In vivo monitoring of the potassium channel KcsA in *Streptomyces lividans* hyphae using immuno-electron microscopy and energy-filtering transmission electron microscopy

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The previous discovery of the *Streptomyces lividans* *kcsA* gene and its overexpression followed by the functional reconstitution of the purified gene product has resulted in new strategies to explore this channel protein *in vitro*. KcsA has evolved as a general model to investigate the structure/function relationship of ion channel proteins. Using specific antibodies raised against a domain of KcsA lacking membrane-spanning regions, KcsA has now been localized within numerous separated clusters between the outer face of the cytoplasm and the cell envelope in substrate hyphae of the *S. lividans* wild-type strain but not in a designed chromosomal disruption mutant ΔK , lacking a functional *kcsA* gene. Previous findings had revealed that caesium ions led to a block of KcsA channel activity within *S. lividans* protoplasts fused to giant vesicles. As caesium can be scored by electron energy loss spectroscopy better than potassium, this technique was applied to hyphae that had been briefly exposed to caesium instead of potassium ions. Caesium was found preferentially at the cell envelope. Compared to the ΔK mutant, the relative level of caesium was $\approx 30\%$ enhanced in the wild-type. This is attributed to the presence of KcsA channels. Additional visualization by electron spectroscopic imaging supported this conclusion. The data presented are believed to represent the first demonstration of *in vivo* monitoring of KcsA in its original host.

INTRODUCTION

The lifecycle of streptomycetes is initiated by the germination of spores. Subsequent extension of the hyphae and branching leads to the formation of an extended filamentous network, the substrate mycelium. Upon depletion of nutrients aerial hyphae arise; within these hyphae, septa are formed, and then the resulting compartments containing genomic DNA develop into spores. These withstand dryness as well as cold conditions and rest in a dormant stage as long as nutrients are not available. Within the natural habitat streptomycetes play a major role in the turnover of organic material as they have a large repertoire of enzymes leading to the degradation of macromolecules including cellulose, lignocellulose, chitin, starch, xylan, proteins and lipids. Hence these enzymes are of great ecological and biotechnological value. Streptomycetes have a huge capacity to synthesize secondary metabolites, many of which have antibacterial, antifungal or cytostatic activities and are being

used in medicine (see reviews by Kutzner, 1981; Schrempf, 1999).

Within *Streptomyces lividans* the *kcsA* gene, which encodes a protein of 16 kDa with two predicted transmembrane helices, has been identified. Multiple alignments had suggested that KcsA has closest kinship to the P-region and its neighbouring transmembrane helices (S5 and S6) of eukaryotic voltage-gated K^+ channel families. Investigations of the *in vitro* reconstituted protein and of a newly established protoplast-vesicle system led to the discovery that KcsA acts as a potassium channel (Schrempf *et al.*, 1995). Being the first discovered bacterial protein functioning as a K^+ channel and the first one which can be obtained in large quantities (Schrempf *et al.*, 1995), it was subsequently crystallized without its C-terminal domain as a tetramer (Doyle *et al.*, 1998). The discovery of the bacterial KcsA channel has opened new avenues for exploring a channel protein. The increasing number of sequenced genomes led to the deduction of a range of bacterial proteins that could also function as ion channels (Derst & Karschin, 1998). Using 3D computer modelling, conserved sequences of transmembrane regions as well as the putative pore

Abbreviations: EELS, electron energy loss spectroscopy; EFTEM, energy-filtering transmission electron microscopy; ESI, electron spectroscopic imaging; IEM, immuno-electron microscopy.

elements within K^+ transporters and channels have been quantified and classified. Together with the analyses of results from mutational studies it has been concluded that K^+ symporter families from prokaryotes and eukaryotes have evolved from the prokaryotic K^+ channel proteins (Durell *et al.*, 1999; Durell & Guy, 2001). The findings were used to clone and overexpress a few additional bacterial proteins (as done for KcsA) and to generate their crystal structure in order to understand the general principle of ion channels (see review by Doyle, 2004). The results of experimental data (Meuser *et al.*, 2001) support the conclusion that the crystal structure of the tetrameric KcsA does not present the 'open state', as initially concluded (Doyle *et al.*, 1998). Hence several implications for ion permeation (including the hydration status of the permeating K^+ ion) need to be reinvestigated (Schrempf, 2005). Thus it will be an important future task to generate the crystal structure of the full-length protein trapped within the open state.

Energy-filtering transmission electron microscopy (EFTEM) allows the acquisition of electron energy loss spectroscopy (EELS) information or elemental maps by electron spectroscopic imaging (ESI), both being based on the detection of inelastically scattered electrons within the sample at an element-specific energy loss. The spatial resolution is in the range of 1 nm, when evaluating a series of energy-filtered images around an edge (Hofer *et al.*, 1999). EELS analysis has been adopted to investigate small biological samples (Somlyo & Shuman, 1982), including granules of vacuoles (Buckling *et al.*, 1998), the apical hair membrane within the cochlea of the guinea pig (Heinrich *et al.*, 1998), root cells of *Allium* (Liu & Kottke, 2003), polyphosphate granules in bacteria (Chavez *et al.*, 2004), and spores of bacilli and different structures of mycorrhizal fungi (Kottke, 1991; Cruz, 2004).

To obtain information about the *in vivo* localization of KcsA within its original host, we generated antibodies and a mutant of *S. lividans* carrying a disruption of its chromosomal *kcsA* gene. Using immuno-electron microscopy (IEM) we show that the KcsA channel is arranged in clusters within the cytoplasmic membrane of hyphae of the wild-type *S. lividans* and that it is absent in the mutant lacking a functional *kcsA* gene. In addition, EFTEM is shown, apparently for the first time, to be applicable to monitoring differences in ion accumulation in bacteria correlated with the presence or absence of ion channels.

METHODS

Strains and plasmids. *Streptomyces lividans* 66 (in the text designated *S. lividans*) (Hopwood *et al.*, 1985) and *Escherichia coli* M15 pREP4 (Qiagen), and the *E. coli* vector pQE32 (Qiagen) and the bifunctional vector pGM160 (Muth *et al.*, 1995) were used.

Media and cultivation of bacteria. *E. coli* colonies were grown on LB agar or in LB liquid medium (Sambrook *et al.*, 1989). Spores of *S. lividans* were inoculated in complete or minimal media (Schlochtermeyer *et al.*, 1992) in baffled Erlenmeyer flasks and

cultivated for 24–36 h. To generate spores, *S. lividans* was grown on complete medium containing agarose until sporulation occurred (Hopwood *et al.*, 1985).

Purchase of chemicals and enzymes. Chemicals for SDS gel electrophoresis were obtained from Serva. Other chemicals were supplied by Sigma.

Cloning of a truncated *kcsA* gene, isolation of the fusion protein and generation of antibodies. The vector pQE32 containing the C-terminal part of *kcsA* gene was described earlier (Schrempf *et al.*, 1995). To generate a truncated gene (encoding only the C-terminal portion) a 217 bp fragment was amplified using Vent polymerase, the primer 1 containing a *SphI* cleavage site and the primer 2 with a designed *HindIII* site. The primers had the following sequence: p1, CCGCGCGCATGCCCTGGTTCGTCGGCCG; p2, CGCACAAGCTTCGCAGACTCATCGGTGC.

The resulting fragment was cleaved with *SphI* and *HindIII* and ligated into the correspondingly cleaved pQE32 vector and transformed into *E. coli* M15 pREP4. The *E. coli* M15 pREP4 containing the correct construct was inoculated in LB medium with ampicillin ($100 \mu\text{g ml}^{-1}$) and kanamycin ($25 \mu\text{g ml}^{-1}$) and during its exponential growth phase (OD_{600} 0.6) induced with 1 mM IPTG for 4 h at 37°C . Cells were disrupted by sonication and cell debris was removed by centrifugation ($14\,000 \text{ g}$). Proteins were purified using Ni^{2+} NTA affinity chromatography. To remove impurities, the procedure was repeated. The isolated protein ($100 \mu\text{g}$) was used to generate antibodies in guinea pigs (EUROGENTEC Belgium). The antisera (from rabbit) were stored in aliquots at -20°C .

Preparation and analysis of membrane and cytosolic fractions of *E. coli*. *E. coli* M15 pREP4 containing the pQE32 construct with the KcsA or the truncated gene (see above) was grown and induced as described above. However, induction was done for 2 h only. After disruption of the cells, the debris was removed (see above). The supernatant was subjected to high-speed centrifugation (10 min , $100\,000 \text{ g}$). The pellet (membrane fraction) was washed and suspended in buffer containing dodecylmaltoside (Splitt *et al.*, 2000). The supernatant represents the cytoplasmic fraction. Corresponding aliquots were subjected to SDS-PAGE. A subsequent Western-blot analysis was pursued with the obtained antibodies (see above chapter).

Generation of the *S. lividans* mutant ΔK . The previously described vector pGM160 (Muth *et al.*, 1995) is a bifunctional *E. coli*/*Streptomyces* vector. Within streptomycetes its replication is temperature-sensitive. It contains an ampicillin-resistance gene (selection in *E. coli*) and (for selection within streptomycetes) the thiostrepton- and gentamicin-resistance gene (*aacC1*). The *aacC1* gene was replaced (after cleavage by *NcoI* and *HindIII*) by a correspondingly cleaved fragment which was constructed in the following fashion: the Omega-hyg fragment (Blondelet-Rouault *et al.*, 1997) with the hygromycin (*hyg*)-resistance gene (Fig. 1) was flanked with one PCR-generated 725 bp fragment (comprising 27 bp of *kcsA* and 698 bp of its upstream region) and another 696 bp fragment (comprising 57 bp of *kcsA* and 639 bp of its flanking downstream region) according to the previously described procedure (Xiao *et al.*, 2002). The resulting pGM160-based construct was transformed into *S. lividans* protoplasts (Hopwood *et al.*, 1985). Thiostrepton-resistant colonies were replica-plated (30°C) onto hygromycin-containing plates. Subsequently, the colonies were replica-plated (twice) on hygromycin-containing plates and incubated at 37°C . Under these conditions the replication of the designed construct ceases. Hence hygromycin-resistant surviving colonies will usually be the result of double crossing over among regions (flanking the Omega-hyg fragment) and those being homologous with the *S. lividans* chromosome (Fig. 1).

Fig. 1. Relative positioning of the *kcsA* gene (black) and the neighbouring genes (light grey) within the chromosomal DNA of *S. lividans* WT, and the Omega-*hyg* fragment (white box) with the hygromycin-resistance gene (*hyg*, in grey) followed by a transcriptional terminator (Ω) flanked by the small residual portions (black) of the *kcsA* gene (named *kcsA'* and '*kcsA*') within the DNA of the mutant ΔK .

EELS of CsCl crystals. Crystals were obtained on a 400-mesh Formvar-nickel grid by floating it on a drop of saturated aqueous solution of CsCl for a few seconds and air drying after removal of excess fluid with filter paper. The peak height was monitored as a function of the amount of crystal material included in the measuring point.

Preparation of mycelia for EELS/ESI measurements. Formvar-coated nickel grids were dipped onto a drop of a 0.1% solution of polylysine and blotted dry on filter paper at room temperature. Mycelia were disaggregated by repeated sucking in a Pasteur pipette, and a 30 μ l drop was pipetted onto a polylysine-coated grid which was then incubated for 10 min at room temperature in a moisture chamber, allowing settling of the mycelia and their adherence to the grid. Residual medium and excess mycelia were removed from the grid with filter paper. All grids were incubated 30 min at room temperature, floating on minimal medium containing 100 mM CsCl. Afterwards they were dipped on a 500 μ l drop of minimal medium containing only 10 mM CsCl for 10 s to avoid precipitation of caesium at and around hyphae. Adhering medium was removed carefully with filter paper and the grids were air-dried.

EELS analysis. The EELS analysis was done on a Zeiss-EM 902A equipped with an in-column Henri–Castain electron energy loss spectrometer. Spectra were drawn with an analogue *x-y* recorder. The cathode was saturated at 80 kV with the cathode-current setting at 1 μ A. The object was positioned in the centre of the fluorescent screen at a magnification of 20 000-fold or 85 000-fold and the condenser was maximally focused. A 100 μ m spectrometer-entrance aperture was positioned in the centre of the optical path, and an EELS registration area of 0.16 or 0.7 μ m in diameter was set (depending on the magnification). After correct positioning of the object, electron beam deflection and condenser focus settings were controlled again in order to achieve maximal brightness. The 0 eV point was positioned exactly on the sensor of the photomultiplier tube (PMT). The slit aperture was inserted, adjusted to an energy window of ~ 15 eV, and positioned to the PMT sensor. Spectra obtained by the PMT were recorded between 720 and 770 eV energy loss. The *x-y* recorder was set to 50 mV in the *x*- and 10 mV in the *y*-dimension. Caesium is recognized from the spectrogram by two peaks at 726 eV and 740 eV energy loss (M_{45} edges) (Ahn & Krivanek, 1983). The height of the first peak (726 eV) was measured in mm as depicted in Fig. 4(a).

Performance of ESI analysis. Specimens were prepared as described for EELS. Analysis was done using EFTEM software (Soft Imaging Systems). Images of 710 eV to 830 eV energy loss were

recorded with a cooled 1024 $\times$ 1024 CCD camera (Proscan CCD 512/1024; Proscan Electronic Systems) and analysed according to the three-windows-white-line method.

Quantitative comparison of EELS data obtained for two strains. Four measuring points of 0.16 μ m were positioned at the inner margins of hyphae as shown in Fig. 5. This procedure was repeated two to four times within one grid window and the mean peak height was calculated. Then the grid window or the grid was changed. In the same fashion, 100 spectra, subdivided into 25 groups of four, were recorded for each strain. Mean peak height and the standard deviation were calculated.

IEM. After fixation in 0.25% glutaraldehyde and 3.7% formaldehyde, bacteria were embedded in Lowicryl K4M (Roth *et al.*, 1981). Ultrathin sectioning was performed using a Reichert–Jung Ultracut E with a diamond knife (Reichert–Jung Diatome 45°). Ultrathin sections of *E. coli* cells or *S. lividans* hyphae were mounted on Formvar-nickel grids (300 mesh). Immuno-labelling was done on ultrathin sections of *E. coli* cells or *S. lividans* hyphae using the above-described anti-KcsA antibodies (first antibody). All incubations were done on 50 μ l drops with the grid on top of the drop, the sections facing downwards. Dilution of the antibodies was done in PBST [PBS containing 0.025% (v/v) Tween 20] supplemented with 0.2% (w/v) BSA. The grids were first incubated for 30 min on drops of PBS containing 2% (w/v) BSA, then 3 h on serum (diluted in PBST 1:50; 1:100 or 1:200) with the primary antibodies. Washing was done on drops of PBST three times for 10 min each. Afterwards the grids were incubated for 45 min on the secondary antibody (goat-anti-guinea pig IgG, coupled to ~ 6 nm gold, Aurion), diluted 1:40 in PBST, and washed further as described above. Grids were stained with 3% (w/v) neutralized phosphotungstic acid for 1 min, rinsed on drops of distilled water and blotted dry on filter paper.

RESULTS

Immunolocalization of KcsA in the heterologous *E. coli* host carrying a functional *kcsA* gene

An *E. coli* host carrying the *kcsA* gene with six histidine codons produces the resulting KcsA-Histag protein, which had previously been found to be predominantly located within the cytoplasmic membrane, from which it can be extracted using detergents (Schrempf *et al.*, 1995; Meuser *et al.*, 1999). The use of the intact KcsA protein (containing its two transmembrane helices) for generating antibodies resulted only in sera with relatively low titres. Therefore, as a prerequisite for immunological studies (for details see Methods), a stretch of the *kcsA* gene corresponding to the C-terminus (60 amino acids) of the KcsA protein was cloned into an *E. coli* pQE32 vector. After optimization of the expression and isolation conditions, the corresponding protein was purified and used to raise antibodies (named anti-C-KcsA).

E. coli carrying the full-length *kcsA* gene under control of the promoter of the *lac* operon (Schrempf *et al.*, 1995), the truncated *kcsA* gene (see above) or the *E. coli* control strain carrying the pQE32 vector were grown under inducing (IPTG) conditions. In extracts of the first strain proteins were detected (Fig. 2b, lane 3) corresponding to the

Fig. 2. Immunodetection of KcsA in *E. coli*. Cells of *E. coli* containing a pQE construct with the full-length (a, lanes 1, 4; b, lanes 1, 5) *kcsA* gene, or with the truncated gene or the vector alone (a, lanes 2, 5; b, lanes 2, 6) were harvested after induction with IPTG. After disruption of the cells, the membrane (a) and cytoplasmic (b) fractions were obtained. After separation by SDS-PAGE, gels were stained with Coomassie (a, lanes 4–7; b, lanes 5–7) or transferred to a nylon membrane and incubated with antibodies raised against the C-terminus of KcsA (a, lanes 1–3; b, lanes 1–4). The purified full-length KcsA_{Histag} protein served as control; note the oligomeric forms (a, lane 7; b, lane 1). (c) Induced *E. coli* cells carrying a pQE construct with the inserted *kcsA* gene or having only the vector as control were embedded in Lowicryl K4M, ultrathin sectioned and afterwards treated with primary anti-KcsA antibodies and subsequently secondary (anti-guinea pig) gold-labelled antibodies. The outer face of the cell wall (black arrowheads), the cytoplasmic membrane (white arrowheads) and the cytoplasmic region (Cpl) are marked.

full-length KcsA (16 kDa). In cytoplasmic extracts from the *E. coli* strain carrying a construct encoding only the C-terminus of KcsA, a protein band corresponding to the C-terminal portion (6.3 kDa) reacted with the antibodies (Fig. 2b, lane 4). Within extracts of the *E. coli* strain having only the vector, no reaction was detectable (Fig. 2b, lane 2). Within the membrane-containing fraction, the dominant portion of the full-length KcsA protein was present as monomers, dimers and oligomers (Fig. 2a, lanes 1 and 4). Due to lack of the transmembrane regions, the truncated KcsA protein (see above) was absent in the membrane fraction obtained (Fig. 2a, lane 3). The data revealed that the generated antibodies were specific for the designed KcsA protein.

For *in situ* studies, the above-described control strain carrying only the vector and the strain containing the full-length *kcsA* gene were grown as described above and embedded in resin. Ultrathin sections (see Methods) were treated with the antibodies raised against the C-terminal region of KcsA (anti-C-KcsA) and with secondary gold-labelled (~ 6 nm) antibodies. Inspection by electron microscopy revealed that the induced *E. coli* cells carrying the

plasmid with the full-length *kcsA* gene reacted with the antibodies. Gold particles bound near to the cytoplasmic membrane and close to the cytoplasm in smaller or larger clusters. Within sections of the control strain, the corresponding labelling was missing (Fig. 2c). The data clearly indicated that the antibodies detected KcsA within the cells.

Immunolocalization reveals a clustered arrangement of KcsA in the cytoplasmic membrane of *S. lividans* hyphae

As described previously (Schrempf *et al.*, 1995), the *kcsA* gene was initially found within the chromosomal DNA of *S. lividans* wild-type (WT). To obtain a designed *S. lividans* mutant carrying an inactivated *kcsA* gene (named ΔK), the WT strain was transformed with a pGM160-based construct which carried the Omega-*hyg* fragment (Blondelet-Rouault *et al.*, 1997) including the hygromycin-resistance gene (*hyg*) with a translational terminator flanked by a few base pairs of the 5' and 3' ends, respectively, of the *S. lividans kcsA* gene connected to its upstream (698 bp) and downstream (639 bp) regions (see Methods and Fig. 1). Chromosomal DNA from several candidates was inspected for double

Fig. 3. Immunolocalization of KcsA in *S. lividans*. (a) WT strain; (b) ΔK strain. *S. lividans* substrate hyphae were embedded in Lowicryl K4M. Ultrathin sections were incubated with primary anti-KcsA antibodies and subsequently secondary (anti-guinea pig) gold-labelled antibodies. Clusters of gold particles at the inner face of the cell wall, where the cytoplasmic membrane is located, are visible in the WT but absent in the ΔK strain. The inner and outer face of the cell envelope are highlighted by white and black arrowheads, respectively. Several areas (numbered squares) have been enlarged to highlight the gold clusters.

Fig. 4. Monitoring of caesium by EFTEM. (a) Reference EELS obtained from a CsCl crystal. A drop of saturated aqueous CsCl solution was air-dried on a Formvar-nickel grid, measuring was done on a crystal of $\sim 1 \mu\text{m}$ size. (b) Hyphae of *S. lividans* showing intact hyphae (dark) and some hollow regions (bright, inset) derived from leakage of the cytoplasm. The proportion of leaked hyphae to intact ones is much smaller in the whole preparations than represented in this image. (c, d) Magnified view of parts of intact (c) and leaked (d) hyphae with the distribution of measuring points for EELS (circles) across them and illustration of the corresponding caesium-specific signals as spectra (above) and as a bar diagram according to the peak heights (below). The margins of the hyphae are marked between arrowheads. (e, f) ESI of the caesium distribution (red) around the margins (framed by arrowheads) of intact (e) or leaked (f) hyphae. The brighter half of the images corresponds to the adjacent support film.

crossover by using the *hyg* and the *kcsA* gene as probes. Several hygromycin-resistant recombinants were found to contain the *hyg* gene (including a terminator) correctly inserted into the *kcsA* gene (Fig. 1). One of them, named ΔK , was used here as negative control in the following immunolocalization experiments.

No labelling was found when whole hyphae of the *S. lividans* WT were treated with the above-described antibodies on grids or in a test tube (data not shown), as epitopes reacting with antibodies are hidden beneath the cell wall *in vivo*. Specific cross-reactivity is found only at the cytoplasmic membrane in sections of hyphae. Therefore a direct overall view on the distribution of KcsA around and along the hyphae cannot be obtained using the immunogold technique. Within sections of the *S. lividans* WT hyphae, epitopes cross-reacting with the antibodies were found to be clustered within the cytoplasmic membrane. No such

labelling was found in the ΔK mutant (Fig. 3). The data clearly demonstrate that KcsA is localized in the *S. lividans* WT within the cytoplasmic membrane within separately spaced clusters. Using this technique, the exact spacing of clusters is difficult to estimate precisely, as not every epitope is expected to be labelled: fixation, dehydration and embedding in the resin probably decrease antigenicity and some epitopes are hidden inside the resin, inaccessible for antibodies. Nevertheless, the data allow the conclusion that *in vivo* the clusters can be at least 50 nm wide, probably wider. The mean distance between the clusters was estimated to be 500 nm, as they were found in about 10 % of the cross-sections of 50 nm thickness.

EFTEM as a tool to analyse caesium distribution in hyphae of *S. lividans* WT

Caesium can be recognized by EELS and the corresponding spectrum is characterized by the M_{45} edges at 726 eV and

Fig. 5. Monitoring of caesium in *S. lividans* strains and evaluation procedure. (a) Analysis was done at various positions (arrowheads) along the hyphae using small measuring points arranged in groups of four, which are depicted as circles within the higher magnified insets. (b) For both the wild-type (WT) and ΔK mutant, relative intensity (height in mm) of caesium-specific peaks of 100 individual spectra was measured (as depicted in Fig. 4a). For both strains, the deduced mean value of the determined relative intensities for all measuring points is shown as a bar diagram with standard deviation. (c) Elemental map of caesium obtained by ESI analysis, using the same grids as for EELS. Caesium deposition (red) is visible at the margins of the hyphae in the WT and ΔK mutant.

740 eV (Ahn & Krivanek, 1983). Spectra achieved from CsCl crystals on reference grids (see Methods) showed these two characteristic peaks (Fig. 4a). The relative intensity (peak height) correlated with the amount of crystal material included in the measuring point (not shown). These peaks were absent in the control, a bare Formvar-nickel grid without CsCl. This experiment showed that the method was suitable to differentiate among varying amounts of caesium being present in the measuring point. Therefore it was subsequently optimized for *Streptomyces* hyphae.

During preparation for transmission electron microscopy, the *S. lividans* hyphae mostly stay associated, forming a

network on the support film. Intact hyphae appear dark. Due to air-drying, all hyphae collapse to a certain degree on the support film and the cytoplasm accumulates towards central regions, retracting from tips and margins. The latter appear comparatively bright as their optical density is lowered due to reduced levels of cytoplasm. Only a few hyphae get disrupted during sample preparation; these appear bright due to leakage of the cytoplasm.

S. lividans was grown under conditions which had been found to allow KcsA synthesis, mounted on grids and exposed to caesium ions (see Methods). After washing, the remaining caesium was monitored by EELS. Measuring

points of 0.7 or 0.16 μm diameter were positioned on the hyphae and, for controlling the background signal, also on the support film next to them. The background was found to be negligible. The large measuring points (0.7 μm) did not always include the exact diameter of the hyphae, as the latter varied between 0.5 and 0.8 μm . To ensure a constant portion of cellular material for every measurement, small measuring points of 0.16 μm were chosen.

Visualization of caesium by ESI revealed higher concentrations of caesium at the margins of hyphae compared to their central regions. This was confirmed by EELS using measuring points distributed in a row across the hyphae: signal intensities were always decreased in the central region, compared to the margins (Fig. 4b, c). In regions where the cytoplasm had leaked out from the hyphae (due to disruption during preparation) the cell envelope remained as a hollow tube, appearing bright due to complete loss of the cytoplasm. ESI revealed that in such areas the signal intensities were almost equal in all regions of the hyphae, as also observed by EELS (Fig. 4d). Artificial intrusion of caesium via disruptions can be ruled out as the signal intensities were not effectively higher in leaked than in intact hyphae. These observations can be explained as follows. During measuring, the electron beam passes the cell envelope, the cytoplasm, and once again the cell envelope. The recorded signal intensity correlates inversely with the amount of cytoplasm. The latter reduces the signal obtained from the cell envelope, due to its mass, and therefore obviously contains only low amounts of caesium in contrast to the cell envelope.

***S. lividans* WT and *kcsA* mutant ΔK differ in the amount of caesium accumulation**

Using ESI and EELS, caesium was detected mostly at the margins of intact hyphae (Fig. 4). Visualization of caesium was possible by ESI; however, for comparable quantification the averaged peak height of many individual EEL spectra was found to be essential. The highest signals were obtained from small measuring points that were positioned centrally onto the margins, including one half of hypha material and one half of adjacent support film (Fig. 4c, measuring points 3 + 7). In comparison, lower signal intensities were obtained by measuring exactly at the outside (including only adjacent support film, measuring points 2 + 8) and also at the inside of the margins (including only hypha material, measuring points 4 + 6). Probably a small fringe of caesium-containing medium surrounds the cells, due to capillary attraction. Based on these results, for all comparative studies the individual measuring points were always positioned exactly at the inner faces of the margins of the hyphae, and inclusion of adjacent support film was avoided. This procedure assured that only caesium directly associated with the cells contributed to the measurements, and any background signal was excluded.

To test whether the existence of KcsA in *S. lividans* hyphae has any effect on accumulation of caesium from the

medium, we compared EELS data obtained from hyphae of the WT and the ΔK mutant after exposure to caesium ions. Hyphae of the *S. lividans* WT and the corresponding *kcsA* disruption mutant ΔK , lacking a functional *kcsA* gene (Fig. 1), were transferred onto grids and exposed to CsCl as mentioned above (see Methods). Based on the results described in the previous paragraph, many small measuring points (0.16 μm) were placed at different intervals along the inner face of margins of the hyphae. As the signal intensity often differed to a certain degree between the two sides of the hyphae, pairs of measuring points were always positioned oppositely (Fig. 5a).

Individual spectra were evaluated for 100 measuring points (0.16 μm) distributed along the hyphae of both *S. lividans* WT and mutant ΔK . Five different grids were used for each strain. The relative signal intensity of each individual spectrum was determined (see Methods). The deduced mean value for the WT was found to be $\approx 30\%$ higher than for the ΔK mutant (Fig. 5b).

DISCUSSION

As a prerequisite to localize KcsA channels within *S. lividans* hyphae, specific antibodies were generated. The C-terminal domain, which has so far no counterpart in other known proteins, has been found to have considerably higher immunogenicity than the full-length protein including its two membrane-spanning helices. Ultrathin sections of induced *E. coli* cells carrying the *kcsA* gene in a multicopy vector (Schrempf *et al.*, 1995) were treated with the anti-C-KcsA antibodies followed by secondary gold-labelled antibodies. Rarely, unspecific gold labelling was detected on the outer face of the cell envelope from *E. coli* carrying the construct with the *kcsA* gene; this feature was shared with the *E. coli* control cells containing only the vector plasmid (Fig. 2c). This control strain lacked, however, specific labelling at the cytoplasmic membrane. Gold labels were frequently found in clusters adjacent to each other within the cytoplasmic membrane and sometimes scattered within neighbouring regions in the cytoplasm (Fig. 2c). These findings are in agreement with previous results (Splitt *et al.*, 2000) and the biochemical studies presented here (Fig. 2a, b), based on which it was shown that within the heterologous host the dominant portion of the full-length KcsA molecules is also extractable as monomers and oligomers from the *E. coli* membrane. Further studies had indicated that the monomers are directly interacting with the inner membrane in which the assembly to oligomeric forms was found to be independent of ATP hydrolysis. The protonmotive force was shown to be essential for the efficiency of oligomerization (van Dalen *et al.*, 2000). Mutational analysis led to the identification of amino acid residues stabilizing the tetrameric assembly (Splitt *et al.*, 2000; Meuser *et al.*, 2001).

Our comparative results (Fig. 2) unambiguously revealed that the generated antibodies were highly specific and

suitable to detect KcsA in ultrathin sections of bacterial cells. Subsequently, longitudinal and cross-sections of hyphae of the *S. lividans* WT strain (containing a functional *kcsA* gene) were treated with antibodies as outlined for the *E. coli* cells. Separate clusters of immunogold conjugates were localized close to the outer surface of the cytoplasm and the inner face of the cell wall (Fig. 3a). This region outlines the cytoplasmic membrane, which is in Gram-positive bacteria in close contact with the inner face of the cell wall. Due to the dimensions (~ 6 nm) of the gold particles, it appears unlikely that each of the four C-termini within the KcsA tetramer, predicted to comprise *in vivo* one channel, will be labelled by a secondary gold-labelled antibody. Thus the identified clusters, usually containing three to seven gold particles, are very likely indicative of areas comprising up to several KcsA channels. Sections of the designed *S. lividans* ΔK mutant, which lacks a functional *kcsA* gene, do not exhibit specific cross-reactivity with the antibodies (Fig. 3b). Taken together, the data demonstrate what we believe to be the first *in vivo* localization of a bacterial K^+ channel protein and verify that KcsA is located in the cytoplasmic membrane. This highly ordered clustering contrasts with the deregulated crowding and very heterogeneous assemblies observed within liposomes to which KcsA had been added (Molina *et al.*, 2006).

For IEM, antibodies can be applied to a specimen before or after embedding and sectioning (i.e. pre-embedding or post-embedding technique). Pre-embedding is generally more sensitive, but is suitable only for surface-accessible antigens. The bacterial cell wall is not permeable for antibodies, which is why membrane-located KcsA could only be labelled by post-embedding. IEM (using anti-peptide antibodies) was also applied to the KCNQ4 K^+ channel within the basal membrane of the hair cells of the mouse cochlea (Kharkovets *et al.*, 2000) and the K^+ channels KCNQ1 and ERG1 within heart cells of rats (Rasmussen *et al.*, 2004). Permeabilized human glioma cells allowed antibodies to access a chloride channel by pre-embedding (Olsen *et al.*, 2003). The number of studies to localize transport proteins within bacteria is relatively low. The documented studies include the detection of a transport system involved in polysaccharide export within *Pseudomonas aeruginosa* via immunolabelling of lipopolysaccharides (Rocchetta & Lam, 1997), and the localization of a proposed peptide transporter in the lipopolysaccharide layer of *Synechocystis* (Bölter *et al.*, 1998).

Inspection of biological samples by EFTEM allows imaging of spatial elemental information. As KcsA is a potassium channel, it would have been reasonable to monitor potassium ions via EFTEM. However, the potassium-specific signal is superimposed with that of carbon and therefore hardly detectable in biological material. A major difficulty in applying EFTEM is the loss of ions during sample preparation, especially during dehydration and embedding for ultrathin sectioning. Tracing of elements is most easily achieved if ions are bound tightly and focused to

distinct cell loci. Metals deposited in electron-dense granules can be analysed by EELS very clearly (Liu & Kottke, 2003). A combination of immunolabelling and EELS allowed mapping of the distribution of endocrine polypeptide hormones and proteins in pancreatic cells (Goping *et al.*, 2003). Recent studies revealed distinct elemental maps within mussels, visualized by ESI (Bleher & Machado, 2004). In *Drosophila*, lead and cerium ions could be analysed by EELS after their precipitation with phosphate which derived from degraded ATP, due to the activity of ATPase-dependent potassium pumps (Bohrmann & Heinrich, 1994).

Within an artificial vesicle system (generated by fusing *S. lividans* protoplasts with liposomes), the KcsA potassium channel activity was found to be blocked by caesium ions (Schrempf *et al.*, 1995). In contrast, the selectivity within the *in vitro* reconstituted KcsA varied according to the different test conditions applied. These data suggest that the conformation of the reconstituted channels differs from that of those within the liposome-protoplast vesicles. This result was not surprising, as the artificial composition of the bilayer varies from those within the protoplasts (generated from the *Streptomyces* hyphae). In addition, the bilayer also lacks other components present in the natural cytoplasmic membrane. Hence it was concluded that the giant protoplast system mimics the physiological conditions most closely (Schrempf *et al.*, 1995; Meuser *et al.*, 1999). Competition of Cs^+ with K^+ had also been documented for ion-uptake systems in plants (Zhu & Smolders, 2000). Based on these data, the *S. lividans* WT hyphae were exposed briefly to medium in which KCl had been replaced by CsCl. It was expected that Cs^+ could enter the vestibule at KcsA channels (or a portion of them) opened during the exposure to Cs^+ .

Our data reveal that in *S. lividans* substrate hyphae, caesium ions are restricted mostly to the cell envelope, the outer and inner face of which can not be discriminated by EFTEM. The mean value (deduced from 100 evaluated areas) for caesium-specific signal intensity was $\approx 30\%$ higher for the *S. lividans* WT than for the ΔK mutant, lacking a functional *kcsA* gene. Hence the determined enhanced value has to be attributed to the presence of KcsA within the WT. To a certain extent, unspecific binding of caesium to the outside of hyphae can be expected for both strains, independent of the presence of KcsA. In addition to this, the accumulation via other as yet uncharacterized transport systems seems likely. These effects are expected to be superimposed in the measurements for both strains.

The observed difference in caesium accumulation may also be due to cation sequestration by additional structures, associated with KcsA. Polyhydroxybutyrate (PHB) and inorganic phosphate (polyP) are widely distributed among pro- and eukaryotic organisms (see review by Reusch, 2000). PHB is a linear polymer of 3-hydroxybutyrate and is an amphiphilic polyester that forms ion-conducting complexes with salts. PolyP is a linear polymer of phosphoryl units and has the capacity for ion exchange and the ability to

discriminate among cations by charge (Reusch, 1999). It has recently been suggested (Zakharian & Reusch, 2004) that KcsA may recruit PHB and polyP to form a conductive core that selects and transports K^+ to the inner face of the selective filter.

In summary, our data reveal for the first time that the combination of IEM investigation and EFTEM is highly valuable for *in vivo* monitoring of ion channels and related proteins in bacteria.

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