due to overexpression and expression of complexes in a non-physiological context. The dynamic localisation of the proteins was investigated only at low time-resolution (frames recorded at 1-minute intervals) and the number of GFP molecules in the mobile patches was not quantified.

We set out to investigate the question further by GFP-tagging of OXPHOS complexes in the plasma membrane of *E. coli*. In contrast to mammalian and plant mitochondria, *E. coli* possess multiple, branched electron transport chains that are employed under various conditions to perform OXPHOS, depending on substrate availability (Uden and Bongaerts, 1997). In our study we investigate the cytochrome *bd*-I complex, which is encoded by the *cydAB* operon. In aerobic respiration, the cytochrome *bd*-I complex is one of two terminal oxidases that catalyse the final transfer of electrons from ubiquinol to molecular oxygen. The complex has been characterized as a heterodimer: subunit 1, encoded by *cydA*, is a 57kDa haem binding protein and a b-type cytochrome; subunit 2, encoded by *cydB*, is a 43kDa haem-binding protein and a d-type cytochrome (Miller and Gennis, 1983). It is maximally expressed in cells under microaerobic conditions, but is relatively repressed under aerobic and anaerobic conditions. Transcription of the other ubiquinol oxidoreductase, the 4-subunit cytochrome *bo* complex, encoded by the *cyoABCD* operon, is however contrastingly induced in aerobic conditions and repressed in microaerobic and anaerobic conditions (Iuchi *et al.*, 1990; Cotter *et al.*, 1990). The two complexes are spectroscopically distinct. Another *bd*-type complex encoded by the *cyx* or *cbd* operon exists in the *E. coli* genome but it does not appear to have respiratory function in wild-type cells (Sturr *et al.*, 1996; Dassa *et al.*, 1991).

Here we have applied live-cell fluorescence imaging techniques to document the organisation and dynamics of functional GFP-tagged cytochrome *bd*-I complexes in living *E. coli* cells on a millisecond time-scale. We engineered an *E. coli* strain, YTL01, which expresses

functional GFP-tagged cytochrome *bd-I* complexes from the native chromosomal locus and is therefore under wild-type genetic control. In YTL01 cells, we see fast-moving patches of GFP fluorescence in the plasma membrane, indicating that the cytochrome *bd-I* complex is localised into mobile membrane domains. Furthermore we are able to track the motion of individual patches and deduce the number of GFP molecules, and hence cytochrome *bd* complexes, present in them, from bleaching kinetics. We suggest that in *E. coli*, patches of the plasma membrane are dedicated to respiratory function by the preference of respiratory complexes to localise within these domains.

RESULTS

Construction of E. coli strain YTL01, expressing CydB-GFP

The C-terminus of the CydB subunit of the cytochrome *bd-I* complex is exposed on the cytoplasmic side of the membrane and appears not to be close to the active site of the complex (Zhang *et al.*, 2004). Thus it is a promising location for addition of a GFP tag. We used as a starting point a *cydB-gfp* fusion construct available from the ASKA Library (Kitagawa *et al.*, 2005). This construct codes for CydB with an N-terminal His-tag and C-terminus tagged with a GFPuv4 (Ito *et al.*, 1999) linked to CydB by a 5 amino-acid spacer. The *cydB-gfpgcn4* gene (without the His-tag) was transferred into the chromosomal *cydB* locus of *E. coli* BW25113 via a suicide vector as detailed in Experimental Procedures and illustrated in Figure 1. Candidate colonies were screened by fluorescence microscopy and colony PCR with primers that flanked the recombination sites. We found one colony with green fluorescence and this was also the only colony to give a PCR product of the expected size. Sequencing of the PCR product showed no mutations in the *cydB* domain (although there is a point mutation in the GFP tag: a C:T substitution at base 231 of the *gfpgcn4* domain converts a histidine residue to tyrosine). Thus, as illustrated in Figure 1, we produced a strain that is genetically identical to BW25113 with the exception of the substitution of *cydB-gfpgcn4* for the wild-type *cydB*.

Characterisation of CydB-GFP expression and function

Figure 2 shows a series of tests for the expression and functionality of CydB-GFP *in vivo*. YTL01 cultures were grown overnight in aerobic batch culture in LB medium and cells were fractionated into membrane, cytoplasmic and periplasmic fractions. Western blots of cell fractions with an anti-GFP antibody show a single band in the membrane fraction at ~50 kDa compared with free GFP at ~28 kDa (Fig. 2B). The observed band is very close to the 56kDa band predicted for the GFP-tagged CydB complex since CydB runs as a 28kDa band in 12.5% acrylamide SDS-PAGE (Kita *et al.*, 1984). No GFP was detected in any of the other YTL01 fractions or any BW25113 fractions (data not shown). Thus there is no indication of any free GFP in the cells: any GFP-fluorescence detected from the cells indicates the position of cytochrome *bd-I* complexes.

Fluorescence microscopy (detailed below) confirms that GFP fluorescence is associated with the plasma membrane. We saw no evidence of GFP association with inclusion bodies or other artefactual aggregates. In preliminary experiments in which we over-expressed CydB-GFP from a plasmid we observed large, bright, immobile patches of GFP fluorescence (not shown). However, we could not detect any such bodies in YTL01 cells.

Levels of cytochrome *bd-I* were quantified by room temperature absorption difference spectra (dithionite-reduced minus air-oxidized) (Green and Gennis, 1983). For these measurements we used cells grown to late exponential/early stationary phase in aerobic batch culture in LB medium. We found that growth to late exponential phase was necessary to give an absorption difference large enough to be quantifiable. However, even under these conditions, which appear to promote the highest levels of cytochrome *bd-I* expression, the difference spectrum is a small signal on a large background of cell absorption and scattering, and therefore we cannot measure cytochrome *bd-I* levels accurately. YTL01 and wild-type cells show a broad absorption peak centred at ~630 nm, consistent with the haem *d* in subunit 2 of the complex, encoded by *cydB* (Green and Gennis, 1983). This peak is absent in the *cydB*-null mutant from the Keio Collection (Baba *et al.*, 2006) (Fig. 2A). These spectra show that cytochrome *bd-I* is present in YTL01 cells at

approximately wild-type levels. Using the extinction coefficient of $27.9\text{mM}^{-1}\text{cm}^{-1}$ for the 628nm - 651nm wavelength pair (Tsubaki *et al.*, 1995), we can estimate that there are approximately 30000 - 40000 cytochrome *bd*-I complexes per cell in wild-type and YTL01 cells under these conditions.

To determine whether the GFP-tagged cytochrome *bd*-I complexes in YTL01 are functional for electron transport, we examined the effect of the respiratory inhibitor hydroxylamine on aerobic respiration in YTL01, wild-type and *cydB*-null cells from mid-exponential phase aerobic batch cultures (Fig. 2C, Table 1). Hydroxylamine has a much larger inhibitory effect on the cytochrome *bo* oxidase than *bd* (Meunier *et al.*, 1995). Oxygen consumption by cells in the presence of hydroxylamine therefore indicates cytochrome *bd*-I activity as cytochrome *bo* is inhibited. As expected, respiration in *cydB*-null cells is sensitive to 30 mM hydroxylamine. By contrast, YTL01 cells, like wild-type cells, are relatively insensitive to hydroxylamine (Fig 2C). This shows that the GFP tag on CydB does not interfere with the electron transport activity of the complex. Furthermore, the growth of YTL01 and wild-type strains in batch culture are similar whereas *cydB*-null cells grow much more slowly (Fig. 2D). Our results show that CydB-GFP is as active as the wild-type protein.

Visualisation of GFP fluorescence

GFP fluorescence in YTL01 cells was visualised using both epifluorescence and Total Internal Reflection Fluorescence (TIRF) microscopy. TIRF microscopy is essentially a modification of epifluorescence microscopy in which the laser excitation beam is directed at a very shallow angle so that it reflects off the interface between a glass coverslip and the aqueous medium containing the biological sample. Under these conditions an evanescent wave penetrates beyond the interface into the sample. The light intensity decays exponentially with distance from the interface, providing a working depth of field which is a function of the wavelength of the laser, and is about 100 nm for our experiments here. Therefore TIRF microscopy effectively gives an optical section of approximately 100nm in thickness at the coverslip-sample interface and is ideal for visualising

fluorophores in bacterial cell membranes, with minimal background from autofluorescence from the cytoplasm (Leake *et al.*, 2006). We used video-rate (25 Hz) image acquisition. Individual frames show the instantaneous distribution of fluorescence within the cell, whereas frame averages show the mean distribution over time.

Cells for fluorescence microscopy were grown to late exponential phase in LB medium, as for the spectroscopic estimation of cytochrome *bd-I* levels described above. However, we transferred the cells to fresh, oxygenated minimal medium a few hours prior to the measurements. The use of minimal medium was necessary to avoid background fluorescence from the LB broth. During fluorescence microscopy the cells were immobilised by adhesion to a polylysine-coated glass cover-slip, and trapped in 5-10 μ l of medium between the coverslip and a microscope slide. Two sides of the flow cell were left open to the air, however the arrangement did not allow us to control oxygen levels in the medium during the 1-2 hour time-span of the fluorescence measurements. Oxygen concentration would have decreased during this time if respiration by the cells were faster than replenishment of oxygen from the atmosphere at the edges of the coverslip. Since cytochrome *bd-I* expression is influenced by the oxygenation of the medium (Cotter *et al.*, 1990) we checked to see if mean cellular GFP fluorescence changed over the time-span of the measurements. However, we could see no significant trend (data not shown).

Frame-averaged images obtained using epifluorescence with the focal plane set at mid-cell height show a halo-like appearance around the perimeter of the cell, indicative of membrane localisation (Fig. 3A). Frame-averaged TIRF images show localisation in the membrane segment within \sim 100 nm of the coverslip surface (Fig. 3B). Observations of single cells in individual image frames at 25 Hz time resolution indicate the presence of fluorescent spots of apparent diameter \sim 400 nm diffusing in the cell membrane (Fig. 3C). Fig. 3C shows a frame with a particularly clear set of bright, distinct fluorescent spots. For a more complete impression of the distribution and dynamics of fluorescence see Supplementary Movie 1. The spots show heterogeneous fluorescence intensity and diffuse in the membrane over a time-scale of tens of milliseconds (Fig. 3C,

Supplementary Movie 1). The heterogeneity in spot brightness indicates that spots consist of clusters containing variable numbers of GFP-tagged cytochrome *bd-I* complexes. Individual spots could sometimes be tracked for over a second before they left the field of view. On this time-scale we could see no direct indication of additional dimmer spots breaking away or coalescing with a given tracked spot. This suggests that there is minimal dissociation or re-association of GFP-tagged complexes within this time window.

To permit detailed analysis on individual fluorescent spots, we improved the imaging contrast further by rapidly photobleaching one of the poles of each cell under investigation using an intense focused laser of width $\sim 1 \mu\text{m}$ (Fig. 3D). This allowed us to monitor Fluorescence Recovery after Photobleaching (FRAP) on a video-rate time-scale fast enough to track individual diffusing spots in either TIRF mode (Supplementary Movie 2) or epifluorescence mode (Fig 3E, Supplementary Movie 3).

Quantitative analysis of fluorescence images

Using robust custom-written tracking software utilising both automated detection and iterative Gaussian masking of pixel intensities (Leake *et al.*, 2006; Leake *et al.*, 2008) the positions of spots could be calculated to within a few nanometres precision (see Supplementary Methods 2 for details). In addition, using a method based on the step-wise photobleaching of individual GFP fluorophores (Leake *et al.*, 2006) we could estimate the number of CydB-GFP molecules in each spot (see Supplementary Methods 3 for details). The distribution is broad and roughly Gaussian with a mean of 76 ± 33 CydB-GFP molecules per spot (Fig. 4). Fourier frequency analysis of the distribution indicates a characteristic spacing of ~ 4 CydB-GFP molecules (Fig. 4 inset). This may indicate that the fundamental structural unit of cytochrome *bd-I* within a cluster is a tetramer. We estimate that there are on average 230 ± 80 clusters per cell. The calculation is detailed in Supplementary Methods 2: it depends on extrapolation from the limited region of the cell membrane that we observe in epifluorescence measurements. Note that in both epifluorescence

images (Fig 3C) and TIRF images (Fig. 3D) only a fraction of the membrane surface is within the field of view. We estimate about 10% in both cases. We can estimate that about $17,500 \pm 9,900$ CydB-GFP molecules per cell are associated directly with the clusters, and $2,300 \pm 830$ CydB-GFP per cell are present in the membrane but not confined in clusters (calculation detailed in Supplementary Methods 3). This leads to an estimated total of roughly $19,800 \pm 9900$ CydB-GFP molecules per cell. We estimate 30,000-40,000 cytochrome *bd* per cell from absorption difference spectra (Fig. 2A). Since this is a little higher than the mean number of GFP molecules per cell it suggests that at least the vast majority of CydB-GFP are fully assembled and redox-active. There is some uncertainty in both estimates of cellular CydB content, but the slight discrepancy in the numbers could also be due to the slightly different treatment of the cells (see Experimental Procedures, and discussion above). However, we could not detect any significant change in mean GFP fluorescence intensity as a function of time during the course of the fluorescence microscopy.

Our main datasets were recorded for cells at room temperature (about 20 °C). However, we also observed a qualitatively similar distribution of GFP fluorescence in cells at 37 °C (not shown). In general, the formation of the CydB-GFP clusters appears very robust. We saw no significant trends in mean GFP per cluster, or mean number of clusters per cell, as a function of time on the coverslip during the 1-2 hour timecourse of the fluorescence measurements (not shown). We saw considerable variation among cells in total GFP fluorescence, indicating widely varying levels of cytochrome *bd-I* expression in individual cells. There was a trend towards increasing GFP per cluster in cells with higher total GFP fluorescence (not shown). However, all cells showed qualitatively the same pattern of clustering of GFP fluorescence. Thus the formation of the cytochrome *bd-I* clusters does not appear particularly sensitive to the concentration of cytochrome *bd-I* in the membrane.

To analyse diffusion of spots in the cell membrane we transformed the spot position coordinates which were recorded in the Cartesian plane of the camera detector into a curvilinear system to represent the unique cell surface based on the length, width and orientation of each

individual cell as measured from the non-fluorescence brightfield image (Leake *et al.*, 2008). We then constructed a mean-squared displacement function (*MSD*) for each track, defined as follows (Gross and Webb, 1988):

$$MSD(\tau) = MSD(n\Delta t) = \frac{1}{N-1-n} \sum_{i=1}^{N-1-n} \{ [x'(i\Delta t + n\Delta t) - x'(i\Delta t)]^2 + [y'(i\Delta t + n\Delta t) - y'(i\Delta t)]^2 \}$$

A track consists of N consecutive image frames with a time interval τ ($n\Delta t$). Averaging all tracks showed deviation from linearity indicating anomalous diffusion behaviour (Fig. 5A). However, tracks could be separated on the basis of diffusive mode into either normal Brownian diffusion or restricted diffusion, using the relative deviation parameter which is defined as unity for pure Brownian diffusion and lower values for restricted diffusion (Kusumi *et al.*, 1993). See Supplementary Methods 4 for further details. The mean normal diffusion *MSD* function was fitted well by a straight line, indicating a diffusion coefficient of $0.05 \pm 0.02 \mu\text{m}^2 \text{s}^{-1}$ (Fig. 5B). The mean restricted diffusion *MSD* function was fitted using a mobility-confinement model (Kusumi *et al.*, 1993), indicating that the effective diameter of the confining zone is $160 \pm 30 \text{ nm}$ (Fig. 5C). Binning individual normal diffusion tracks on the basis of estimated CydB-GFP stoichiometry indicated an approximately inverse relationship between the stoichiometry and measured diffusion coefficient (Fig. 5D).

Figure 6A (black histogram) shows the apparent diameter of the fluorescent spots (defined as twice the fitted $1/e^2$ Gaussian radius) measured using TIRF illumination to avoid defocusing artefacts from epifluorescence. There is a broad spread, with a mean of $370 \pm 80 \text{ nm}$. However, the real dimensions of the membrane patches must be considerably smaller than this, since the blurring due to limited optical resolution is very significant at these scales. When fluorescence from a single GFP molecule is imaged in our microscope, it appears as a spot with mean diameter $270 \pm 30 \text{ nm}$ (Fig. 6A, grey histogram) which approximates to the point-spread function of the microscope. The difference between the two diameters gives a rough estimate of the real physical dimensions of the clusters (since fluorophores at the edge of the patch will produce fluorescence blurred out to a radius of about 135 nm beyond the boundary of the patch). Thus we can estimate a mean patch

diameter of about 100 nm. Surprisingly, the variation in apparent spot diameter was independent of the number of GFPs per spot (Fig. 6B), thus the number of cytochrome *bd*-I complexes per spot is not directly related to the area of the membrane patch.

DISCUSSION

Our results show that the cytochrome *bd*-I terminal oxidase in *E. coli* is concentrated in mobile domains in the membrane. This is qualitatively similar to the report of Johnson *et al.* (2004) on the dynamic localisation of succinate dehydrogenase and ATP-synthase in *Bacillus subtilis*. However, our methodology yields a level of quantitative detail on organisation and dynamics that is unprecedented for any respiratory protein. The GFP-fusion protein is expressed from its native locus, meaning that transcriptional regulation of the construct is under wild-type control. This eliminates the possibility that our observations are artefacts due to overexpression and/or expression in a non-physiological context. Furthermore, we find no significant physiological differences between wild-type and YTL01 cells. This strongly suggests that the observed distribution of cytochrome *bd*-I complexes is physiologically relevant, since we would expect abnormal distribution of the complex to result in abnormal function.

The live-cell imaging apparatus used is sensitive enough to distinguish the fluorescence signal of single GFP molecules from background electronic noise without frame averaging or long exposure times (Leake *et al.*, 2006). This sensitivity is mainly conferred by the electron-multiplying charge-coupled device (EMCCD) camera technology used but is further enhanced by using the apparatus in TIRF mode, where selective excitation of fluorophores close to the coverslip removes “pollution” of the signal from out-of-focus fluorescence and cytoplasmic autofluorescence. In addition, FRAP-style pre-bleaching removes considerable background fluorescence and results in a significantly improved imaging contrast. The high-sensitivity of the apparatus allows for images with meaningful signal-to-noise ratios to be captured at video rate, allowing us to observe events that happen on a short (tens of milliseconds) timescale and resolve mobile structures without

blurring. Using these high-speed imaging techniques in combination with single-particle tracking analysis, we have not only been able to observe clustering of the cytochrome *bd*-I complex, but also we could 1) quantitatively estimate the number of complexes within a cluster to a precision of single molecules; 2) obtain a distribution of cluster sizes within a single cell; 3) accurately characterise the mobility of the observed clusters with spatial resolution of a few nanometres and 4) estimate the real diameter of the clusters.

The mobile assemblages of the cytochrome *bd*-I complex are on a much larger scale than respiratory supercomplexes identified by biochemical means. The largest biochemically-characterised supercomplexes contain only four to six electron transport complexes (Boekema and Braun, 2007). By contrast, the assemblages that we observe *in vivo* contain on average about 76 cytochrome *bd*-I complexes. The assemblages are too small for us to be able to measure their physical dimensions accurately, but we can estimate a mean diameter of ~ 100 nm. This would correspond to a membrane area of ~ 8000 nm². Since no structure is available for cytochrome *bd*-I we cannot be sure of the membrane area occupied by a single complex. However, we can estimate this based on the predicted 17 transmembrane alpha helices of the cytochrome *bd*-complex (Zhang *et al.*, 2004). By extrapolation from the transmembrane portion of fumarate reductase, which has six membrane-spanning alpha helices and a diameter of ~ 3.8 nm (Iverson *et al.*, 1999) we can estimate an area of ~ 32 nm². So 76 cytochrome *bd*-I complexes would occupy a membrane area of ~ 2400 nm², only about 30% of the mean area of the membrane patches. Furthermore, apparent spot width does not increase with the number of cytochrome *bd*-I complexes (Fig. 6B), and there is a broad distribution of apparent widths of spots with the same number of CydB-GFP molecules. This suggests that the fluorescent spots observed mark patches of membrane containing rather loose assemblages of cytochrome *bd*-I complexes, as opposed to cytochrome *bd*-I multimers held together by strong, stable dimer-dimer interactions. By our estimates, these patches are large enough to contain other proteins as well. Preliminary results from GFP-tagging suggest that other *E. coli* OXPHOS complexes including NDH-2 (Lenn *et al.*, unpublished) and Complex I,

Complex II and Complex V (Thorsten Friedrich, personal communication) are also heterogeneously distributed in the membrane. We cannot yet be sure whether these OXPHOS complexes are all located in the same respiratory patches, or whether each complex is concentrated in separate patches. Careful co-localisation studies will be needed to answer the question. However, we can estimate that, in an average cell, the cytochrome *bd-I* patches will occupy about 23% of the membrane area. It seems unlikely that there could be space in the membrane for similar, separate patches of the other OXPHOS complexes, plus the numerous other protein structures that must be accommodated in the membrane. This suggests that co-localisation of other OXPHOS complexes within the cytochrome *bd-I* patches is likely.

At this stage we can only speculate about the forces that hold the cytochrome *bd-I* assemblages together. A network of protein-lipid and protein-protein interactions is likely to be involved. It is already known that cardiolipin is involved in the organisation of respirasomes (Zhang *et al.*, 2002; Pfeiffer *et al.*, 2003) and that membrane lipids are heterogeneously distributed in the *E. coli* plasma membrane (Matsumoto *et al.*, 2006). With specific regard to the *E. coli* cytochrome *bd-I* complex, its clustering may also involve YhcB, which has been identified as a putative third subunit of the complex by BN-PAGE (Stenberg *et al.*, 2005) but has also been shown to have no role in the assembly or enzymatic activity of the complex (Mogi *et al.*, 2006).

The existence of OXPHOS supercomplexes, discrete protein superstructures made up of various electron transport enzymes, in eukaryotic mitochondria and prokaryotes suggests that such structures may be characteristic of all respiratory membranes. While OXPHOS supercomplexes have not been identified in *E. coli*, this work emphasizes the theme that respiratory complexes are not freely diffusing “lone-rangers” in bioenergetic membranes. In addition to this, by counting GFP molecules, we show that there are patches in the cell membrane of *E. coli* where large numbers of cytochrome *bd-I* complexes congregate. Even though we have visualised just one respiratory complex here, our observations in combination with the general apparent prevalence of OXPHOS supercomplexes lead us to suggest as a working hypothesis that respiration occurs in “respirazones”

– membrane patches dedicated to respiratory activity. If respirasomes do exist in *E. coli*, we propose that multiple respirasomes congregate in "respirazones" (Lenn *et al.*, 2008).

The respirazone hypothesis predicts the compartmentalisation of membranes such that respiratory electron transport (and the associated redox sensing of the quinone pool) is segregated from other membrane functions by the concentration of respiratory enzymes within mobile domains. This is distinct from previous hypotheses about the organisation of OXPHOS complexes in that it considers the organisation of these complexes across the whole of the membrane surface rather than at the level of proteins and quinols.

We suggest that the physiological significance of respirasomes may be that membrane compartmentalisation, particularly of a membrane that is multifunctional as in *E. coli*, improves the specificity of reactions of the quinol respiratory intermediates. In an uncompartimentalised membrane, reduced quinols could non-specifically reduce membrane components, which could be structurally damaging and would be wasteful of respiratory substrates. If the ATPase is also associated with respirazones, the efficiency of the proton circuit could also be enhanced by the close proximity of proton pumps and sinks as proton diffusion at the membrane surface leads to decay of the proton motive force with increasing distance from the proton pump (Cherepanov *et al.*, 2003). Further studies will be required to determine the full protein composition of respirazones and the extent to which quinone diffusion and the proton-motive force are localised to specific patches in the membrane. It also remains to be determined whether or not all the inhomogeneously-distributed electron transport complexes participate equally in electron transport.

This work displays the potential of current fluorescence imaging technology on both biological and physical fronts. The high sensitivity of EMCCD technology and the improved contrast of both TIRF microscopy and FRAP-style pre-bleaching are combined here to resolve a unique combination of structural and dynamic detail in living cells, giving a new cellular perspective of a well-understood biochemical process.

On a more general level, our work shows that the plasma membrane of *E. coli* is heterogeneous on the 100 nm-scale, with randomly distributed patches containing concentrations of a particular membrane protein. These patches are mobile on the sub-second timescale. The bacterial plasma membrane appears to be a highly dynamic compartmentalised fluid.

EXPERIMENTAL PROCEDURES

Construction of E. coli strain YTL01

Strains and plasmids used are catalogued in Supplementary Table 1, and the construction of the plasmid pYTL01 is illustrated in Supplementary Figure 1. PCR primers used are given in Supplementary Table 2. *E. coli* BW25113 (wild-type) and Keio:cydB (*cydB*-null), strains were obtained from NIG, Japan (Baba *et al.*, 2006). The *E. coli* strain YTL01 was made in this study by a method based on that of Philippe *et al.* (2004). Here the *cydB-gfpuv4* fusion gene from the *cydB-gfp* ASKA plasmid (Kitagawa *et al.*, 2005) and the 533-bp sequence downstream of the *cydAB* operon from BW25113 genomic DNA were PCR amplified separately and then fused by overlap PCR (Shevchuk *et al.*, 2004). The resultant construct contained an Sph1 restriction site in *cydB* and a Pst1 restriction site, which was added by PCR with overhanging primers to the 3' end of the downstream 533bp sequence. The construct and pDS132 were sequentially digested with Pst1 and Sph1 and the approximately 2.4kb digestion product of the construct was ligated into the digested pDS132 to produce pYTL01, which was amplified and maintained in *pir*+ *E. coli* strain BW25141 (Datsenko and Wanner, 2000). Allelic exchange was effected between the chromosome of BW25113 and pYTL01 by chloramphenicol-sucrose counter-selection (Figure 1). The wild-type BW25113 (*pir*-) was transformed with pYTL01 by electroporation. Chloramphenicol resistant colonies, arising by integration of the pYTL01 into the chromosome of strain BW25113, were grown overnight in liquid culture without selection and plated onto sucrose plates to select for sucrose-resistant candidates where pYTL01 has been excised from the chromosome through a second recombination event. The desired sucrose-resistant-chloramphenicol-sensitive phenotype of

candidate recombinants was verified by replica-plating and candidate colonies were screened for the desired genotype by colony PCR with primers designed to anneal outside the sites of recombination. PCR products were sequenced to check that the GFP was correctly inserted and for point mutations

Growth of cells

The YTL01, BW25113 and Keio:cydB strains were grown to the growth phase required for each experiment in aerobic batch culture in Luria-Bertani (LB) broth (Sambrook *et al.*, 2001) at 37°C unless otherwise stated. The growth medium for Keio:cydB was supplemented with kanamycin (50µg/ml).

Growth curves

Growth of *E. coli* strains in LB batch culture was monitored by inoculating 100 ml of LB (with 50 µg/ml kanamycin where appropriate) in a 250ml conical flask with enough overnight culture to give cultures with identical initial OD₆₀₀ values of 0.06. Cultures were shaken at 200rpm at 37°C and the OD₆₀₀ was measured in a WPA Biowave II spectrophotometer at regular intervals.

Spectroscopy

Dithionite reduced-minus-air oxidised absorption spectra of whole cells in late exponential/early stationary phase were recorded by a Hitachi U3310 dual-beam spectrophotometer. Cells were grown to late exponential/early stationary phase in aerobic batch culture in LB medium and harvested by centrifugation. The pellets were resuspended in 20% (w/v) Ficoll (Sigma F4375) solution and the samples placed into quartz cuvettes. The cell suspensions were oxidised by bubbling with air and a baseline spectrum was then recorded. A few grains of sodium dithionite (Sigma 157953) were then added to the cuvette and after a few minutes, when the spectrum was

stable, the oxidised-minus-reduced spectrum was recorded. Cell concentrations were determined by OD₆₀₀ values calibrated by a hemocytometer count.

Respiratory electron transport

O₂ uptake of cell cultures was measured at 37 °C in a Clarke-type oxygen electrode (OxyLab 2, Hansatech, King's Lynn, UK). 1 ml of cell suspension from mid-exponential phase aerobic LB batch culture was placed into the electrode chamber, aerated and sealed from the atmosphere. Hydroxylamine (Sigma 467804) was added to a final concentration of 30mM in the reaction chamber when cells had reached a steady respiratory rate but had not yet used up all the oxygen in the medium.

SDS-PAGE and Immunoblotting

Cells were grown overnight in liquid LB batch culture and fractionated as previously described (Randal and Hardy, 1986), to obtain cytoplasmic, periplasmic and membrane fractions. Proteins from each of the fractions were separated by SDS-PAGE (12.5% acrylamide) and semi-dry electroblotted onto a Hybond-P PVDF membrane (Amersham Biosciences). Washed blots were probed with anti-GFP antibodies (Abcam ab6556) and horseradish peroxidase anti-rabbit IgG conjugates and imaged using the Amersham ECL™ detection system (GE Healthcare) in a Typhoon™ 9200 Variable Mode Imager (GE Healthcare).

Microscopy

YTL01 cells were prepared for microscopy by adding 100 µl of overnight LB culture to 1ml of minimal medium (M63-glucose (Atlas, 1996)) and incubating at room temperature without shaking in 1.5 ml microcentrifuge tubes for 3-4 hours. Cells were then adhered to polylysine-coated cover slips and imaged at room temperature in a flow cell containing M63-glucose medium. Cells were imaged with a home-built inverted fluorescence microscope (Leake *et al.*, 2006; Lo *et al.*, 2006; Lo

et al., 2007; Supplementary Methods 1). The microscope was used in both TIRF and epifluorescence modes with an excitation wavelength of 473 nm. Fluorescence was imaged at 25 Hz with a pixel width of about 50 nm using a 128x128-pixel, cooled, back-thinned electron-multiplying charge-coupled-device camera (iXon DV860-BI, Andor Technology, UK). Before switching to TIRF mode, the focal plane of the objective lens was set about 100 nm from the coverslip surface by focussing at the midpoint of fluorescent latex beads 200nm in diameter. Images were sampled continuously, typically for about 50 s. Where appropriate, pre-bleaching was performed by application of an intense focused 473nm laser spot of diameter $\sim 1 \mu\text{m}$ to one pole of the cell, typically for about 0.5 s. This permitted single-particle tracking and quantitative stoichiometric measurements of fluorescent spots diffusing into the bleached region.

Data analysis

Images were subjected to automated tracking using custom-written code which measured the intensity, position and number of diffusing spots in single cells (Supplementary Methods 2), and CydB-GFP stoichiometry estimated by utilising edge-detection algorithms (Leake *et al.*, 2003; Leake *et al.*, 2004) and Fourier analysis to measure the step-wise photobleaching of GFP (Leake *et al.*, 2006; Leake *et al.*, 2008; Supplementary Methods 3). Position data were used to measure diffusion for each spot and characterise anomalous behaviour (Supplementary Methods 4).

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LEGENDS TO FIGURES

Figure 1. Construction of *Escherichia coli* YTL01: Insertion of GFP into the BW25113 chromosome downstream of the *cydB* locus.

Figure 2. Characterisation of *E. coli* YTL01. (A) Reduced-minus-oxidised difference spectra of YTL01 cells in early stationary phase. (B) Immunoblot with anti-GFP antibodies: Lane 1) YTL01 membrane fraction; Lane 2) GFP standard. (C) Oxygen electrode traces showing the effect of 30mM hydroxylamine on respiration of *E. coli* cultures. Arrows indicate the addition of hydroxylamine to a final concentration of 30mM. ΔR values are the mean percentage change in respiratory rate upon addition of hydroxylamine of 5 replicate experiments, with standard deviation given in parentheses. (D) Representative growth of *E. coli* batch cultures: BW25113 (○), YTL01 (5) and Keio:*cydB* (∇).

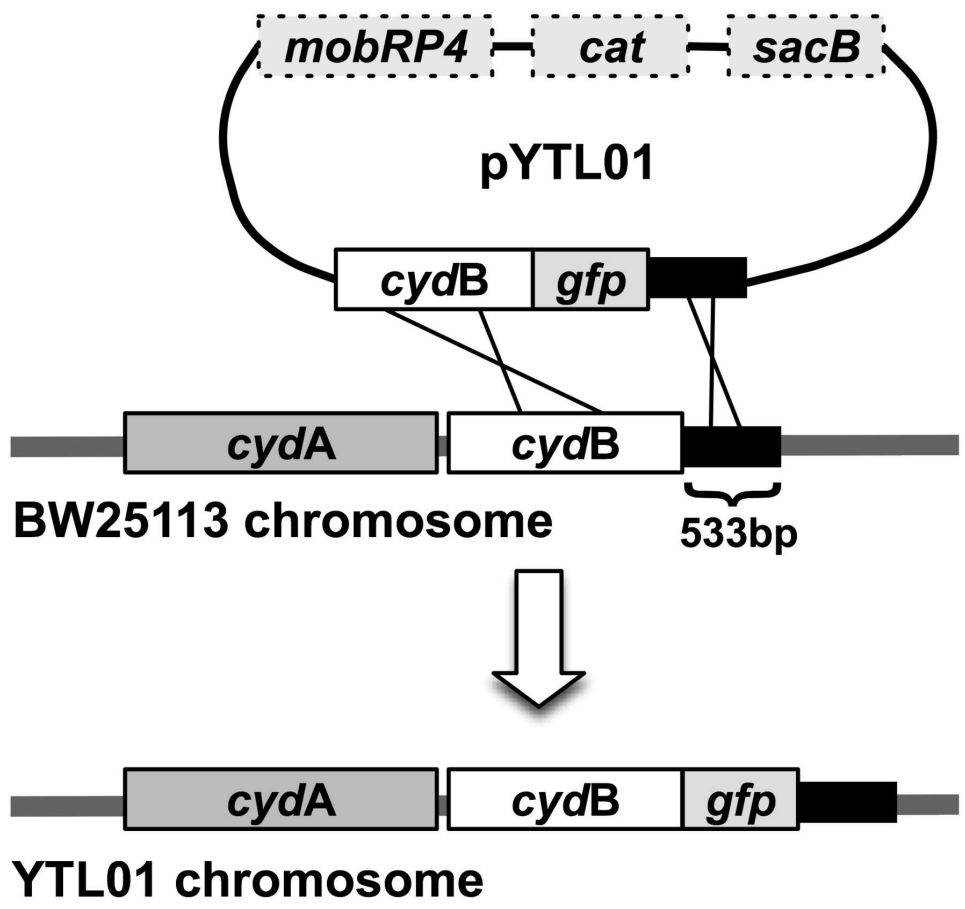
Figure 3. Visualisation of GFP fluorescence in *E. coli* YTL01. (A) Brightfield (left panel) and epifluorescence images (right panel, frame-averaged over 2 s) of same cell, focal plane 500 nm from coverslip surface. (B) TIRF images of a cell (frame-averaged over 2s) and (C) individual 40 ms frame (auto-detected fluorescent spots indicated (arrows), focal plane 100 nm from coverslip surface. (D) Epifluorescence images before and after bleaching one end of the cell. The circle indicates the laser focus width. Images 0 s and 10 s after bleaching are frame-averaged over 2 s. (E) same cell 5 s after FRAP bleach showing individual 40 ms frame with auto-detected fluorescent spots (arrows).

Figure 4. Stoichiometry of CydB-GFP complexes. Distribution estimated using step-wise photobleaching (Leake *et al.*, 2006; Leake *et al.*, 2008) of GFP of tracked fluorescent spots. The

power spectrum of the distribution (inset) indicates a periodicity of ~four CydB-GFP molecules per complex.

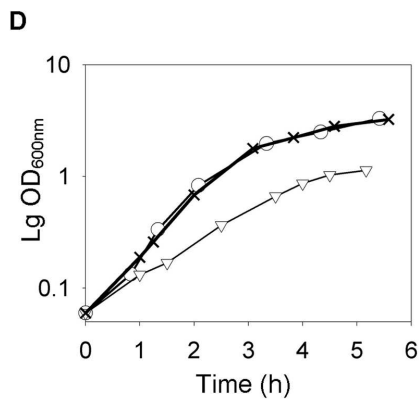
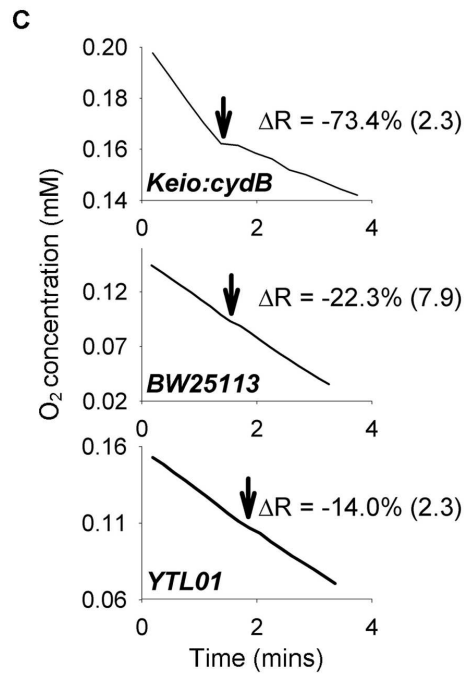
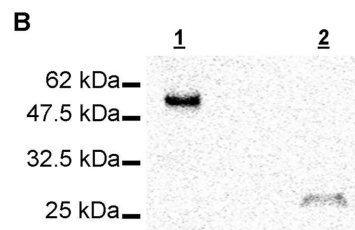
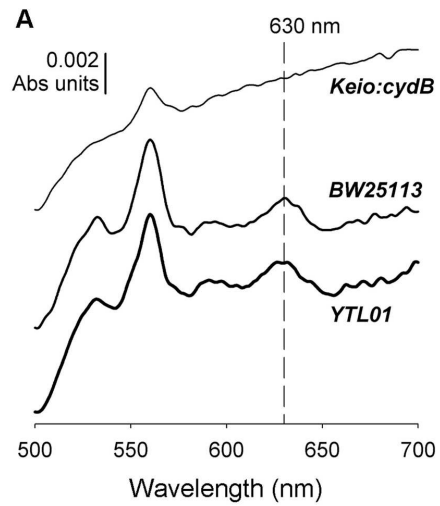
Figure 5. Mobility of tracked CydB-GFP complexes. (A) Estimate of mean-squared displacement (*MSD*) assuming two-dimensional diffusion over the cell membrane surface (mean of 103 tracks, s.d. error bounds indicated). (B) *MSD* (black, dotted error bounds) for normal diffusion tracks such that relative deviation parameter >0.3 , showing mean of 61 tracks with linear fit. (C) *MSD* (black, dotted error bounds) for confined diffusion tracks such that relative deviation parameter ≤ 0.3 , showing mean of 43 tracks with restricted diffusion fit (Kusumi *et al.*, 1993) for which asymptotic values of *MSD* approach $L^2/6$ where L is the effective diameter of a confinement zone. (D) Variation of estimated *MSD* with stoichiometry (grey), with binned values indicated (black).

Figure 6. A. Variation in fluorescent spot diameter using TIRF illumination on purified, surface-immobilised GFP (Leake *et al.*, 2006) indicated in grey, compared against fluorescent spots observed *in vivo* in YTL01 cells (black). B. Variation of spot width with estimated number of CydB-GFP molecules per complex (grey, binned value black with s.d. error bars) with linear fit.



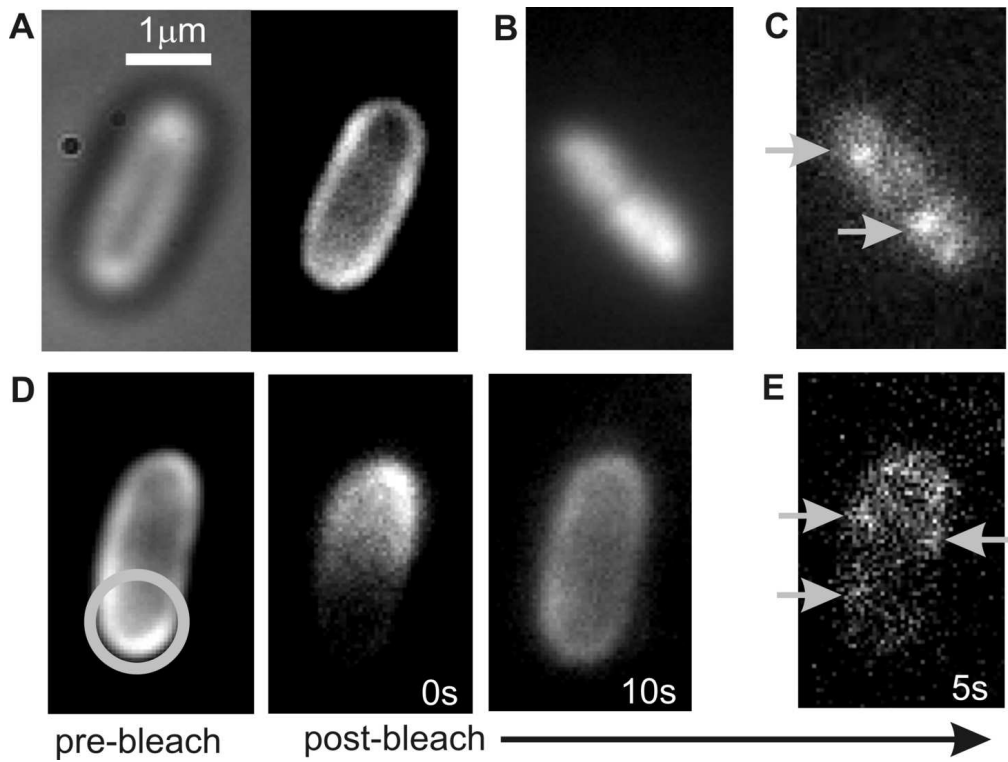
Construction of Escherichia coli YTL01
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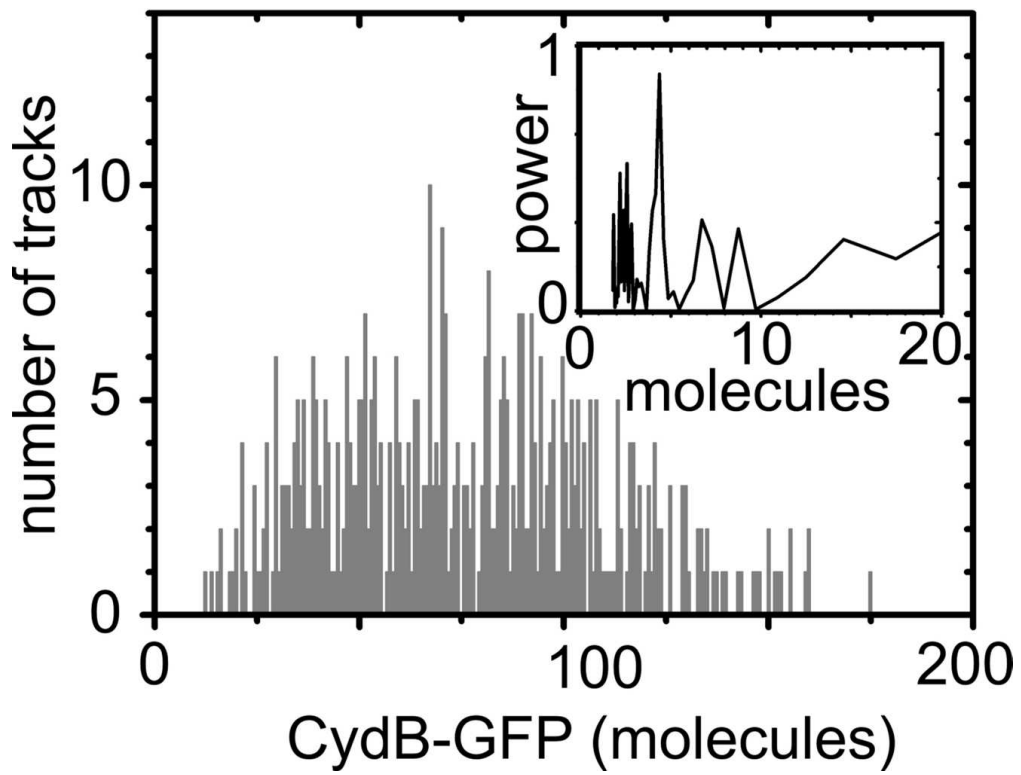
Characterisation of *E. coli* YTL01
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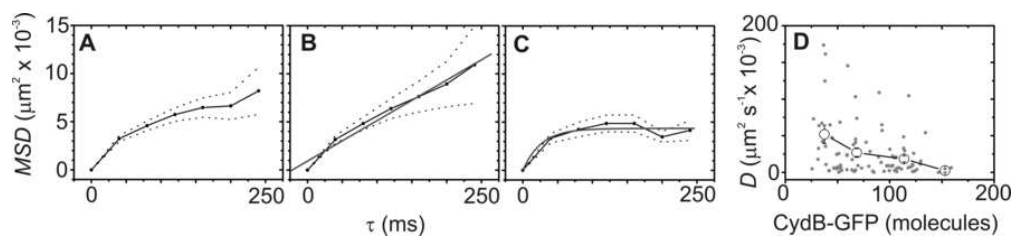
Visualisation of GFP fluorescence in *E. coli* YTL01
61x46mm (600 x 600 DPI)

review



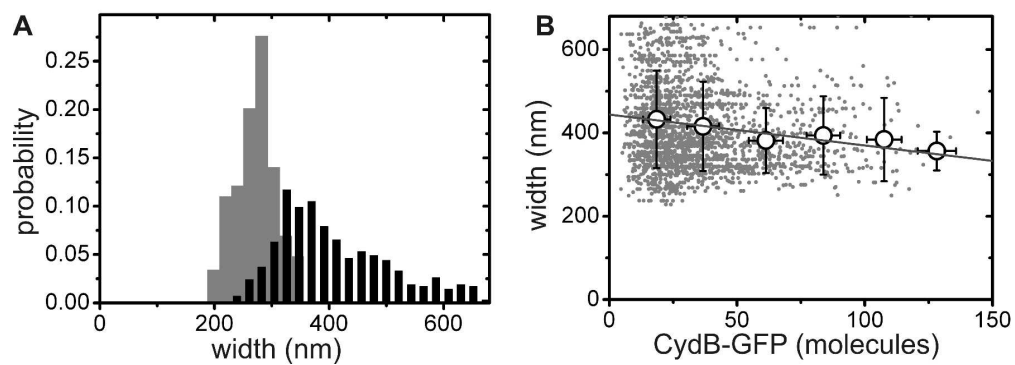
Stoichiometry of CydB-GFP complexes
56x43mm (600 x 600 DPI)

Review



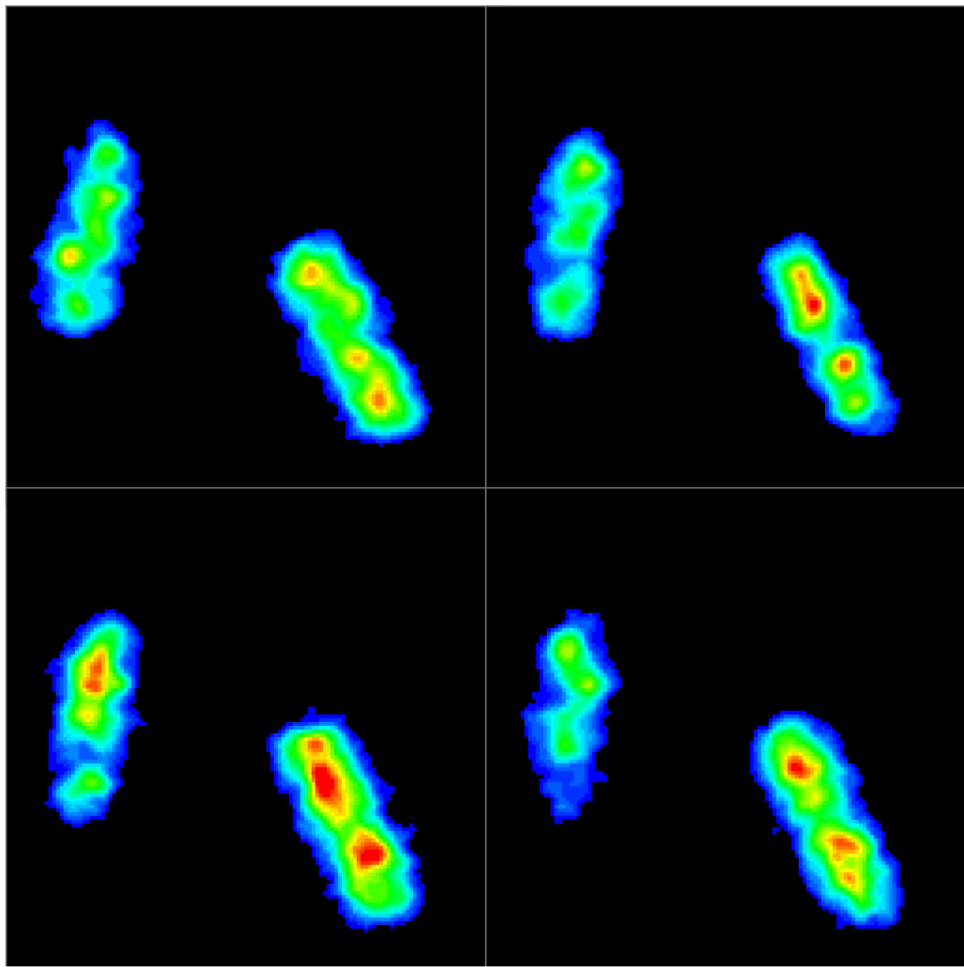
Mobility of tracked CydB-GFP complexes
37x8mm (600 x 600 DPI)

For Peer Review



Variation in fluorescent spot diameter
151x54mm (599 x 599 DPI)

Peer Review



99x100mm (600 x 600 DPI)

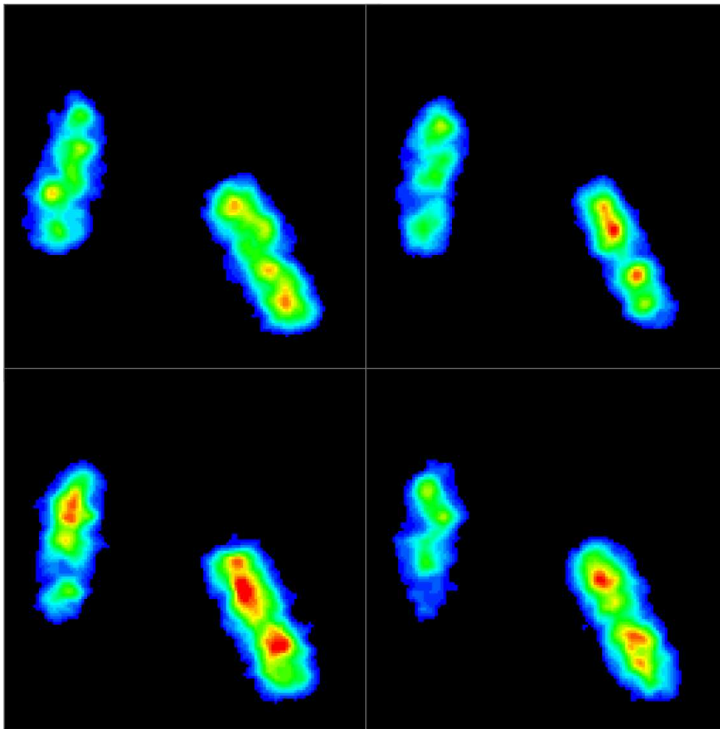


"Clustering and dynamics of cytochrome *bd*-I complexes in the *Escherichia coli* plasma membrane *in vivo*"

T. Lenn, M.C. Leake, C.W. Mullineaux

Description of Proposed Cover Illustration

False-colour snapshot fluorescence images showing the distribution of a GFP-tagged oxidase complex in the plasma membranes of two *Escherichia coli* cells. The complex is concentrated in highly-mobile membrane patches.



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