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Multiple essential roles for EzrA in cell division of *Staphylococcus aureus*

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Summary

In *Bacillus subtilis*, EzrA is involved in preventing aberrant formation of FtsZ rings and has also been implicated in the localization cycle of Pbp1. We have identified the orthologue of EzrA in *Staphylococcus aureus* to be essential for growth and cell division in this organism. Phenotypic analyses following titration of EzrA levels in *S. aureus* have shown that the protein is required for peptidoglycan synthesis as well as for assembly of the divisome at the midcell and cytokinesis. Protein interaction studies revealed that EzrA forms a complex with both the cytoplasmic components of the division machinery and those with periplasmic domains, suggesting that EzrA may be a scaffold molecule permitting the assembly of the division complex and forming an interface between the cytoplasmic cytoskeletal element FtsZ and the peptidoglycan biosynthetic apparatus active in the periplasm.

Introduction

Cell division is a fundamental process that is required for growth of all bacterial populations. In almost all bacteria, division is initiated by polymerization of the tubulin homologue FtsZ into the Z-ring at the midcell. The Z-ring subsequently acts as a scaffold for the recruitment of downstream cell division proteins and their assembly into the divisome. This large macromolecular complex spans the cytoplasmic membrane and includes cytosolic components that interact with FtsZ [FtsA, ZapA, ZipA (*Escherichia coli*), EzrA and SepF (*Bacillus subtilis*)] and those with large periplasmic domains that are involved in the manufacture of septal peptidoglycan (FtsQ/DivIB, FtsB/DivIC, FtsL, FtsI/PBPs) (Errington *et al*., 2003).

Although the process of cell division is conserved among prokaryotes, it is likely that a diversity of cell division components exists to reflect the range of microbes. While key components of the divisome, such as FtsZ, are highly conserved throughout bacterial species, others have diverged significantly (Angert, 2005). Bacteria display a wide variety of cell shapes, ranging from simple spheres and rods to more elaborate curves and spirals. Each morphology presents a different set of challenges for the cell division machinery and therefore, the set of cell division proteins in each species is likely to be influenced by bacterial shape and envelope structures. For example, while the min system is necessary to prevent aberrant division at cell poles in rod-shaped *B. subtilis* and *E. coli*, cocci lack cell poles and in most of these species MinC is absent (Margolin, 2001).

Staphylococcus aureus is a Gram-positive human pathogen of increasing importance due to the incidence of nosocomial bacteraemia and the spread of antibiotic resistance among clinical isolates (Lowy, 2003). Cell division components make attractive targets for the development of novel anti-staphylococcal agents, although the process of division in this organism is largely unexplored because much of the work in the field of prokaryotic division has concentrated on the model organisms *E. coli* and *B. subtilis*. *S. aureus* is closely related to *B. subtilis* and therefore some of the mechanisms of cell division are likely to be conserved between the two organisms. However, the species differ in their cell shape: *S. aureus* is a spherical coccus, whereas *B. subtilis* is a rod. Rod-shaped bacteria alternate between two modes of cell wall peptidoglycan synthesis. Between division cycles, peptidoglycan synthesis occurs in a helical pattern along the lateral cell walls resulting in elongation (Daniel and Errington, 2003). When the cell has doubled in length, and following chromosome segregation, peptidoglycan synthesis occurs at the midcell leading to septum formation and division. Spherical *S. aureus* cells do not have an elongation phase of growth and instead synthesize peptidoglycan only at the septum (Pinho and Errington, 2003). Once the septum is formed, it is subsequently cleaved and becomes the nascent hemispherical poles of

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the daughter cells. This septal peptidoglycan synthesis is dependent upon the cell division apparatus. When FtsZ is depleted, cell wall peptidoglycan synthesis is delocalized resulting in cell enlargement and lysis (Pinho and Errington, 2003). *S. aureus* also differs from *B. subtilis* in that it does not sporulate and therefore has only one mode of cell division. The apparently simple cell cycle of *S. aureus* suggests that it could be a useful organism in which to study the mechanisms of prokaryotic division.

Homologues of all genes essential for division in *B. subtilis* are found to be conserved in *S. aureus*. A high density transposon screen for putative essential genes of *S. aureus* identified *ezrA* to be putatively essential in this organism (Chaudhuri *et al*., 2009), despite not being required for growth and division of *B. subtilis* (Levin *et al*., 1999). The *ezrA* gene is conserved throughout low-GC-content Gram-positive bacteria and is essential for growth of ovococcal *Streptococcus pneumoniae* (Thanassi *et al*., 2002), suggesting that functional redundancy of EzrA is reduced in cocci. Although the gene is non-essential in *B. subtilis*, the *ezrA* mutation is synthetically lethal in the absence of any one of the cell division genes, *zapA, noc, gpsB* or *sepF* (Gueiros-Filho and Losick, 2002; Wu and Errington, 2004; Hamoen *et al*., 2006; Claessen *et al*., 2008).

The role of EzrA in cell division is not well understood although it is generally considered to be a negative regulator of FtsZ ring formation. The *ezrA* gene was first identified in *B. subtilis* via the ability of nonsense mutations to restore viability of a temperature-sensitive *ftsZ* mutation at the non-permissive temperature (Levin *et al*., 1999). In the absence of EzrA, the frequency of Z-ring formation is increased and the critical concentration of FtsZ required for Z-ring formation is reduced (Levin *et al*., 1999). *In vitro*, EzrA interacts directly with FtsZ to inhibit polymerization and bundling of FtsZ protofilaments by reducing the affinity of FtsZ for GTP and the activation of GTP hydrolysis (Haeusser *et al*., 2004; Chung *et al*., 2007; Singh *et al*., 2007).

Although much data indicate EzrA to be a negative regulator of cell division, the protein is also required for efficient division in *B. subtilis*, suggesting that it may also have a positive role. In the absence of *ezrA* expression, cell length is increased indicating a delay in cell division (Levin *et al*., 1999; Chung *et al*., 2004; Claessen *et al*., 2008), and some non-septate filaments are observed (Chung *et al*., 2004). Time-lapse microscopy of live cells has shown that in the absence of EzrA, the disassembly of the Z-ring is delayed (Chung *et al*., 2004).

EzrA has also been suggested to have a role in cell elongation in *B. subtilis*. Cells lacking EzrA have a reduced diameter, similar to those affected in lateral wall synthesis (Claessen *et al*., 2008). Double mutants of *gpsB* and *ezrA* are defective in both elongation and division with disturbed localization of PBP-1 (Claessen *et al*., 2008). As a result, GpsB and EzrA have been proposed to have partially redundant roles in the localization cycle of PBP-1, with EzrA promoting recruitment to the septum and GpsB facilitating removal from the completed cell pole (Claessen *et al*., 2008).

Putative divisome components of *S. aureus* were identified on the basis of homology with cell division components of *B. subtilis*. A bacterial two-hybrid screen was used to investigate the interaction of likely divisome members and found the orthologue of *B. subtilis* EzrA to be a promiscuous partner. We have used conditional mutant analysis to determine the requirement for EzrA in growth of *S. aureus*. Phenotypic analysis of the conditional mutant strain under varying levels of *ezrA* expression has revealed EzrA to have several distinct roles in co-ordinating the cell division process.

Results

Complex interactions between S. aureus *divisome components*

A bacterial two-hybrid screen (Karimova *et al*., 1998) was used to map the complement of pairwise interactions between proteins that are known or are thought to participate in divisome formation in *S. aureus* (FtsZ, FtsA, EzrA, GpsB, SepF, Pbp1, Pbp2, Pbp3, DivIB, DivIC, FtsL, FtsW) and the results are shown in Fig. S1. An orthologue of *B. subtilis* RodA, an elongation-specific homologue of FtsW (Henriques *et al*., 1998), was also tested.

A level of β-galactosidase activity against MUG at least fourfold higher than that measured for negative control cells (BTH101 pUT18C pKT25) is considered to indicate a positive interaction (Karimova *et al*., 2005). A complex web of interactions was identified (Fig. 1), similar to those found among divisome components of *E. coli* and *S. pneumoniae* (Karimova *et al*., 2005; Maggi *et al*., 2008). Nearly all proteins were observed to interact with many partners, suggesting that multiple interactions stabilize the division complex. Of the observed interactions, most have been previously found in *E. coli, S. pneumoniae* and/or *B. subtilis*: the interaction between FtsZ and FtsA is well characterized and is conserved in most bacteria (Ma and Margolin, 1999; Yan *et al*., 2000); self-interaction of FtsA is well documented (e.g. Yan *et al*., 2000; Yim *et al*., 2000; Feucht *et al*., 2001), and its association with division-specific penicillin-binding proteins has been shown in *E. coli* (Tormo *et al*., 1986; Karimova *et al*., 2005) and *S. pneumoniae* (Maggi *et al*., 2008); *in vitro* association of recombinant *S. pneumoniae* DivIB, DivIC and FtsL has been reported (Noirclerc-Savoye *et al*., 2005) and interaction of the proteins has also been shown by co-immunoprecipitation and two-hybrid analyses

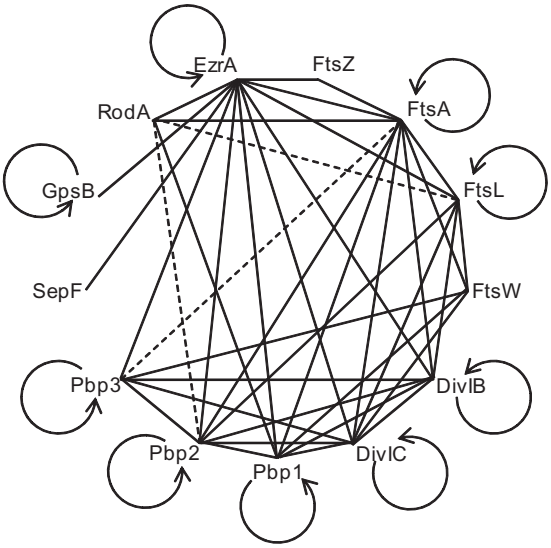

Fig. 1. Interaction web among the cell division proteins as determined by two-hybrid analysis. Positive interactions are shown by a solid line and putative interactions with a dashed line. Homodimerization is indicated by a circular arrow.

(Buddelmeijer and Beckwith, 2004; Karimova *et al*., 2005; Daniel *et al*., 2006); the interaction of DivIB, DivIC and FtsL with penicillin-binding proteins has been shown by two-hybrid analysis (Karimova *et al*., 2005; Daniel *et al*., 2006); BACTH analysis of *E. coli* FtsW indicated direct interactions with FtsL and division-specific PBPs (Karimova *et al*., 2005); two-hybrid interaction data have been verified in *S. pneumoniae* by co-immunoprecipitation of FtsL and FtsW (Maggi *et al*., 2008) and pull-down and peptide-based assays in *Mycobacterium tuberculosis* have shown and interaction between FtsW and division-specific PBPs (Datta *et al*., 2006). The conservation of an interaction in phylogenetically distant species indicates likely biological significance. The high degree of similarity between the interaction webs of division proteins in *S. aureus, E. coli* (Di Lallo *et al*., 2003; Karimova *et al*., 2005) and *S. pneumoniae* (Maggi *et al*., 2008) suggests the existence of a common core bacterial division complex. Importantly, several novel interactions were also observed: self-association of GpsB has not been reported in *B. subtilis*; FtsA was found to interact with FtsW and DivIC; the interactions of RodA with components of the division apparatus indicate that in an organism that does not carry out elongation, the protein has a potential role in peptidoglycan synthesis at the septum; and EzrA was observed to interact directly with nearly all divisome components.

EzrA-T18~T25-SepF was considered to be a positive interaction although the β-galactosidase activity was slightly less than this cut-off value, because it was significantly greater ($P = 0.0037$) than the negative control (10.35 ± 0.98 MUG units) and resulted in blue colonies on plates containing X-gal. Several other plasmid combinations gave conflicting results in solid and liquid assays. T18-FtsA~T25-DivIC showed negative β-galactosidase activity against MUG but blue colonies on plates containing X-gal. T18-FtsL~T25DivIB showed a positive β-galactosidase activity in the liquid assay but white colonies on plates containing X-gal. Reciprocal analysis (T18-DivIC~T25-FtsA and T18-DivIB~T25-FtsL respectively) showed positive β-galactosidase activity in both liquid and solid assays and so interaction between FtsA and DivIC and between FtsL and DivIB in the BACTH assay was considered to be positive and the difference between quantitative and qualitative assays suggested to be as a result of protein instability. T18-FtsL~T25-GpsB and T18-FtsA~T25-GpsB resulted in a β-galactosidase activity against MUG that was slightly higher than the cut-off for a positive interaction but white colonies on plates containing X-gal. Reciprocal analysis showed negative β-galactosidase activity against MUG and X-gal, and therefore interaction between FtsL and GpsB and between FtsA and GpsB was considered negative. T18-Pbp2~T25-RodA, T18-FtsA~T25-Pbp3 and T18-FtsL~T25-RodA resulted in blue colonies on plates containing X-gal although negative β-galactosidase activity against MUG and negative results in reciprocal analysis (or reciprocal analysis could not be performed). Interactions between Pbp2 and RodA, FtsA and Pbp3, and between FtsL and RodA in BACTH analysis are therefore unclear.

The functional role of EzrA is not well understood, but the multiple interactions of this protein indicated that EzrA is a core component of the cell division machinery in *S. aureus*. Strong positive interactions were observed between EzrA-T18 and T25 fusions to FtsA and proteins with large periplasmic domains that have a demonstrated or potential involvement in peptidoglycan synthesis: DivIB, FtsL and DivIC, and the penicillin-binding proteins Pbp1, Pbp2 and Pbp3. Weaker, but significant ($P < 0.01$), interactions were also observed between EzrA-T18 and T25 fusions to the cytoplasmic Z-ring components FtsZ, GpsB and SepF and to RodA (Fig. S1). Our data also revealed EzrA's self-interaction. Fewer interactions were observed between EzrA-T25 and T18 fusions to the same proteins: only weak interactions with EzrA-T18 and T18-DivIB were detected (Fig. S1), presumably as a result of reduced functionality of EzrA-T25 compared with EzrA-T18 or the low copy number of the plasmid carrying the *ezrA-T25* gene.

Most of the observed interactions of EzrA are novel, although some are conserved in *B. subtilis*. Nickel-affinity purification of His-tagged FtsA resulted in co-purification of EzrA, SepF and FtsZ in *B. subtilis* (Ishikawa *et al*., 2006), suggesting an association between these proteins. Direct interaction of EzrA with FtsZ has been observed via

size exclusion chromatography (Haeusser *et al*., 2004), and with GpsB and Pbp1 via bacterial two-hybrid analysis (Claessen *et al*., 2008). The C-terminal region of *B. subtilis* EzrA contains four coiled-coil motifs (Levin *et al*., 1999). Secondary structure prediction of the *S. aureus* protein sequence using the COILS program (Lupas *et al*., 1991) showed five putative coiled-coil regions distributed along the length of the cytoplasmic domain. It is likely that at least some of the interactions of EzrA are mediated via these domains, but the absence of an interaction between EzrA-T18 and a T25 fusion to the leucine zipper of GCN4 (data not shown) indicated that interactions observed by BACTH analysis were not the result of non-specific hydrophobic association of overexpressed coiled-coil proteins.

EzrA is required for growth of S. aureus

A high-density transposon screen for genes required for growth of *S. aureus* revealed the orthologue of *ezrA*, SAOUHSC_01827, to be putatively essential in this organism (Chaudhuri *et al*., 2009). In order to confirm essentiality and validate *ezrA* as a potential novel antibacterial target, a conditional mutation was constructed in *S. aureus* using pAISH1 (Aish, 2003). The resulting strain, VF79, carries a truncated copy of *ezrA* under the control of the native promoter with a transcriptional fusion to *lacZ*, and the full-length copy of the gene under the control of P_{Spac}. The 5′ fragment of *ezrA* encodes a protein lacking the C-terminal 291 amino acids which forms two of the predicted coiled-coil domains (Fig. S2A). The absence of the C-terminal 166 amino acids in the orthologous *B. subtilis* protein results in an *ezrA*-mutant phenotype (Levin *et al*., 1999) and therefore it is assumed that the truncated *ezrA* gene in VF79 is non-functional, although it may retain some of its functionality. In order to obtain minimal expression from P_{Spac} in the absence of inducer, *lacI* was constitutively overexpressed from a multicopy plasmid, pGL485 (Cooper *et al*., 2009). Bioinformatic analysis of the *S. aureus ezrA* chromosomal region (Fig. S2B) indicated that the gene is not part of an operon and is unlikely to have polar effects on downstream genes.

IPTG-dependent growth of strain VF79 (P_{Spac}–*ezrA* pGL485; Fig. 2B) showed that EzrA is essential for growth of *S. aureus*. The reduced growth rate of VF79 (P_{Spac}–*ezrA* pGL485) compared with wild type, under fully induced conditions, indicates that the level of transcription of *ezrA* from P_{Spac} is incorrect for normal growth, which is consistent with high cellular levels of EzrA in *B. subtilis* (Haeusser *et al*., 2004).

S. aureus *EzrA colocalizes with FtsZ to form a ring at midcell*

EzrA is an integral membrane protein with a single 22-amino-acid transmembrane helix at the extreme N-terminus and a large cytoplasmic domain. In *B. subtilis*, EzrA localizes to the division site in an FtsZ-dependent manner (Levin *et al*., 1999). The N-terminal membrane spanning domain of EzrA is not required for localization, although it is required for EzrA function (Haeusser *et al*., 2004). Seven conserved residues, the QNR patch, at the C-terminus of EzrA are necessary for septal localization but not for inhibition of FtsZ assembly, implying either that EzrA interacts with FtsZ at more than one site, or that recruitment of EzrA via the QNR patch is mediated through another component of the divisome (Haeusser *et al*., 2007).

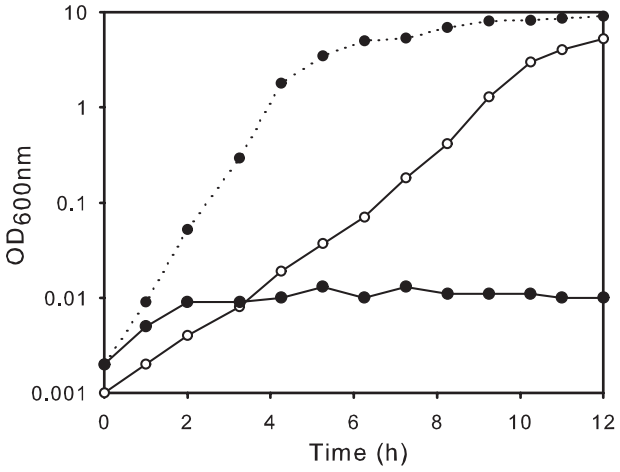

Fig. 2. EzrA is required for growth of *S. aureus*. VF79 (P_{Spac}–*ezrA* pGL485; solid line) and control VF17 (SH1000 pGL485; dotted line) cells were grown in the presence (open circles) or absence (filled circles) of 1 mM IPTG and growth was followed as OD_{600}. Strain VF17 (SH1000 pGL485) was unaffected by IPTG and therefore only growth in the absence of the inducer is shown.

Two-hybrid analysis indicated EzrA to be a component of the *S. aureus* divisome. In order to confirm this, its subcellular localization was determined using GFP fusion analysis. Strain JGL227 expresses *ezrA–GFP*+ from the *ezrA* promoter and the native gene from IPTG-inducible Spac promoter (P_{Spac}) (Yansura and Henner, 1984). EzrA–GFP+ was able to support growth of *S. aureus* in the absence of native *ezrA* expression, via inducer depletion, showing the fusion to be functional (data not shown). In 86% of JGL227 cells, EzrA–GFP+ was concentrated in a ring-like structure at the nascent division site (Fig. 3A). EzrA localization to the midcell has also been very recently shown in *S. aureus* RN4220 (Pereira *et al*., 2010). Simultaneous localization of EzrA–GFP+ and FtsZ via immunofluorescence in a *spa* mutant background showed the two proteins to colocalize to the nascent division site (Fig. 3B).

To determine the hierarchy of localization between EzrA and FtsZ, a strain (VF87) was constructed that carried the EzrA–GFP+ fusion in which *ftsZ* was under the control of

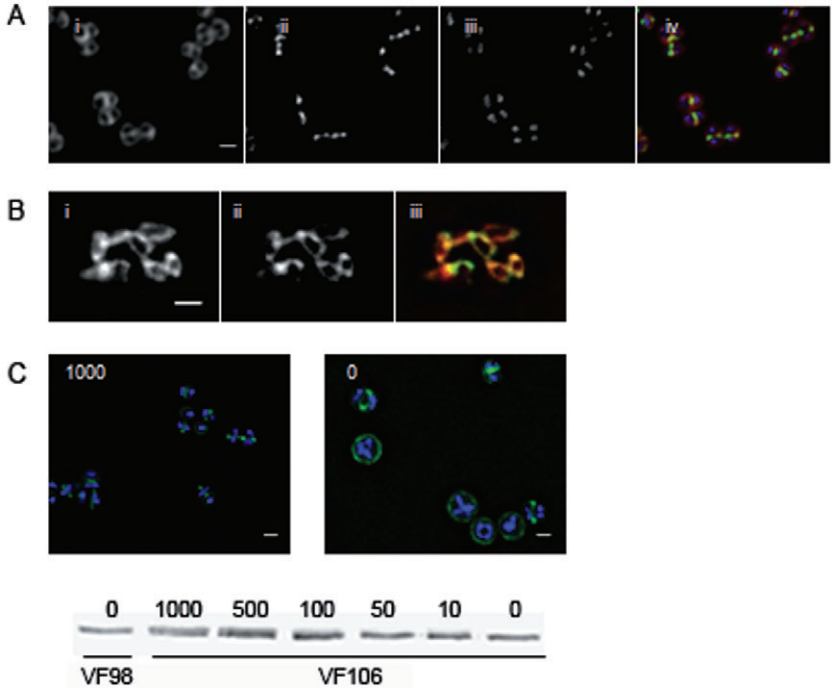

Fig. 3. A. Subcellular localization of EzrA–GFP+ (ii; green in overlay iv). Cell membranes were stained with FM464 (i; red in overlay iv) and DNA with Hoechst 33342 (iii; blue in overlay iv). Scale bar = 1 μm.
B. Colocalization of FtsZ (i; red in overlay iii) and EzrA–GFP+ (ii; green in overlay iii). FtsZ was detected by immunofluorescence. EzrA–GFP+ was observed as a complete ring, rather than double dots corresponding to a slice through a ring as seen in (A), due to flattening of cells following lysostaphin digestion of the cell wall. Scale bar = 1 μm.
C. Localization of EzrA–GFP+ in VF 87 (P_{Spac}–*ftsZ ezrA–GFP*+ pGL485) after 180 min in the presence of 1000 μM IPTG to induce *ftsZ* expression (1000) or in the absence of IPTG to deplete cells of FtsZ (0). Combined fluorescence microscopy images show EzrA–GFP+ in green and Hoechst 33342-stained nucleoids in blue. Scale bar = 1 μm.
D. Levels of FtsZ in VF106 (*spa::tet* P_{Spac}–*ftsZ* pGL485) and the control strain VF98 (*spa::tet* pGL485) following 180 min growth at varying levels of *ftsZ* induction were detected by immunoblot of total protein extracts. Lanes are labelled according to the IPTG concentration (μM) during growth. Protein quantities were equalized on the basis of the OD_{600} of the culture from which they were taken.

P_{Spac}. In cells with *ftsZ* expression induced by IPTG, EzrA–GFP+ localized to the site of division, similar to wild-type cells (Fig. 3C). When cells were examined after 3 h growth in the absence of IPTG induction, cell enlargement was observed [mean diameter in 1 mM and 0 mM IPTG was 1.00 ± 0.12 μm ($n = 76$) and 1.61 ± 0.40 μm ($n = 249$), respectively, and the percentage of cells with diameter greater than 1.4 μm was 5.3% and 73% respectively] as has been previously shown to occur following FtsZ depletion, due to delocalization of peptidoglycan synthesis (Pinho and Errington, 2003). The proportion of cells showing a ring of EzrA–GFP+ at midcell was reduced from 82% in the presence of IPTG to 45% in the absence of *ftsZ* induction. However, 35% of cells with a diameter greater than 1.4 μm ($n = 181$) showed septal localization of EzrA, indicating that successful recruitment of EzrA to midcell can occur in cells in which peptidoglycan synthesis is delocalized. Western blot analysis of a strain carrying the IPTG-inducible *ftsZ* mutation in a *spa* mutant background (VF106) was performed to determine levels of FtsZ following growth for 3 h in 0–1 mM IPTG. Due to an increase in cell size following FtsZ depletion and normalization of samples according to OD_{600}, conclusions cannot be made regarding the amount of FtsZ per cell. However, FtsZ was clearly present in the absence of inducer, despite almost all cells displaying a large cell phenotype (Fig. 3D). There is a threshold level of *ftsZ* expression below which *S. aureus* cannot divide and above which cells divide normally (Pinho and Errington, 2003). Results therefore suggest that in the absence of IPTG induction, cellular levels of FtsZ were below the threshold required for division, but sufficient for detection by Western blot, as a result of protein stability and possible transcriptional leakage from P_{Spac}. As recruitment of EzrA to the septum of *B. subtilis* is FtsZ-dependent, it seems likely that the observed midcell localization of EzrA in some cells of FtsZ-depleted *S. aureus* is mediated by this residual FtsZ protein.

Titration of ezrA *expression results in variation in cell size*

In order to investigate the essential role of EzrA in *S. aureus* cell division, the cellular morphology of VF79

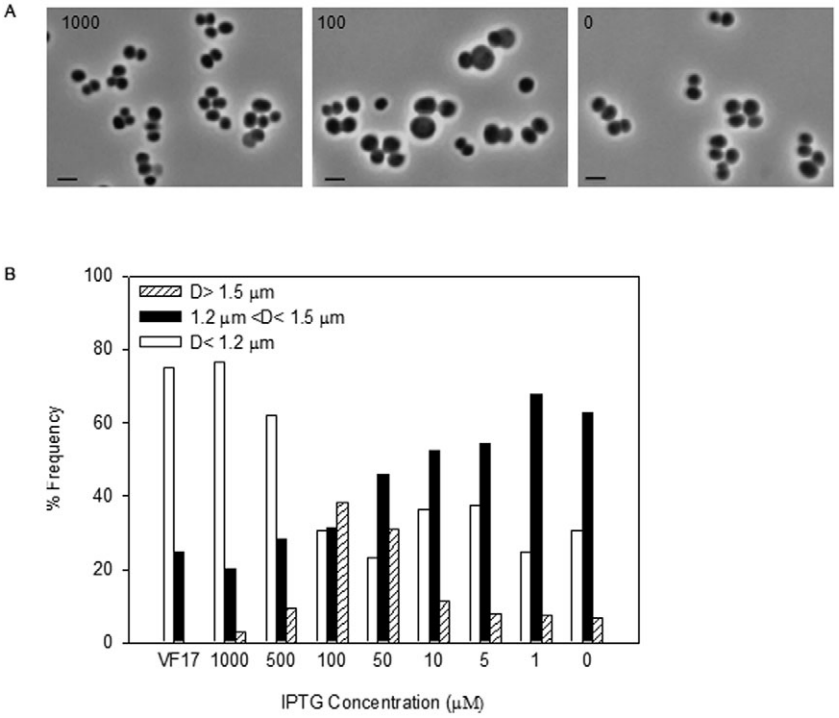

Fig. 4. A. Phase-contrast images of VF79 (P_{Spac}–*ezrA* pGL485) after 120 min growth in the presence of 1000, 100 or 0 μM IPTG. Scale bar = 1 μm.
B. Frequency of phenotypes of VF79 (P_{Spac}–*ezrA* pGL485) grown in different inducer concentrations. VF79 (P_{Spac}–*ezrA* pGL485) was grown for 120 min in the presence of 0–1000 μM IPTG. Cells were given one of three phenotypes on the basis of their diameter (D). The frequency of phenotypes of VF17 (SH1000 pGL485) grown in the absence of IPTG is also shown. In each case, 250 cells were measured.

(P_{Spac}–*ezrA* pGL485) was examined following growth in the presence of various concentrations of the inducer IPTG. Cell diameter was observed to be affected by the level of *ezrA* induction (Fig. 4A). Wild-type VF17 (SH1000 pGL485) cells showed a normal size distribution (mean diameter = 1.11 ± 0.13 μm, $n = 250$). In the presence of 1 mM IPTG, the size distribution of VF79 (P_{Spac}–*ezrA* pGL485) was very similar to wild type (mean diameter = 1.12 ± 0.16 μm, $n = 250$). In the absence of IPTG induction, cell diameter was greater than wild type (mean diameter = 1.28 ± 0.16 μm, $n = 250$). At 100 μM IPTG, a much greater range in cell size was measured. Some cells had diameters similar to those observed for wild-type cells and those without *ezrA* expression, but a high proportion of very large cells up to 3.16 μm in diameter was seen (Fig. 4A).

In order to quantify the frequency of different cellular phenotypes at each inducer concentration, cut-off diameters were chosen to define three phenotypes. Wild-type size cells (diameter less than 1.2 μm) were seen predominantly at high (500–1000 μM) IPTG concentrations, very large cells (diameter greater than 1.5 μm) were seen mainly at intermediate (50–100 μM) IPTG concentrations, and intermediate size cells (diameter between 1.2 and 1.5 μm) were seen mainly at low (0–10 μM) IPTG concentrations (Fig. 4B). It can therefore be assumed that intermediate size cells are the result of an absence of *ezrA* expression, very large cells are the result of higher, though insufficient, *ezrA* expression and small cells result from greatest inducer-controlled or wild-type *ezrA* expression.

EzrA is required for GpsB localization

The enlarged cell phenotype of *S. aureus* partially depleted of EzrA indicated that cells cannot divide in the absence of sufficient protein. In order to elucidate the cause of this, and of the less dramatic morphological phenotype of cells grown in the absence of *ezrA* induction, the effect of EzrA depletion on localization of other divisome components was studied.

In *B. subtilis*, an *ezrA/gpsB* double mutant is synthetically lethal, with defects in both cell division and cell elongation, and it has been suggested that GspB and EzrA act together to orchestrate the shift in localization of PBP1 between elongation and division sites (Claessen *et al.*, 2008). GpsB localizes to the lateral cell wall during elongation and to the septum during division, but cells fail to recruit GpsB to the division site in the absence of EzrA (Claessen *et al.*, 2008). BACTH analysis showed that the direct interaction between EzrA and GpsB in *B. subtilis* (Claessen *et al.*, 2008) is conserved in *S. aureus* and therefore it was proposed that EzrA depletion may disrupt GpsB localization in *S. aureus*.

The wild-type localization of GpsB in *S. aureus* was determined using a C-terminal GFP+ fusion. Fluorescence was observed as a ring or disc at the septum in almost

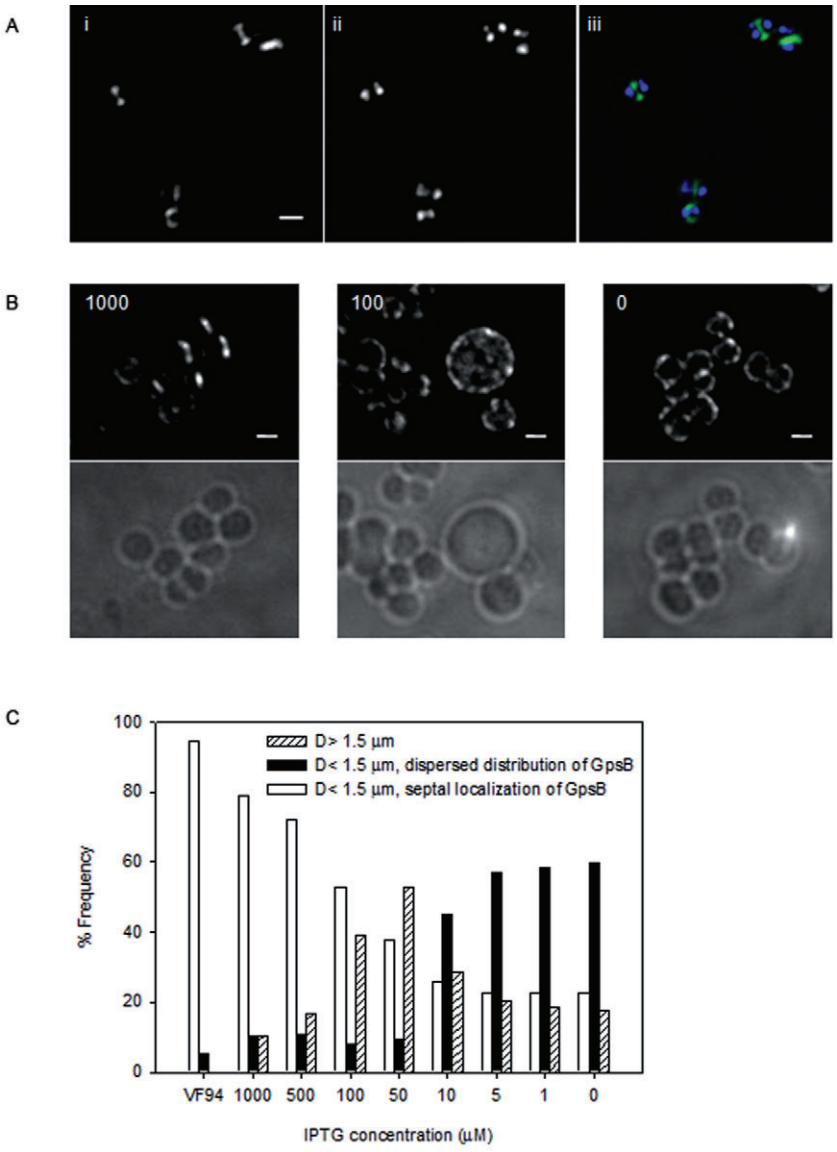

Fig. 5. A. Localization of GpsB–GFP+ (i; green in overlay iii) in JGL228 (*gpsB–GFP*+). Nucleoids were stained with Hoechst 33342 (ii; blue in overlay iii). Scale bar = 1 μm.
B. Localization of GpsB–GFP+ in VF89 (P_{Spac}–*ezrA gpsB–GFP*+ pGL485) grown for 120 min in 1000, 100 or 0 μM IPTG. Upper images show the GpsB–GFP+ signal and lower images show the corresponding phase-contrast image. Scale bar = 1 μm.
C. Frequency of cellular phenotypes of VF89 (P_{Spac}–*ezrA gpsB–GFP*+ pGL485) grown in 0–1000 μM IPTG for 120 min. Cells were given one of three phenotypes on the basis of their diameter (D) and localization of GpsB–GFP+. The control strain VF94 (*gpsB–GFP*+ pGL485) showed septal localization of GpsB in the presence and absence of IPTG, therefore data for cells grown in the absence of IPTG are shown. The number of cells measured was 89, 109, 130, 115, 159, 146, 88, 123 and 114 respectively.

all cells (Fig. 5A), indicating that in the absence of an elongation phase, GpsB localizes to the division site. The GpsB–GFP+ fusion was also used to determine the localization of GpsB in a strain in which *ezrA* expression was under the control of P_{Spac}, following growth in various concentrations of inducer. The size distribution of EzrA-depleted cells was similar to that reported above (Fig. 4), but use of GpsB–GFP+ as a marker allowed clear distinction between small cells (diameter less than 1.5 μm) that were wild type and those depleted of EzrA. At high IPTG concentrations the majority of cells had septal localization of GpsB, similar to wild type. At IPTG concentrations below 10 μM, GpsB was delocalized in most cells, with a weak GpsB–GFP+ signal distributed around the cell periphery. At 50 μM and 100 μM IPTG, many large cells (diameter greater than 1.5 μm) were observed, in which GpsB–GFP+ was usually localized to one or more intense foci (Fig. 5B). This requirement of EzrA for recruitment of GpsB to the divisome may be mediated via the direct interaction between the two proteins.

EzrA is required for assembly of the S. aureus *divisome at midcell*

Delocalization of GpsB under conditions of low *ezrA* expression suggested that the divisome cannot assemble at midcell in the absence of EzrA. In order to test this hypothesis, the localization of Pbp2 was determined in an *ezrA* conditional mutant with varying levels of IPTG induction. Pbp2 is one of four penicillin-binding proteins in *S. aureus* and is essential for growth of methicillin-

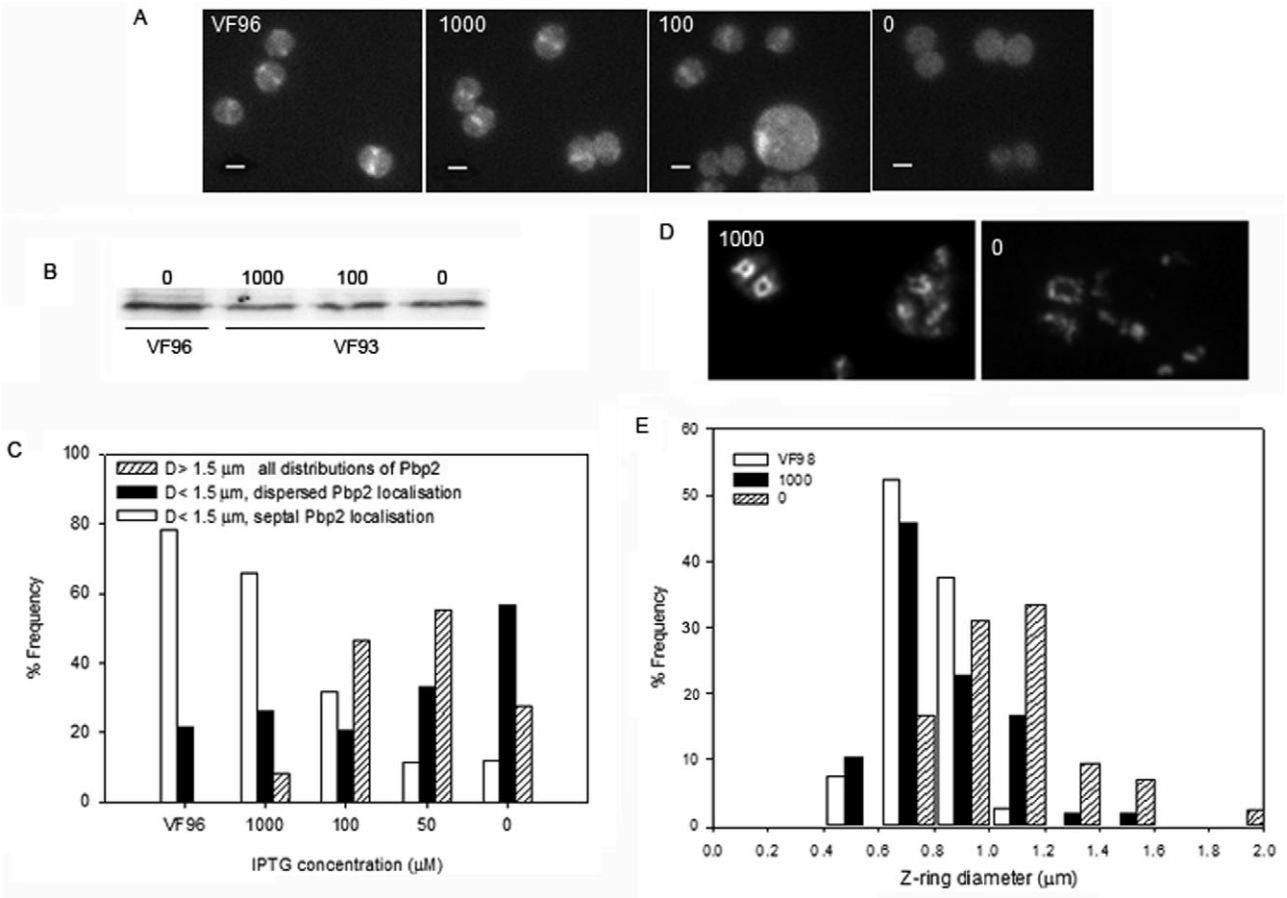

Fig. 6. A. Localization of GFP–Pbp2 in VF96 (P_{xyl}–*GFP–pbp2* pGL485) and VF93 (P_{Spac}–*ezrA* P_{xyl}–*GFP–pbp2* pGL485) grown for 120 min in 1000, 100 or 0 μM IPTG. The control strain VF96 (P_{xyl}–*GFP–pbp2* pGL485) showed septal localization of Pbp2 in the presence and absence of IPTG, therefore cells grown in the absence of IPTG are shown. Scale bar = 1 μm.
B. Levels of GFP–Pbp2 in VF93 (P_{Spac}–*ezrA* P_{xyl}–*GFP–pbp2* pGL485) and VF96 (P_{xyl}–*GFP–pbp2* pGL485) following 120 min growth in 1000, 100 or 0 μM IPTG were detected by immunoblot of total protein extracts using anti-GFP. Lanes are labelled according to the IPTG concentration (μM) during growth. Protein quantities were equalized on the basis of the OD_{600} of the culture from which they were taken.
C. Frequency of cellular phenotypes of VF93 (P_{Spac}–*ezrA* P_{xyl}–*GFP–pbp2* pGL485) grown in 0–1000 μM IPTG for 120 min. Cells were given one of three phenotypes on the basis of their diameter (D) and localization of GFP–Pbp2. Data for VF96 (P_{xyl}–*GFP–pbp2* pGL485) grown in the absence of IPTG are also shown. The number of cells measured was 102, 69, 92, 121 and 103 respectively.
D. Immunolocalization of FtsZ in VF105 (*spa::kan* P_{Spac}–*ezrA* pGL485) after 120 min in the presence of 1000 μM IPTG (1000) or in the absence (0) of IPTG to deplete cells of EzrA. Scale bar = 1 μM.
E. Frequency of Z-ring diameters of VF98 (*spa::tet* pGL485) and VF105 (*spa::kan* P_{Spac}–*ezrA* pGL485) grown for 120 min in the presence of 1000 μM IPTG (1000) or in the absence (0) of IPTG determined by FtsZ immunolocalization. The number of Z-rings measured was 48, 42 and 40 cells respectively.

sensitive strains of this organism (Pinho *et al*., 2001). Penicillin-binding proteins catalyse the last stages of cell wall peptidoglycan assembly and Pbp2 possesses both *trans*-glycosylation and *trans*-peptidation activity. In spherical *S. aureus*, peptidoglycan synthesis takes place only at the septum, to which Pbp2 is localized (Pinho and Errington, 2003).

When *ezrA* expression was fully induced there was septal localization of GFP–PBP2 in most cells, similar to wild type (Fig. 6A). Fluorescence was observed as a line across the cell at the septum, or as two spots corresponding to a ring at the division site. In the absence of *ezrA* induction, cells were usually small without localized GFP–Pbp2. The fluorescence of the GFP–Pbp2 fusion is low and therefore a delocalized signal cannot be distinguished from background fluorescence. Western blot analysis was used to confirm that the cellular levels of GFP–Pbp2 are unaltered following EzrA depletion (Fig. 6B) and therefore that the absence of GFP–Pbp2 at the septum is due to delocalization. In the enlarged cells observed predominantly at intermediate inducer concentrations, the fluorescence signal was dispersed either across the whole cell (39% of cells with diameter > 1.5 μm, $n = 56$) or as a ring at midcell (61% of cells with diameter > 1.5 μm, $n = 87$). The frequency of the different cellular phenotypes is shown in Fig. 6C.

FtsZ is the first known protein to be recruited to midcell and localization of all known division proteins is dependent on it (Errington *et al*., 2003). In order to determine whether FtsZ can localize to midcell in the absence of EzrA, immunofluorescence of VF105 (*spa::kan* P_{Spac}–*ezrA* pGL485) in the presence and absence of IPTG was performed using α-*S. aureus* FtsZ. In a subpopulation of VF98 (*spa::tet* pGL485) and VF105 (*spa::kan* P_{Spac}–*ezrA* pGL485) grown in the presence and absence of IPTG, it was found that FtsZ localized in a ring at midcell (Fig. 6D). However, FtsZ localization could not be observed in many cells. The proportion of cells with FtsZ localization could not be quantified due to cell lysis as a result of the lysostaphin treatment required to allow access of antibodies to the cytoplasm. In VF105 cells grown in the absence of *ezrA* induction, greatly enlarged cells were observed less frequently during immunofluorescence microscopy than expected, perhaps due to increased susceptibility to lysostaphin treatment as compared with smaller cells. Measurement of Z-ring diameter in the subset of cells with FtsZ localization showed Z-rings to be smaller in VF98 (*spa::tet* pGL485) and VF105 (*spa::kan* P_{Spac}–*ezrA* pGL485) cells grown in the presence of IPTG (mean diameter 0.82 ± 0.21 μm and 0.77 ± 0.11 μm respectively) than VF105 cells grown in the absence of *ezrA* induction (mean diameter 1.03 ± 0.26 μm) (Fig. 6E). This could be due to Z-ring constriction in growing and dividing EzrA-containing cells, or may represent cell enlargement in the absence of *ezrA* expression.

EzrA is required for peptidoglycan synthesis in S. aureus

Depletion of EzrA in *S. aureus* resulted in delocalization of the cell division machinery. Previous studies have shown that depletion of FtsZ also leads to delocalization of Pbp2; however, dispersed peptidoglycan synthesis causes cell enlargement (Pinho and Errington, 2003). It was therefore suggested that despite delocalized division machinery, EzrA-depleted cells do not enlarge due to a requirement for EzrA in peptidoglycan synthesis. The enlargement of cells with an intermediate level of *ezrA* induction was proposed to be due to dispersed, EzrA-dependent peptidoglycan synthesis. In order to test this hypothesis, the localization of peptidoglycan biosynthesis was determined in a *S. aureus ezrA* conditional mutant following growth in various inducer concentrations, using a fluorescent derivative of vancomycin (Van-FL) to label nascent cell wall. Cells were depleted of EzrA in medium supplemented with 0.125 M D-serine for 90 min, and then depletion was continued for a further 30 min in medium without D-serine to allow incorporation of peptidoglycan precursors with D-ala-D-ala termini into nascent cell wall. Cells were stained *in vivo* with Van-FL, which binds to the D-ala-D-ala termini in the newly formed cell wall.

For wild-type *S. aureus* and the P_{Spac}–*ezrA* strain under conditions of high *ezrA* induction, the majority of cells showed septal peptidoglycan synthesis (Fig. 7). Different stages of septum formation could be observed: some cells showed a ring of fluorescence at the division site, corresponding to a ring of new peptidoglycan and others showed a fluorescent line across the cell, corresponding to a disk of newly synthesized peptidoglycan that is the septum. This phenotype is similar to that previously reported for wild-type *S. aureus* (Pinho and Errington, 2003). In the absence of *ezrA* induction, the majority of cells were not labelled by Van-FL, indicating that peptidoglycan synthesis does not occur. This confirms that the small size of EzrA-depleted cells with delocalized penicillin-binding proteins is due to a requirement for EzrA in cell wall synthesis. At intermediate inducer concentrations, variation in cell size and Van-FL staining patterns was observed. Small cells showed either no Van-FL labelling, similar to cells grown in the absence of IPTG, or septal Van-FL labelling, similar to wild type. Enlarged cells showed dispersed Van-FL labelling around the cell periphery, but in most cells a ring of nascent peptidoglycan was also seen at midcell (observed as two brighter dots in a single-Z section). In contrast to wild-type cells with midcell localization of Van-FL, completed septa (observed as a line of fluorescence across the cell) were not present in the enlarged cells, showing that higher levels of EzrA are required for septum formation.

Discussion

Bacterial two-hybrid analysis has shown the *S. aureus* divisome to consist of many pairwise interactions. Results showed that there is a close net of at least 49 homo- or heterodimeric protein associations and nearly all components of the division machinery were observed to interact with multiple partners, suggesting that the bacterial divisome is stabilized by many interactions. SepF was the only protein found to interact with a single partner, EzrA, although its interaction with FtsZ in *B. subtilis* is known (Hamoen *et al*., 2006). The high degree of similarity between the interaction webs of division proteins in *S. aureus, E. coli* (Di Lallo *et al*., 2003; Karimova *et al*., 2005) and *S. pneumoniae* (Maggi *et al*., 2008) suggests the existence of a common core bacterial division complex. Novel interactions observed for *S. aureus* proteins were predominantly those of the recently identified and less well-conserved division proteins EzrA, GpsB and SepF as well as RodA, a protein involved in lateral cell wall synthesis during elongation of rod-shaped organisms. The spherical bacterium *S. aureus* does not carry out elongation and synthesizes peptidoglycan only

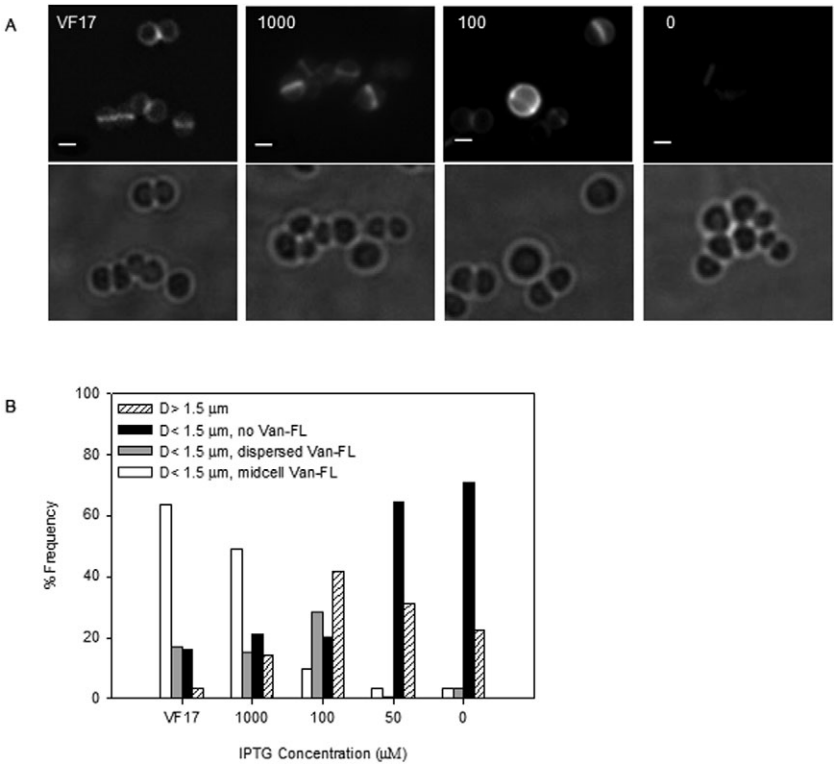

Fig. 7. A. Van-FL labelling of nascent cell wall synthesis in VF17 (SH1000 pGL485) and VF79 (P_{Spac}–*ezrA* pGL485) grown for 120 min in 1000, 100 or 0 µM IPTG. IPTG concentration had no effect on the phenotype of VF17 (SH1000 pGL485) therefore results in the absence of IPTG are shown. Upper images show the fluorescence signal and lower images show the corresponding phase-contrast image. Scale bar = 1 µm.
B. Frequency of cellular phenotypes of VF79 (P_{Spac}–*ezrA* pGL485) grown in 0–1000 µM IPTG for 120 min determined by Van-FL staining. Cells were given one of four phenotypes on the basis of their diameter (D) and localization of Van-FL. Results for VF17 (SH1000 pGL485) grown in the absence of IPTG are also shown. The number of cells measured was 118, 118, 123, 147 and 147 respectively.

at the septum (Pinho and Errington, 2003) where the non-essential *rodA* gene product of *S. aureus* (Chaudhuri *et al*., 2009) may play a minor role.

Protein interaction studies showed *S. aureus* EzrA to interact with cytoplasmic proteins (FtsZ, FtsA, SepF, GpsB), an integral membrane protein (RodA) and proteins with most of their bulk on the outside of the cell membrane (FtsL, DivIC, DivIB, PBP1, PBP2, PBP3). EzrA could therefore act in division at the interface between Z-ring assembly and constriction in the cytoplasm and synthesis of septal peptidoglycan in the periplasm. *S. aureus* EzrA carries only an N-terminal methionine residue on the extracellular side of the membrane and so interaction with outer cell division proteins is likely to occur through cytoplasmic or transmembrane domains. In *B. subtilis*, the transmembrane domain of PBP1 is sufficient for its interaction with EzrA (Claessen *et al*., 2008).

The construction of a *S. aureus* conditional-lethal mutation of *ezrA* shows that this gene is essential for growth in this organism. Phenotypic analysis of EzrA-depleted cells indicated two distinct roles for the protein in peptidoglycan synthesis and cell division. In *B. subtilis, ezrA* is non-essential (Levin *et al*., 1999); however, in its absence several other non-essential genes become required for division (Gueiros-Filho and Losick, 2002; Wu and Errington, 2004; Hamoen *et al*., 2006; Claessen *et al*., 2008) suggesting some functional redundancy. EzrA is essential in both *S. aureus* and *S. pneumoniae* (Thanassi *et al*., 2002), indicating that *ezrA* is less redundant in these cocci than in the rod-shaped *B. subtilis*.

Delocalization of the divisome markers GpsB and Pbp2 in EzrA-depleted *S. aureus* revealed that EzrA is required for assembly of the division machinery at midcell. The presence of intact Z-rings in some EzrA-depleted cells suggests that FtsZ localization is independent of EzrA. In cells partially depleted of EzrA, peptidoglycan synthesis continues causing cell enlargement and shows that division cannot occur without sufficient EzrA. The high abundance of EzrA (Haeusser *et al*., 2004), large-size and likely coiled-coil domains suggests that the protein has a structural role in cell division. EzrA could act as a scaffold that is required to recruit the 'late' division proteins FtsL, DivIC, DivIB, PBP1, PBP2 and GpsB to midcell via their direct interaction with EzrA. EzrA may also act to stabilize the divisome through interaction with multiple components. FtsL is a coiled-coil protein thought to have a structural role in stabilization of the divisome (Sievers and Errington, 2000) and in *B. subtilis* overexpression of FtsL is able to compensate for an *ezrA* null mutation (Kawai and Ogasawara, 2006), indicating some functional overlap between the two proteins. The ability of *B. subtilis* EzrA to increase the GTPase activity of FtsZ and destabilize FtsZ polymers (Haeusser *et al*., 2004; Chung *et al*., 2007; Singh *et al*., 2007) also suggests that EzrA might be required to promote septum formation through depolymerization of the Z-ring.

Visualization of cell wall synthesis using a fluorescent derivative of vancomycin showed that peptidoglycan synthesis does not occur in the absence of *ezrA* expression. In FtsZ-depleted *S. aureus* cells, delocalization of PBP2 results in dispersed cell wall synthesis and cell enlargement (Pinho and Errington, 2003). In contrast, cells do not enlarge in the absence of EzrA despite PBP2 dispersion because cell wall synthesis does not take place. The involvement of EzrA in peptidoglycan biosynthesis has not been previously shown, although it has been suggested in *B. subtilis* because *ezrA* mutation leads to reduced cell diameter (Claessen *et al*., 2008).

The absence of a putative catalytic site suggests that EzrA does not have a direct role in peptidoglycan biosynthesis and instead it is likely that EzrA-dependent formation of the division complex is required for PBP activity. PBPs may require association with peptidoglycan hydrolases or enzymes providing peptidoglycan precursors in order to synthesize new cell wall, or may require allosteric activation through interaction with components of the divisome (Eberhardt *et al*., 2003). The low abundance of PBPs (Pucci and Dougherty, 2002) suggests that chance interactions of PBPs with other proteins involved in cell wall synthesis due to stochastic movement within the cell membrane are unlikely and that formation of a peptidoglycan-synthesizing complex is probably required. In wild-type *S. aureus*, peptidoglycan synthesis takes place only at the division site (Pinho and Errington, 2003) and the divisome is therefore the only peptidoglycan-synthesizing complex of *S. aureus*. Immunofluorescence of FtsZ-depleted *S. aureus* shows that delocalized PBP2 is not homogeneously distributed throughout the membrane, and instead forms discrete foci that result in cell enlargement and patches of thickened cell wall (Pinho and Errington, 2003). Although the fluorescence of the GFP fusion was insufficient to detect similar foci of PBP2 in enlarged *S. aureus* cells partially depleted of EzrA, multiple foci of GpsB–GFP were observed. These foci are likely to represent assemblies of divisome components into complexes that are not correctly targeted to midcell in the absence of FtsZ or sufficient EzrA. Foci of GpsB–GFP were not observed in EzrA-depleted cells, consistent with the hypothesis that EzrA is required for formation of a stable complex of cell division proteins.

In contrast to traditional on/off strategies to determine gene essentiality, titration of *ezrA* expression levels has allowed the multiple roles of the protein in cell division to be distinguished. Null mutation of *B. subtilis ezrA* results in seemingly contradictory phenotypes: extra Z-rings indicate a negative role for the protein in Z-ring assembly (Levin *et al*., 1999; Haeusser *et al*., 2004; Chung *et al*., 2007; Singh *et al*., 2007); however, the delay in cell division suggests a positive role for EzrA in the process (Levin *et al*., 1999; Chung *et al*., 2004; Claessen *et al*., 2008), and cells show a reduced cell diameter (Claessen *et al*., 2008). Variation of *ezrA* expression in *S. aureus*, an organism in which the gene is non-redundant, has shown EzrA to have essential roles in divisome assembly and peptidoglycan synthesis. This allows rationalization of some of the phenotypes observed in a *B. subtilis*: delayed division is likely to be a result of defective divisome assembly and reduced cell diameter a result of defective lateral cell wall peptidoglycan synthesis. This study highlights the previously unexplored benefits of conditional expression analysis as well as revealing EzrA to be an important multifunctional component of the bacterial division apparatus.

Experimental procedures

Bacterial strains, plasmids and oligonucleotides

The strains and plasmids used in this study are listed in Table S1. Table S2 shows the oligonucleotide sequences used.

Growth conditions and media

Staphylococcus aureus strains were grown at 37°C in brain heart infusion broth (BHI; Oxoid). For growth of *S. aureus* on solid media, 1.5% (w/v) agar was added. When required, antibiotics were added at the following concentrations: 30 μg ml^{-1} chloramphenicol, 10 μg ml^{-1} tetracycline or 5 mg ml^{-1} erythromycin with 25 μg ml^{-1} lincomycin for selection of *cat*, *tet* and *erm* markers respectively.

Transformations of *S. aureus* strain RN4220 were performed as described by Schenk and Laddaga (1992). Phage transductions using Φ11 were performed as described previously (Novick and Morse, 1967). DNA manipulation and *E. coli* transformations were performed according to the method of Sambrook and Russell (2001).

Bacterial two-hybrid analyses

To screen for interaction of EzrA with various proteins involved in cell wall synthesis or cell division, the *ezrA*-coding sequence was amplified by PCR using primers GLUSH302M5′ and VRF78 and cloned into the bacterial two-hybrid vectors pUT18 (Karimova *et al*., 1998) and p25-N (Claessen *et al*., 2008), resulting in C-terminal fusions of EzrA to the T18 or T25 domain of the adenylate cyclase enzyme respectively. Inserts and plasmids were restriction digested using EcoRI and BamHI prior to ligation. In addition, the coding sequences of *divIB*, *ftsA*, *ftsL*, *divIC*, *ftsW*, *pbp1*, *pbp2*, *pbp3*, *gpsB*, *sepF* and *rodA* were amplified by PCR using the primers shown in Table S2, and following digestion with either PstI/KpnI (*divIB*, *ftsA*, *ftsL* and *pbp2*) or BamHI/EcoRI (*pbp1*, *divIC*, *pbp3*, *gpsB* and *sepF*) were ligated into pKT25 and pUT18C cut with the same enzymes, creating N-terminal fusions. Cloning of *pbp1* and *pbp3* into the high-copy-number plasmid pUT18C could not be achieved, presumably due to toxicity of the resulting fusions. The coding

sequence of *ftsZ* was amplified using primers GLUSH302J5′ and VF77 cloned into p25-N and pUT18 resulting in C-terminal fusions.

To assay for pairwise interactions using the bacterial two-hybrid system, β-galactosidase activity of BACTH strains was measured qualitatively on solid media using the substrate X-Gal. Cells from an overnight culture were washed three times by centrifugation and resuspension in dH_2O. Ten microlitres of a 1:100 dilution were spotted onto minimal medium agar plates (Daniel *et al.*, 2006) containing 100 μg ml^{-1} amp, 50 μg ml^{-1} kan and 150 μg ml^{-1} X-Gal. Plates were incubated at 30°C for 36 h. To quantify interactions, liquid culture samples were assayed for β-galactosidase activity against MUG (4-methylumbelliferyl-β-D-galactopyranoside), using a method based on that of Youngman (1990). *E. coli* BTH101 carrying both T18 and T25 fusion plasmids was grown to exponential phase in minimal medium (Daniel *et al.*, 2006) at 30°C. Triplicate samples of 100 μl were collected and centrifuged at 13 000 r.p.m. for 5 min. Supernatant was discarded and cell pellets stored at −80°C. The cell pellets were thawed at room temperature for 5 min and then resuspended in 0.5 ml of ABT [5.884 g l^{-1} NaCl, 10.51 g l^{-1} K_2HPO_4, 5.44 g^{-1} KH_2PO_4, 0.1% (v/v) Triton X-100]. Fifty microlitres of 4 mg ml^{-1} MUG was added and the samples were incubated at 25°C for exactly 60 min before the reaction was stopped by addition of 0.5 ml of 42.394 g l^{-1} Na_2CO_3. A fluorimeter (Victor^{2}™, Wallac) was used to measure the fluorescence of a 1:100 dilution of each sample (355/460 nm, 0.1 s). A calibration curve was used to determine the amount of MU produced by the β-galactosidase activity in each sample. One MUG unit is defined as the amount of β-galactosidase that catalyses the hydrolysis of 1 pmol of MUG per minute, per ml of culture, per unit of optical density at 600 nm (OD_{600}).

Construction of an ezrA *inducible strain*

A fragment of 864 bp containing the ribosomal binding site and the first 273 codons of the *ezrA* gene from *S. aureus* SH1000 was amplified by PCR using the primers VRF75 and VRF76, which contain EcoRI and BamHI restriction sites respectively. The PCR fragment was cloned into pAISH1 downstream of the IPTG-inducible Spac promoter (Yansura and Henner, 1984), using the above restriction enzymes. The resulting plasmid, pVF23, was introduced into *S. aureus* RN4220 by electroporation, resulting in transformants with only one full copy of *ezrA*, under the control of P_{Spac}. The correct single-cross-over chromosomal insertion was confirmed by PCR. The chromosome region containing the plasmid insertion was transferred into the SH1000 background by Φ11 transduction, and transformants were verified by PCR.

In order to reduce the basal level of *ezrA* expression from P_{Spac} in the absence of IPTG, the LacI repressor was overproduced from a multicopy plasmid, pGL485 (Cooper *et al.*, 2009).

Protein depletion

IPTG-inducible strains were grown to exponential phase (OD_{600} approximately 0.5) in 50 ml of BHI containing 30 μg ml^{-1} chloramphenicol and either 200 μM IPTG for P_{Spac}–*ezrA* strains or 100 μM IPTG for P_{Spac}–*ftsZ* strains. Cells were washed three times by centrifugation at 5000 r.p.m., 20°C for 10 min and resuspension in BHI pre-warmed to 37°C. The suspension was then used to inoculate 50 ml of fresh BHI containing 30 μg ml^{-1} chloramphenicol and various IPTG concentrations. Cultures were inoculated to OD_{600} 0.001 for essentiality studies and OD_{600} 0.05 for phenotypic studies. For phenotypic analysis, cultures were incubated for 120 min to allow depletion of EzrA before imaging.

Construction of an ezrA–*GFP fusion strain*

A PCR fragment including the entire *ezrA* gene was amplified from SH1000 genomic DNA with primers GLUSh248B5′ and GLUSh248B3′. The PCR product was digested with KpnI and EagI and inserted into pMUTIN–GFP+ (Kaltwasser *et al.*, 2002), previously digested with the same enzymes to create plasmid pGL514. To construct a tetracycline-resistant *ezrA–GFP*+ fusion plasmid, the Spac promoter and *ezrA–GFP*+ gene were excised from pGL514 by restriction digestion with PacI and BsiWI and were ligated into the tetracycline-resistant plasmid backbone of pVF23 cut with the same enzymes, to create plasmid pVF34.

The resultant plasmids, pGL514 and pVF34, were used to transform *S. aureus* RN4220. Integration of either plasmid via a single-cross-over event at the chromosomal *ezrA* locus resulted in an intact copy of *ezrA* under the control of P_{Spac} and the *ezrA–GFP*+ fusion under the control of the native *ezrA* promoter.

Generation of anti-FtsZ antibodies

Anti-FtsZ polyclonal antibodies were obtained from a rabbit immunized with a purified his-tagged form of the *S. aureus* FtsZ (BioServ UK, UK).

Microscopic imaging

For fluorescence microscopy, cells from a mid-exponential-phase culture were fixed with formaldehyde and glutaraldehyde as described previously (Pinho and Errington, 2003). Cell membrane and DNA were stained *in vitro* with 0.24 μg ml^{-1} FM4-64 and 7.5 μg ml^{-1} Hoechst 33342, respectively, at room temperature for 15 min. Cells were washed in PBS and then mounted directly onto poly-L-lysine slides. Fluorescence images were acquired using an Olympus IX70 deconvolution microscope and SoftWoRx 3.5.0 software (Applied Precision). Images were analysed using ImageJ (http://rsbweb.nih.gov/ij/).

Fluorescent vancomycin staining

VF79 was depleted of EzrA for 90 min as described above, except that 0.125 M D-serine (Sigma) was included in the media to allow incorporation of this amino acid into the cell wall. Control samples of VF17 were also grown under the same conditions. Cells were then harvested by centrifugation at 5000 r.p.m. for 10 min at 20°C and resuspended in the

same volume of BHI containing 30 μg ml^{-1} chloramphenicol and the same IPTG concentration but without D-serine. Cultures were incubated for a further 25 min to allow incorporation of D-alanine into the cell wall. A mixture of equal amounts of vancomycin and BODIPY FL-conjugated vancomycin (Molecular Probes) was added to the culture to a concentration of 0.5 μg ml^{-1} and incubated for 5 min at 37°C, 250 r.p.m. Cells were harvested and prepared for microscopy as described above.

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