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The BAM complex subunit BamE (SmpA) is required for membrane integrity, stalk growth and normal levels of outer membrane β-barrel proteins in *Caulobacter crescentus*

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The outer membrane of Gram-negative bacteria is an essential compartment containing a specific complement of lipids and proteins that constitute a protective, selective permeability barrier. Outer membrane β-barrel proteins are assembled into the membrane by the essential hetero-oligomeric BAM complex, which contains the lipoprotein BamE. We have identified a homologue of BamE, encoded by *CC1365*, which is located in the outer membrane of the stalked alpha-proteobacterium *Caulobacter crescentus*. BamE associates with proteins whose homologues in other bacteria are known to participate in outer membrane protein assembly: BamA (CC1915), BamB (CC1653) and BamD (CC1984). *Caulobacter* cells lacking BamE grow slowly in rich medium and are hypersensitive to anionic detergents, some antibiotics and heat exposure, which suggest that the membrane integrity of the mutant is compromised. Membranes of the Δ*bamE* mutant have normal amounts of the outer membrane protein RsaF, a TolC homologue, but are deficient in CpaC*, an aggregated form of the outer membrane secretin for type IV pili. Δ*bamE* membranes also contain greatly reduced amounts of three TonB-dependent receptors that are abundant in wild-type cells. Cells lacking BamE have short stalks and are delayed in stalk outgrowth during the cell cycle. Based on these findings, we propose that *Caulobacter* BamE participates in the assembly of outer membrane β-barrel proteins, including one or more substrates required for the initiation of stalk biogenesis.

INTRODUCTION

The outer membrane of Gram-negative bacteria is an important determinant of cell integrity, physiology and virulence, by virtue of its component lipids and proteins (Nikaido, 2003). The inner leaflet of the outer membrane contains phospholipids, while the outer leaflet contains glycolipids, primarily lipopolysaccharide (LPS). Lipoproteins, which are anchored in a membrane via amino-terminal lipid groups, can reside in either the cytoplasmic membrane or the outer membrane, and are sorted to the correct compartment by the LolAB system (Narita *et al.*, 2004). Integral outer membrane proteins (OMPs) span the membrane as β-barrels composed of amphipathic β-strands. OMPs include porins (Delcour, 2002), TonB-dependent receptors (Braun & Endriss, 2007), TolC homologues (Koronakis *et al.*, 2004), which transport proteins and small molecules, and secretins (Bayan *et al.*, 2006), which are outer membrane channels for type II secretion systems and type IV pili.

The mechanism for targeting and assembling OMPs was largely unknown until 2003, when researchers studying *Neisseria meningitidis* found that the OMP Omp85 is essential for viability and for maintaining normal levels of folded, oligomerized β-barrel proteins in the outer membrane (Voulhoux *et al.*, 2003). The Omp85 homologue in *Escherichia coli* was later discovered to be part of a hetero-oligomeric complex (the BAM complex) that assembles β-barrel proteins into the outer membrane (Knowles *et al.*, 2009). The BAM complex contains the essential β-barrel protein BamA (formerly YaeT in *E. coli* and Omp85 in *N. meningitidis*) and the lipoproteins BamB (YfgL), BamC (NlpB), BamD (YfiO) and BamE (SmpA) (Sklar *et al.*, 2007; Wu *et al.*, 2005). In *E. coli* and *N. meningitidis*, BamA and BamD are essential for viability, and depletion of either one causes several OMPs to accumulate in their unfolded or monomeric forms (Doerrler & Raetz, 2005; Malinverni *et al.*, 2006;

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Abbreviations: ECF, extracytoplasmic function; OMP, outer membrane protein; PYE, peptone-yeast extract.

A supplementary table providing information about strains and plasmids is available with the online version of this paper.

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Volokhina *et al.*, 2009; Voulhoux *et al.*, 2003; Werner & Misra, 2005). BamB, BamC and BamE are not essential for viability in *E. coli* (Bouvier *et al.*, 1991; Rolhion *et al.*, 2005; Ruiz *et al.*, 2005; Sklar *et al.*, 2007), and BamB is absent from neisserial genomes (Volokhina *et al.*, 2009). In single *bamB*, *bamC* or *bamE* mutants, one or a few OMPs are incorrectly assembled, while double mutants have more severe OMP assembly defects or are inviable (Ruiz *et al.*, 2005; Sklar *et al.*, 2007; Volokhina *et al.*, 2009; Wu *et al.*, 2005). The *N. meningitidis* BAM complex contains an additional protein, RmpM (Volokhina *et al.*, 2009), which also associates with the porins PorA and PorB and the TonB-dependent receptors TbpA and LbpA (Jansen *et al.*, 2000; Prinz & Tommassen, 2000). Analysis of *Neisseria rmpM* mutants suggests that this protein stabilizes OMP complexes rather than participating in OMP assembly (Volokhina *et al.*, 2009).

Caulobacter crescentus is a Gram-negative α-proteobacterium that lives in dilute aquatic environments (Poindexter, 1964). Although the *Caulobacter* outer membrane proteome has been characterized (Phadke *et al.*, 2001), no proteins involved in the biogenesis of outer membrane components have been studied in this organism. *Caulobacter* has chiefly been the subject of research on cell cycle regulation and cell polarity (Collier & Shapiro, 2007). Each *Caulobacter* cell division is asymmetrical, yielding a flagellated swarmer cell that cannot initiate chromosome replication and a cell with a polar stalk that immediately begins a new round of chromosome replication and cell division. The swarmer progeny re-enters the cell division cycle when it differentiates into a stalked cell and initiates DNA replication (Iba *et al.*, 1977; Stove & Stanier, 1962).

The investigation of OMP assembly in *Caulobacter* is likely to yield new insights, first because this organism encodes and expresses a distinctive set of OMPs (Ireland *et al.*, 2002; Molloy *et al.*, 2001; Nierman *et al.*, 2001; Phadke *et al.*, 2001). *Caulobacter* lacks genes for the trimeric porins OmpF/C and for the maltoporin LamB, which are highly expressed in *E. coli*. However, the genome encodes several proteins with homology to the monomeric porin OmpA as well as approximately 65 TonB-dependent receptors, which function in energy-dependent nutrient uptake (Postle & Larsen, 2007). TonB-dependent receptors transport siderophores (Pugsley & Reeves, 1976), cobalamin (Bassford & Kadner, 1977), maltodextrins (Neugebauer *et al.*, 2005) and sucrose (Blanvillain *et al.*, 2007), and members of this protein family may transport a wide variety of carbohydrates in plant pathogens and aquatic bacteria (Blanvillain *et al.*, 2007). TonB-dependent receptors may also be particularly important for growth in dilute environments where passive diffusion through porins cannot satisfy the cell's nutritional requirements.

In addition, OMP assembly has not previously been studied in stalked bacteria. The *Caulobacter* stalk is an extension of the cell envelope that is devoid of cytoplasmic components and contains membrane proteins involved in nutrient uptake (Ireland *et al.*, 2002; Poindexter & Cohen-Bazire, 1964). Stalk biogenesis is initiated once in the life of the cell, during differentiation of the swarmer progeny into a stalked cell, and stalk growth continues throughout the life of the cell (Stove & Stanier, 1962). Stalks become elongated under conditions of phosphate limitation (Gonin *et al.*, 2000; Schmidt & Stanier, 1966), and isolated stalks take up the fluorogenic organophosphate compound fluorescein diphosphate (Wagner *et al.*, 2006). Stalk growth could be mediated by the same proteins that build the cell envelope as a whole, but there may also be a dedicated pathway for the assembly of stalk-specific proteins.

Although little is known about the proteins directly involved in building the stalk, two regulatory pathways have been found to promote stalk growth. The alternative sigma factor σ^{54} (encoded by *rpoN*) and the response regulator TacA direct the transcription of genes needed for stalk biogenesis, and *rpoN* and *tacA* mutants are stalkless in rich medium (Biondi *et al.*, 2006; Brun & Shapiro, 1992; Skerker *et al.*, 2005). However, the need for σ^{54} and TacA can be overcome by growing cells in low-phosphate medium (Biondi *et al.*, 2006; Brun & Shapiro, 1992). Under these conditions, a second regulatory pathway, possibly mediated by the response regulator PhoB, promotes stalk outgrowth (Gonin *et al.*, 2000). In addition to these regulatory pathways, some proteins needed for elongation of the entire cell are involved in stalk biogenesis, including penicillin-binding protein 2, RodA and MreB (Seitz & Brun, 1998; Wagner *et al.*, 2005).

Here, we identify a protein in *C. crescentus* that is homologous to *E. coli* BamE and *Pseudomonas aeruginosa* OmlA, and is encoded by *CC1365*. *Caulobacter* BamE is located in the outer membrane and is associated with homologues of other BAM complex proteins. The Δ*bamE* mutant grows slowly in rich and minimal media and is hypersensitive to heat, detergents and antibiotics. The membranes of cells lacking BamE contain normal levels of the TolC homologue RsaF (Toporowski *et al.*, 2004), but contain greatly reduced levels of an aggregate of the secretin CpaC (Viollier *et al.*, 2002), as well as three predicted TonB-dependent receptors. These results are consistent with the phenotypes of *bamE* or *omlA* mutants in other species (Lewis *et al.*, 2008; Ochsner *et al.*, 1999; Sklar *et al.*, 2007). We further show that BamE is required for the timely initiation of stalk biogenesis during the *Caulobacter* cell division cycle. Together, our data strongly suggest that *Caulobacter* BamE functions in the assembly of OMPs which mediate membrane integrity, nutrient uptake and stalk biogenesis.

METHODS

Bacterial strains, plasmids and culture conditions. Strains and plasmids used are listed in Supplementary Table S1, available with the online version of this paper. All experiments were performed using derivatives of *C. crescentus* strain CB15N (Evinger & Agabian, 1977) grown to mid-exponential phase. Plasmids were mobilized from *E. coli* to *C. crescentus* by conjugation using *E. coli* strain S17-1 (Ely,

1991). The sequences of primers used for amplification or introduction of restriction sites are available upon request. CB15N strains were grown in peptone-yeast extract (PYE; Ely, 1991), minimal medium (M2G; Ely, 1991) or M5G low-phosphate medium (10 mM PIPES, pH 7, 1 mM NaCl, 1 mM KCl, 0.05 % NH_4Cl, 0.01 mM Fe/EDTA, 0.2 % glucose, 0.5 mM $MgSO_4$, 0.5 mM $CaCl_2$ and 0.03 mM phosphate) at the indicated temperatures. *E. coli* strains were grown in Luria Broth at 37 °C. Solid and liquid media were supplemented with antibiotics as described by Reisinger *et al.* (2007).

To construct the strain Δ*bamE* (Δ*CC1365*), we replaced the *bamE* open reading frame with a tetracycline resistance cassette flanked by FRT sites (McLeod *et al.*, 1986) by two-step homologous recombination using pJT11. We transformed this strain (KR1694) with pBH474, which encodes Flp recombinase (House *et al.*, 2004). Gentamicin-resistant colonies were selected, grown overnight in PYE medium without antibiotic selection and plated on PYE/3 % (w/v) sucrose to obtain colonies that had lost pBH474. Sucrose-resistant colonies were screened for those that had regained sensitivity to gentamicin and tetracycline.

For 3 × FLAG fusions, we used PCR to generate a *Bam*HI site either after the last codon or after codon 115 of *bamE* and cloned each fragment into *Spe*I/*Bam*HI-digested pSK84, containing the 3 × FLAG coding sequence. We digested these intermediate vectors with *Spe*I and *Eco*RV and ligated each insert into pMR10 to create pJH4, carrying *bamE*: : *3 × FLAG* or pLB40, carrying *bamEΔC*: : *3 × FLAG*. The 3 × FLAG tag adds the amino acid residues DYKDHDGDYKDHDIDYKDDDDK to the C terminus of each protein.

Cell fractionation. KR1735 was grown in PYE/kanamycin medium to an OD_{660} of 0.4. The cell pellet from 100 ml culture was harvested by centrifugation (10 min, 4 °C, 9000 r.p.m., Sorvall GSA rotor). Cells were resuspended in 3 ml TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and lysed by two passages through a French pressure cell at 6000 p.s.i. Unbroken cells were removed by centrifugation (10 min, 4 °C, 5000 r.p.m., Sorvall SS-34 rotor) and the supernatant was loaded onto a two-step gradient composed of 0.3 ml 65 % and 1.0 ml 25 % sucrose (w/v) in TE. After centrifugation (2.5 h, 4 °C, 50 000 r.p.m., Beckman SW60 Ti rotor), the top 3 ml was collected as a soluble fraction containing both cytoplasm and periplasm, the next 500 μl was discarded as cellular debris and the next 500 μl containing total cellular membranes was mixed with 1.5 ml TE. The membrane sample was loaded onto a discontinuous gradient composed of 0.5 ml 65 %, 0.5 ml 55 %, 1 ml 50 %, 2 ml 45 %, 2 ml 40 %, 2 ml 35 % and 1.5 ml 30 % sucrose (w/v) in TE and centrifuged (17 h, 4 °C, 36 000 r.p.m., Beckman SW41 rotor). After centrifugation, 400 μl samples were withdrawn from the top of the gradient, and 20 μl of each even-numbered fraction was assayed for total protein content using quick-start Bradford dye reagent (Bio-Rad) according to the manufacturer's instructions. Protein-containing fractions were analysed by SDS-PAGE and Western blotting using anti-McpA antiserum (Alley *et al.*, 1992; 1 : 30 000), anti-FLAG M2 antibody (Sigma; 1 : 5000), anti-CpaC antiserum (Viollier *et al.*, 2002; 1 : 2000) and anti-RsaF antiserum (Toporowski *et al.*, 2004; 1 : 10 000).

Isolation of the BAM complex. Cell pellets of strains KR1717, KR1735 and KR2473 grown to mid-exponential phase in PYE/kanamycin (30 ml culture at OD_{660}=0.3) were resuspended using 1.0 ml buffer A (50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 0.5 % dodecyl maltoside). Cells were lysed by adding 10 μl of 10 mg lysozyme ml^{-1} and 2 μl benzonase nuclease (Novagen) and rotating end-over-end at room temperature for 15 min. Lysates were cleared by centrifugation for 10 min at 16 000 ***g***. Anti-FLAG M2 agarose was prepared, added to cleared lysates and washed according to the manufacturer's instructions (FLAG immunoprecipitation kit, Sigma). To elute immunoprecipitates, 20 μl 2 × SDS sample buffer was added, and samples were incubated for 5 min at 100 °C. To determine the efficiency of immunoprecipitating BamE–3 × FLAG or BamEΔC–3 × FLAG, we analysed 10 μl of each cleared lysate or flow-through fraction and 0.5 μl of each immunoprecipitate by SDS-PAGE and Western blotting with anti-FLAG M2 antibody (Sigma; 1 : 5000).

Identification of proteins by mass spectrometry. To identify proteins that co-purified with BamE–3 × FLAG or BamEΔC–3 × FLAG, or proteins that were deficient in the membranes of Δ*bamE* cells, we analysed samples by separating using SDS-PAGE and staining with GelCode Blue (Thermo Scientific) according to the manufacturer's instructions. Proteins in each gel slice were excised and digested as described by Jimenez *et al.* (1998). Mass spectrometry was performed on peptides from band 1 of Fig. 5(d) and all BamE-associated bands (see Fig. 3) by the QB3 Proteomics/Mass Spectrometry Laboratory at UC Berkeley. Each sample was loaded onto a 10 cm nano LC column packed in a 100 μm inner diameter glass capillary with an emitter tip. The column consisted of Polaris c18 5 μm packing material (Varian). The column was directly coupled to an electrospray ionization source mounted on a Thermo-Finnigan Deca XP Plus ion trap mass spectrometer. The programmes SEQUEST (Eng *et al.*, 1994) and DTASelect (Tabb *et al.*, 2002) were used to identify peptides. Statistical cutoffs for peptide identification were set at levels shown to give very low rates of false positive identifications (Elias *et al.*, 2005). Peptides from bands 2 and 3 of Fig. 5(d) were analysed by the HHMI Mass Spectrometry Laboratory at UC Berkeley. The buffer was removed with C18 ZipTips (Millipore) and the resulting peptides were analysed by MALDI in reflector mode on an ABI 4800 TOF-TOF mass spectrometer (Applied Biosystems). A peptide mass fingerprint search of the NCBI database identified the principal component of band 3 as CC2010. The MS/MS spectra of three of the tryptic peptides obtained from that band were acquired and matched the theoretical tryptic peptide sequences, SSITQDFISR, [*m*/*z* 1153.6 (MH^+, monoisotopic)], YNINPSNTGNLR (*m*/*z* 1163.5) and LTRPEPETTQAYDLGYR (*m*/*z* 2010.0) from CC2010. A similar search of the mass spectrum of band 2 identified it as primarily CC2819. Two of the tryptic peptides obtained from band 2 were consistent with the theoretical tryptic peptides FNNVGVNLVWSHLDDVVDDNPASR, (*m*/*z* 2682.3) and GFGVNPVPR (*m*/*z* 942.5) from that protein.

Chemical sensitivity tests. Exponential phase cells of each strain grown in PYE medium were diluted to OD_{660}=0.25; 100 μl was added to 4 ml PYE medium containing 0.3 % agar at 42 °C. The agar was poured onto a PYE/kanamycin plate to solidify at room temperature. Ten microlitres of each tested compound was pipetted onto a sterile filter paper disc (3M) and each disc was transferred onto the overlaid agar using sterile forceps. Plates were incubated upright at 30 °C for 24 h and the zone of growth inhibition between each disc and the lawn of cells was measured.

Isolation of surface RsaA protein. Surface-exposed RsaA protein was extracted with acid as described by Koronakis *et al.* (2000). RsaA was visualized using SDS-PAGE and Western blotting using anti-RsaA antiserum (Toporowski *et al.*, 2004; 1 : 10 000).

Stalk length measurements. To measure stalk lengths in PYE medium, cultures were grown at 30 °C to OD_{660} 0.2–0.4. To measure stalk lengths in M5G-low phosphate medium, cells were initially grown in M2G medium to OD_{660} 0.4–0.6, then diluted 1 : 500 into M5G low phosphate medium and grown at 30 °C to OD_{660} 0.1–0.3. Cells were immobilized on agarose pads (1 % w/v agarose in distilled water), and differential interference contrast images were acquired as described by Reisinger *et al.* (2007). Pixel lengths of stalks were measured using the line region tool in Metavue (Universal Imaging) and all cells displaying a stalk were scored. Pixel lengths were converted to micrometres by photographing and measuring the

distances between scored lines of a Petroff Hausser Counter (catalogue no. 3900, Hausser Scientific) in the same manner.

Time-lapse microscopy of synchronized cells. Wild-type and mutant cells were grown in 50 ml M2G to OD_{660} 0.3–0.5 and harvested by centrifugation (10 min at 12 000 r.p.m., Sorvall SS34 rotor). We resuspended each cell pellet in 5 ml M2 salts (6.0 mM Na_2HPO_4, 3.9 mM KH_2PO_4, 9.3 mM NH_4Cl), added 5 ml Percoll (Sigma) and aliquoted the mixture into microcentrifuge tubes. After centrifuging for 20 min at 4 °C at 10 000 r.p.m. in a microcentrifuge, we harvested and pooled the swarmer cell bands. Isolated swarmer cells were washed twice in M2 salts and resuspended in 400–600 μl M2G medium. We spotted 0.6 μl onto an agarose pad [1 % (w/v) agarose in M2G medium], sealed the edges with Valap (equal weights lanolin, petroleum jelly and paraffin) and photographed the same field of cells at 30 min intervals. For each time point, we determined the percentage of cells that had grown a visible stalk and the percentage of cells that had divided. Cells on the agarose pad that failed to grow (i.e. were the same length at the beginning and end of the time-lapse) were not counted.

RESULTS

CC1365 has homology to the lipoprotein BamE

The *C. crescentus* gene *CC1365* was originally predicted to encode a conserved hypothetical protein (Nierman *et al.*, 2001) and was identified in a bioinformatics study as a signature protein of the α-proteobacteria (Kainth & Gupta, 2005). However, proteins closely related to CC1365 encoded in other α-proteobacterial genomes have been annotated as members of the SmpA/OmlA family of lipoproteins (Badger *et al.*, 2006; DelVecchio *et al.*, 2002), which is present in the alpha-, beta- and gammaproteobacteria. We aligned the amino acid sequence of CC1365 with BamE and OmlA proteins that have been studied experimentally from the organisms *E. coli* (Sklar *et al.*, 2007), *Salmonella enterica* serovar Typhimurium (Lewis *et al.*, 2008), *P. aeruginosa* PAO1 (Ochsner *et al.*, 1999) and *Xanthomonas axonopodis* pv. *citri* (Vanini *et al.*, 2008) using Kalign (Lassmann & Sonnhammer, 2005). These proteins share significant homology over the first approximately 107 aa of CC1365 (Fig. 1), corresponding to the Pfam domain SmpA_OmlA (PF04355). The regions of highest homology also correspond to distinct secondary structural elements in the solution structure of *X. citri* OmlA (Fig. 1; Vanini *et al.*, 2008), including residues predicted to form the hydrophobic core of each protein (M41, L51, L55, W69, Y71 and F96 of CC1365). The N-terminal 22 aa of CC1365 are predicted to function as a lipoprotein secretion signal and lipid attachment site (Prosite PDOC00013). This sequence specifies lipid modification on the side chain of a conserved cysteine residue (Fig. 1, green shading), cleavage by signal peptidase II just before the cysteine and subsequent lipid modification of the α-amino group of the N-terminal cysteine (Sankaran *et al.*, 1995). Based upon these conserved sequence features, we hereafter refer to CC1365 as *Caulobacter* BamE.

The OmlA proteins and *Caulobacter* BamE have non-homologous C-terminal extensions that are not found in *E. coli* or *S.* Typhimurium BamE (Fig. 1). BamE/OmlA homologues in alpha- and betaproteobacteria typically contain C-terminal extensions of approximately 60–100 aa beyond the region of conservation. Among the gammaproteobacteria, BamE homologues in the enterobacteriales

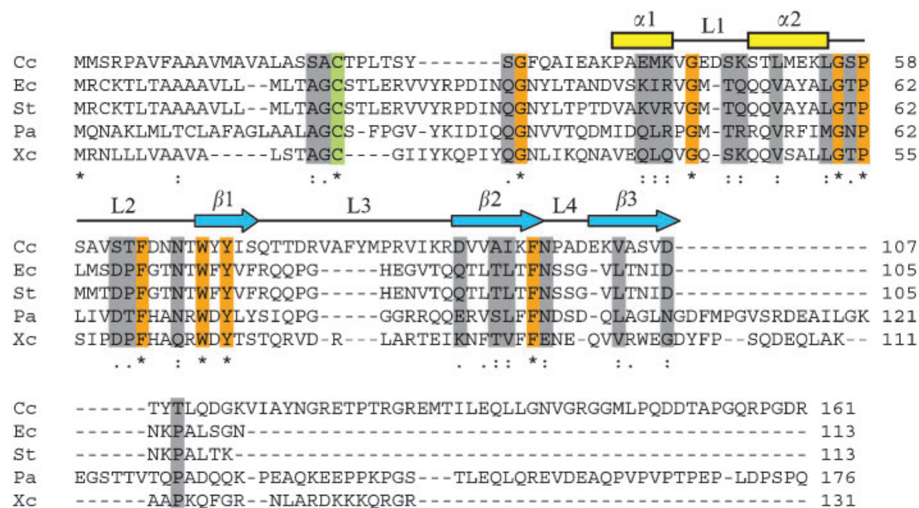

Fig. 1. Sequence alignment of BamE/OmlA homologues generated by Kalign (Lassmann & Sonnhammer, 2005). *C. crescentus* CC1365 (Cc) was aligned with BamE/OmlA homologues that have been studied experimentally from *E. coli* (Ec), *S. enterica* serovar Typhimurium (St), *P. aeruginosa* (Pa) and *X. axonopodis* pv. *citri* (Xc). Identical (orange shading, asterisks) and similar (grey shading, dots) amino acid residues are highlighted. The conserved cysteine residue where signal cleavage and lipid modification are predicted to occur is indicated (green shading). Secondary structure elements indicated above the sequence correspond to the solution structure of *X. citri* OmlA (Vanini *et al.*, 2008).

contain only a few amino acids beyond the SmpA_OmlA domain, while homologues in other groups possess tails up to 70 aa in length. When the C-terminal extension of *Caulobacter* BamE (beginning with T109) is used as a BLAST query (Altschul *et al.*, 1990) against α-proteobacterial genomes, it identifies the same SmpA_OmlA proteins (and hypothetical proteins) as the full-length protein sequence, indicating that the C-terminal extension is conserved among alphaproteobacterial BamE proteins. In contrast, the C-terminal extension of *Caulobacter* BamE identifies no sequences of significant homology in beta- or gamma-proteobacterial genomes.

BamE is located in the *Caulobacter* outer membrane

Lipoproteins of Gram-negative bacteria are modified by three acyl chains on an N-terminal cysteine residue, and they can be anchored in either the cytoplasmic or the outer membrane (Narita *et al.*, 2004). Rules for sorting lipoproteins have been established in some gammaproteobacteria (Lewenza *et al.*, 2006; Narita & Tokuda, 2007; Seydel *et al.*, 1999), but no similar studies have been undertaken in alphaproteobacteria. To determine where BamE resides in the cell envelope, we deleted the chromosomal copy of *bamE* and expressed a version of BamE tagged at the C terminus with a 3 × FLAG epitope for detection on Western blots. BamE–3 × FLAG complements the chemical sensitivity (Table 2) and stalk length defects (Fig. 6a, h) of the deletion mutant, indicating that the fusion protein is functional. We harvested total cell membranes of this strain, separated them by sucrose density-gradient centrifugation, and probed the resulting fractions with anti-FLAG antibodies and antisera that recognize the inner membrane chemoreceptor McpA (Alley *et al.*, 1992), the outer membrane secretin for the type IV pilus, CpaC (Skerker & Shapiro, 2000; Viollier *et al.*, 2002), and the TolC homologue RsaF, which secretes the crystalline S-layer protein RsaA (Toporowski *et al.*, 2004).

McpA levels peaked in lower-density fractions and diminished steadily in the higher-density fractions (Fig. 2), consistent with the migration of inner-membrane proteins (Clancy & Newton, 1982). In contrast, BamE–3 × FLAG abundance peaked in the high-density fractions, characteristic of OMPs. Both CpaC monomers (50 kDa) and the high-molecular-mass species CpaC* (~110 kDa) were most abundant in high-density fractions. CpaC* is recognized by purified anti-CpaC antibodies and represents an aggregate of CpaC monomers (Viollier *et al.*, 2002). Anti-RsaF antiserum recognizes two homologous proteins, $RsaF_a$ and $RsaF_b$, each of which can secrete RsaA (Toporowski *et al.*, 2004). $RsaF_a$ was distributed in both the inner and outer membrane fractions, while $RsaF_b$ was present exclusively in the high-density outer membrane fractions. *Caulobacter* BamE resides chiefly in the outer membrane, along with CpaC and RsaF. The slightly different distributions of the OMPs may be due to different assembly pathways or kinetics.

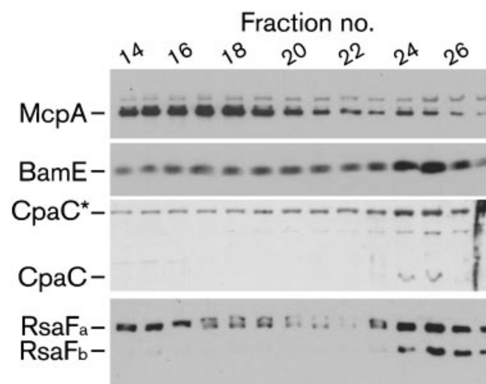

Fig. 2. Total membranes of Δ*bamE*/p*bamE*::*3×FLAG* were separated by sucrose density centrifugation, and protein-containing fractions were analysed by SDS-PAGE and Western blotting with antibodies against McpA, CpaC, RsaF and the FLAG epitope.

Caulobacter BamE associates with other proteins implicated in OMP assembly

In *E. coli*, BamE is one subunit of a hetero-oligomeric complex (the BAM complex) responsible for assembling β-barrel proteins into the outer membrane (Sklar *et al.*, 2007; Wu *et al.*, 2005). Within the BAM complex, BamB interacts directly with BamA, while BamC, D and E depend on each other for interaction with BamA (Malinverni *et al.*, 2006; Sklar *et al.*, 2007; Vuong *et al.*, 2008). Because *Caulobacter* lacks a BamC homologue (Gatsos *et al.*, 2008), we wondered if BamE (and BamD) could still associate with BamA.

We solubilized whole cells of strain KR1717 (Δ*bamE*/p*bamE*) or strain KR1735 (Δ*bamE*/p*bamE*::*3 × FLAG*), incubated the cleared lysates with anti-FLAG M2 agarose (Sigma), and eluted bound proteins. Western blots using anti-FLAG M2 antibodies showed that BamE–3 × FLAG was completely removed from the initial cell lysate by this procedure (data not shown). Immunoprecipitates were analysed by SDS-PAGE and stained with GelCode Blue. FLAG-tagged BamE specifically coprecipitated with proteins of ~85 and ~30 kDa (Fig. 3, compare lanes 1 and 2). At ~50 kDa, a faint doublet appeared, in which the lower band was non-specific, while the upper band was present only in the immunoprecipitate containing FLAG-tagged BamE (Fig. 3, compare lanes 1 and 2). The ~85 kDa band was identified by mass spectrometry as *Caulobacter* BamA (CC1915), while the ~30 kDa band corresponded to BamD (CC1984). Peptides from BamB (CC1653) were predominant in the 50 kDa region associated with BamE–3 × FLAG, while this protein was absent from the 50 kDa region associated with untagged BamE. Thus, *Caulobacter* contains a protein complex including homologues of BamA, BamB, BamD and BamE, despite lacking a homologue of BamC.

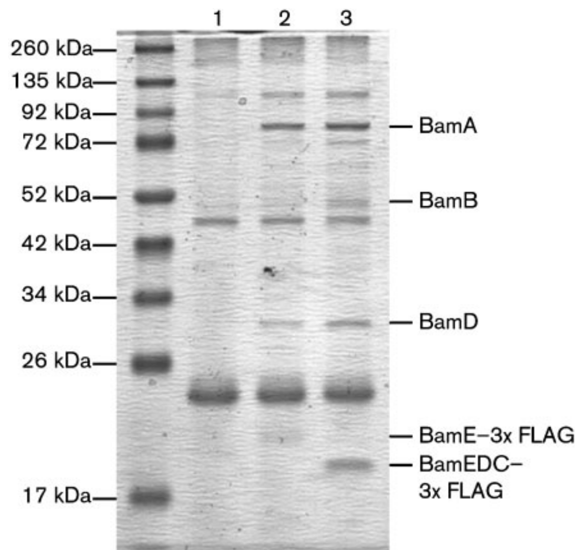

Fig. 3. Equal amounts of anti-FLAG M2 immunoprecipitates from strains KR1717 (Δ*bamE*/p*bamE*, lane 1), KR1735 (Δ*bamE*/p*bamE*::*3×FLAG*, lane 2) and KR2473 (Δ*bamE*/p*bamEΔC*::*3×FLAG*, lane 3) separated on SDS-PAGE and stained with GelCode Blue. Bands labelled BamA, BamB and BamD were excised from the gel and digested with trypsin, and the resulting peptides were identified by mass spectrometry.

Growth defect of the Δ*bamE* mutant

We measured the growth in rich (PYE) and minimal (M2G) media of the wild-type strain CB15N/pMR10, the mutant Δ*bamE*/pMR10 and Δ*bamE* containing the complementing low-copy plasmid p*bamE*. Δ*bamE*/pMR10 grew more slowly than either the wild-type strain or the complemented mutant in both media, but the growth rate discrepancy was reduced in M2G (Table 1). Further, Δ*bamE*/pMR10 cells grew at approximately the same rate in both rich and minimal media (Table 1). Thus, *Caulobacter* BamE is particularly important to sustain rapid growth on rich medium. Unlike the *Caulobacter* Δ*bamE* mutant, *P. aeruginosa*, *E. coli* and *S.* Typhimurium strains lacking OmlA or BamE have wild-type growth rates in both rich and minimal media (Lewis *et al.*, 2008; Ochsner *et al.*, 1999; Sklar *et al.*, 2007). However, other combinations of mutations that affect components of the BAM complex in *E. coli* are only tolerated when the growth rate is reduced by propagation on minimal media or at low temperature (Ruiz *et al.*, 2005; Wu *et al.*, 2005).

Heat and chemical sensitivity of the Δ*bamE* mutant

The *P. aeruginosa* mutant lacking OmlA is not thermosensitive (Ochsner *et al.*, 1999), but the *S.* Typhimurium *bamE* mutant grows more slowly than a wild-type strain at 46 °C (Lewis *et al.*, 2008). To determine if BamE is

Table 1. Doubling times (h) of wild-type, Δ*bamE* and complemented strains

During exponential growth, the OD_{660} and c.f.u. ml^{-1} were measured for each indicated strain. The doubling time was calculated for each experiment, and the mean ± SD for three independent experiments is reported.

Strain	PYE		M2G	
	OD_{660}	c.f.u. ml^{-1}	OD_{660}	c.f.u. ml^{-1}
CB15N/pMR10	1.67 ± .07	1.67 ± .12	2.46 ± .11	2.35 ± .07
Δ*bamE*/pMR10	2.72 ± .34	2.91 ± .35	2.82 ± .10	2.86 ± .21
Δ*bamE*/p*bamE*	1.71 ± .12	1.62 ± .02	2.44 ± .10	2.66 ± .11

necessary for *Caulobacter* survival at elevated temperatures, we incubated the strains CB15N/pMR10, Δ*bamE*/pMR10 and Δ*bamE*/p*bamE* at 42 °C and measured the optical density (660 nm) and viable cell count of each culture. CB15N/pMR10 and Δ*bamE*/p*bamE* increased in optical density during the incubation (Fig. 4a), but the number of viable cells ml^{-1} decreased by approximately 100-fold (Fig. 4b). Δ*bamE*/pMR10 underwent only a minimal increase in optical density (Fig. 4a) and decreased approximately 10 000-fold in viability (Fig. 4b). Thus, in *Caulobacter*, BamE contributes to survival during exposure to high temperatures.

Mutants in other species lacking components of the BAM complex are hypersensitive to some chemical agents due to membrane permeability defects (Fardini *et al.*, 2009; Lewis *et al.*, 2008; Ochsner *et al.*, 1999; Ruiz *et al.*, 2006; Volokhina *et al.*, 2009). We tested the sensitivity of the wild-type and Δ*bamE* *Caulobacter* strains to a variety of detergents and antibiotics. Δ*bamE* cells displayed enhanced sensitivity to the anionic detergents deoxycholate and SDS, but not to the nonionic detergents Tween 20 and Triton X-100 (Table 2). Growth of Δ*bamE* cells was selectively inhibited by 0.5 M EDTA and by the antibiotics nalidixic acid, carbenicillin and rifampicin (Table 2), which target DNA gyrase, peptidoglycan transpeptidation and RNA polymerase, respectively (Raleigh *et al.*, 2002). Hypersensitivity to each chemical was complemented by p*bamE* (Table 2). The Δ*bamE* mutation in *Caulobacter* causes hypersensitivity to antibiotics which affect disparate processes in different cellular compartments, consistent with a role for BamE in generating or maintaining the outer membrane permeability barrier.

Each species whose *bamE*/*omlA* mutant has been tested for chemical sensitivity defects displays a different basal level of sensitivity to different detergents and antibiotics (Fardini *et al.*, 2009; Fuangthong *et al.*, 2008; Lewis *et al.*, 2008; Ochsner *et al.*, 1999). For example, wild-type *Xanthomonas campestris* pv. phaseoli, *P. aeruginosa* and *C. crescentus* are growth-inhibited by 10 % SDS, but *S.* Typhimurium is unaffected by 20 % SDS. Furthermore, the *bamE*/*omlA* mutants of *P. aeruginosa* and *C. crescentus* are much more

Table 2. Susceptibilities of strains to detergents and antibiotics

ND, Not done.

Compound*	Zone of growth inhibition (mm)†				
	CB15N/ pMR10	Δ*bamE*/ pMR10	Δ*bamE*/ p*bamE*	Δ*bamE*/p*bamE*:: 3×*FLAG*	Δ*bamE*/p*bamE*Δ*C*:: 3×*FLAG*
10 % Tween 20	0	0	0	ND	ND
10 % Triton X-100	0	0	0	0	ND
10 % deoxycholate	2	4	2	2	2
10 % SDS	7	11	8	8	9
0.5 M EDTA	5	11	5	5	7
2 mg oxytetracycline ml^{-1}	13	14	13	13	ND
1 mg chloramphenicol ml^{-1}	15	15	14	13	ND
10 mg nalidixic acid ml^{-1}	1	10	1	1	2
50 mg carbenicillin ml^{-1}	3	8	4	3	5
3 mg rifampicin ml^{-1}	11	18	11	11	15
5 mg streptomycin ml^{-1}	8	10	8	9	ND

*Ten microlitres of each compound was spotted onto a sterile disc placed on PYE top agar containing 5×10^7 cells.
†Zones were measured after 24 h growth at 30 °C. Each value is the mean of at least three experiments.

sensitive to SDS than their respective wild-type strains, but the *omlA* mutant of *X. campestris* is growth-inhibited to the same extent as the wild-type strain. It is therefore difficult to make broad statements about the effects of individual chemicals on the loss of BamE/OmlA. However, we noted two trends: (i) whether the species in question was growth-inhibited by non-ionic detergents or not, the *bamE*/*omlA* mutant of the same species was not affected to a greater extent than the wild-type (Fuangthong *et al.*, 2008; Lewis *et al.*, 2008; Ochsner *et al.*, 1999); and (ii) all species tested were growth-inhibited by rifampicin, and all *bamE*/*omlA* mutants were more sensitive to rifampicin than the wild-type strains (Fardini *et al.*, 2009; Lewis *et al.*, 2008; Ochsner *et al.*, 1999). Chemical sensitivity is influenced by the specific complement of porins and outer membrane transporters present in each wild-type strain, as well as which molecules are affected by the loss of BamE/OmlA, so it is not surprising to find distinct phenotypes in different species.

Membranes of Δ*bamE* cells are deficient in some OMPs

When proteins of the BAM complex are removed from *N. meningitidis*, *E. coli* or *S.* Enteritidis by mutation or depletion, OMPs of various types are reduced in abundance or accumulate in premature forms (Fardini *et al.*, 2009; Ruiz *et al.*, 2005; Sklar *et al.*, 2007; Voulhoux *et al.*, 2003; Wu *et al.*, 2005). Depletion of BamA or BamD causes profound effects on OMP biogenesis, as only one OMP, PulD, has been reported to be normally assembled when BamA is depleted (Collin *et al.*, 2007). In the case of BamC, a non-essential member of the complex in *E. coli*, the TolC protein is correctly assembled in the deletion mutant, but OmpA, LamB and OmpF/C are not (Charlson *et al.*, 2006; Ruiz *et al.*, 2005; Wu *et al.*, 2005). An *E. coli bamE* mutant has reduced OmpA levels and accumulates an unfolded form of LamB (Sklar *et al.*, 2007), but no other OMP defects have been reported. The non-uniform dependence of OMPs upon subunits of the BAM complex could indicate that some components are not strictly necessary for OMP assembly, but improve the efficiency of the process. Alternatively, individual OMPs may be channelled to the central component, BamA, using different subsets of the accessory lipoproteins, BamB–E.

We separated the membrane and soluble fractions of *Caulobacter* cells containing or lacking BamE to determine if known *Caulobacter* OMPs are present in reduced levels in the Δ*bamE* mutant. The $RsaF_a$ and $RsaF_b$ proteins are found exclusively in the *Caulobacter* membrane fraction, and they are present in similar amounts in wild-type and Δ*bamE* cells (Fig. 5a). $RsaF_a$ and $RsaF_b$ are expected to function as trimers, similar to TolC (Koronakis *et al.*, 2000). To determine if the RsaF proteins in the Δ*bamE* mutant are competent to secrete RsaA, we isolated surface-exposed RsaA protein by low-pH extraction (Koronakis *et al.*, 2000). Surface RsaA levels were similar in cells of CB15N/pMR10, Δ*bamE*/pMR10 and Δ*bamE*/p*bamE* (Fig. 5b), suggesting that RsaF is assembled into functional trimers in the absence of BamE.

We observed CpaC monomers in both the soluble and membrane fractions, in equal amounts in the wild-type and Δ*bamE* strains (Fig. 5c), indicating that Δ*bamE* cells can still express CpaC monomers and target them to the cell envelope. The high-molecular-mass form CpaC* was present only in the membrane fraction, and the level of CpaC* was greatly reduced in the mutant Δ*bamE* (Fig. 5c, lanes 3 and 4). These results suggest that BamE is necessary

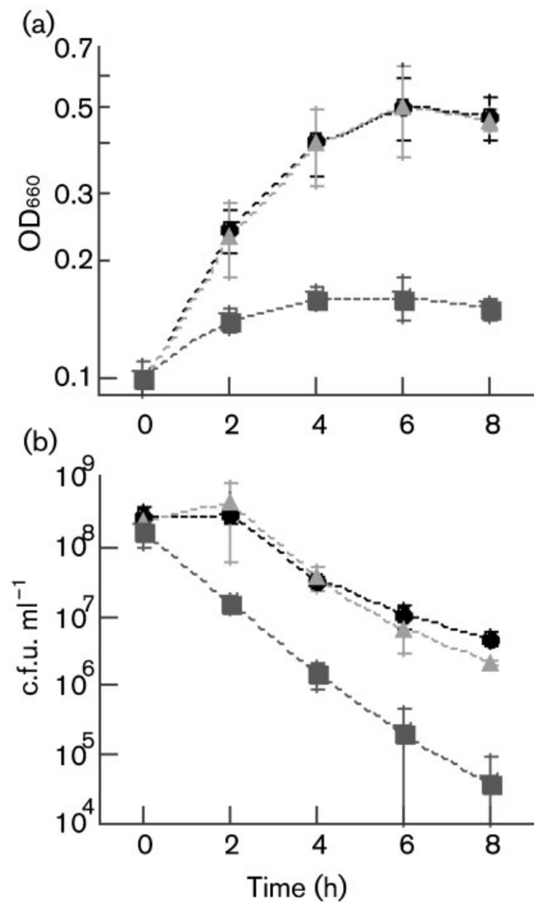

Fig. 4. Cells lacking BamE have decreased growth and viability during heat exposure. Strains were grown to OD_{660}=0.2–0.5 in PYE/kan medium at 30 °C, diluted to OD_{660}=0.1, then incubated at 42 °C for the duration of the experiment. At the indicated times, samples were withdrawn from each culture, and OD_{660} (a) and viable cells (b) were measured. CB15N/pMR10, ●; Δ*bamE*/pMR10, ■; Δ*bamE*/p*bamE*, ▲. Error bars, ± SD.

for the assembly or maintenance of CpaC multimers in the *Caulobacter* outer membrane.

To detect additional proteins affected by the loss of BamE, we analysed the soluble and membrane fractions of wild-type and Δ*bamE* cells by SDS-PAGE and staining with GelCode Blue. Both strains had roughly the same composition of soluble proteins (Fig. 5d, lanes 1 and 2). Unlike the *P. aeruginosa omlA* and *S.* Enteritidis *bamE* mutants that have little or no change in their OMP profiles (Fardini *et al.*, 2009; Ochsner *et al.*, 1999), Δ*bamE* membranes were severely deficient in three prominent, high molecular mass proteins (Fig. 5d, lanes 3 and 4). These three proteins were excised from the gel lane containing wild-type membranes, digested with trypsin and identified by mass spectrometry.

Each band was primarily composed of an OMP homologous to TonB-dependent receptors. Band 1 was identified as CC3013, band 2 as CC2819 and band 3 as CC0210. (For example, 83 % of the *Caulobacter* peptides identified in band 1 were derived from CC3013, covering 49.9 % of the CC3013 protein sequence. The next most abundant protein, CC0288 contributed only 10 % of the *Caulobacter* peptides in band 1, yielding 14.7 % sequence coverage.) All three proteins were identified in a proteomic study of *Caulobacter* OMPs (Phadke *et al.*, 2001), and CC0210 and CC2819 were also identified in a study of proteins enriched in *Caulobacter* stalks (Ireland *et al.*, 2002). Finally, CC3013 and CC0210 were identified as OMPs that are abundant during growth on rich medium but not on minimal medium (Neugebauer *et al.*, 2005). Because the levels of CpaC* and three abundant TonB-dependent receptors are reduced in Δ*bamE* membranes, and because *Caulobacter* BamE associates with other BAM complex homologues, we propose that it participates in the assembly of these OMPs.

BamE is required for normal stalk biogenesis

In PYE medium, stalks of the mutant Δ*bamE* were shorter than those of wild-type cells, and this morphological defect was corrected by supplying *bamE* on a low-copy plasmid (Fig. 6b, d). The mean stalk lengths (μm) in PYE, calculated from measurements of >250 stalks for each strain, were 2.5 ± 0.8 for CB15N/pMR10, 1.4 ± 0.4 for Δ*bamE*/pMR10 and 2.6 ± 0.7 for Δ*bamE*/p*bamE*.

Under conditions of phosphate limitation, wild-type *Caulobacter* cells grow elongated stalks (Gonin *et al.*, 2000; Schmidt & Stanier, 1966) which enhance nutrient uptake (Wagner *et al.*, 2006). Some strains, such as the mutants *ctrA401* (Quon *et al.*, 1996), *rpoN* (Brun & Shapiro, 1992) and *tacA* (Biondi *et al.*, 2006; Skerker *et al.*, 2005), are stalkless in rich medium but build polar stalks in low-phosphate medium. In M5G low-phosphate medium, the stalks of Δ*bamE* cells were longer than in PYE medium (Fig. 6c and f), indicating that this mutant can sense and respond to phosphate limitation. However, stalks of the mutant strain were still much shorter than those of wild-type cells or of the mutant complemented by p*bamE* (Fig. 6a and e–g). The stalk length distributions for the wild-type and complemented strains grown in low-phosphate medium are broad because measurements were performed using mixed cultures, which contain newly born stalked cells as well as older cells that have been engaged in stalk elongation for several generations.

In micrographs of the mutant Δ*bamE*/pMR10 grown in low-phosphate medium, we observed debris that resembled broken stalks (Fig. 6f, white arrowheads). Short stalks could result from a defect in stalk growth, from a normal rate of growth followed by stalk breakage, or from a combination of breakage and impaired growth. The cells in these images were grown in shaking cultures, where shear forces could promote stalk breakage. To eliminate the effects of stalk breakage and to determine whether stalk growth is initiated at the proper time during differenti-

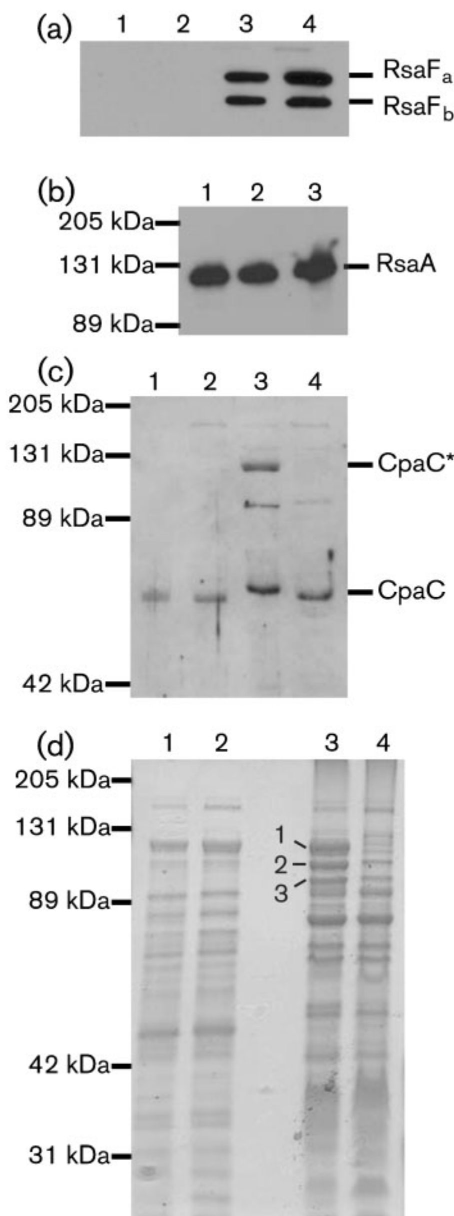

Fig. 5. Levels of CpaC* and of three abundant membrane proteins are reduced in Δ*bamE* membranes. (a, c, d) Equal amounts of soluble and membrane proteins were analysed by SDS-PAGE. Lanes: 1, CB15N/pMR10 soluble; 2, Δ*bamE*/pMR10 soluble; 3, CB15N/pMR10 membrane; 4, Δ*bamE*/pMR10 membrane. RsaF$_a$ and RsaF$_b$ (a) and CpaC and CpaC* (c) were detected by Western blotting; proteins were visualized by GelCode Blue staining (d), and three indicated protein bands (1–3) were excised for trypsin digestion and analysis by mass spectrometry. (b) Surface-exposed RsaA was extracted from CB15N/pMR10 (lane 1), Δ*bamE*/pMR10 (2) and Δ*bamE*/p*bamE* (3) and detected by SDS-PAGE and Western blotting.

ation, we performed time-lapse microscopy on cells growing on agarose pads. We isolated swarmer cells of the wild-type and Δ*bamE* strains and immobilized them on agarose pads containing minimal medium (M2G). We photographed the cells at 30 min intervals, noting the time of division and the first appearance of a stalk on each cell in the field. In these experiments, 33 % of wild-type cells divided completely by 180 min, and ~60 % of cells had a visible stalk at this time (Fig. 7). Δ*bamE* cells took 210 min to achieve 30 % cell division, consistent with their slower growth in liquid cultures, but at this time, no cells had visible stalks (Fig. 7). We know that stalk growth is not completely inhibited in the mutant because liquid cultures contain cells with visible, albeit short, stalks (Fig. 6). Therefore, the short stalk phenotype of Δ*bamE* cells is caused at least in part by slow or delayed stalk growth.

The C-terminal extension of BamE is important for optimal BamE function, but not for stability of the BAM complex

To determine the importance of the C-terminal segment of BamE beyond the conserved BamE/OmlA domain, we truncated the protein after amino acid 115 and appended the 3 × FLAG epitope. The resulting protein, BamEΔC–3 × FLAG, was expressed in the Δ*bamE* mutant at levels comparable to the wild-type, tagged protein (data not shown). Cells expressing the truncated BamE protein were less sensitive to several chemicals than the Δ*bamE* mutant, but more sensitive than the mutant expressing full-length BamE (Table 2). When grown in M5G-low phosphate medium, cells expressing BamEΔC–3 × FLAG grew extended stalks, similar to cells containing full-length versions of BamE (Fig. 6a, i). Thus, BamEΔC–3 × FLAG complements different defects of the Δ*bamE* mutant to different extents.

A previous study of the *E. coli* BAM complex identified alleles of *bamB* (*yfgL*) that caused little or no phenotypic defect, yet weakened the interaction between BamB and BamA in co-immunoprecipitation experiments, suggesting that the affected amino acids are important for BAM complex stability (Vuong *et al.*, 2008). To generate intermediate defects in chemical sensitivity (Table 2), either the function of *Caulobacter* BamEΔC or the stability of the resulting BAM complex could be compromised. We used anti-FLAG M2 agarose to isolate BamEΔC and its associated proteins from lysates of the strain Δ*bamE*/p*bamEΔC*: : *3 × FLAG*. BamA, BamB and BamD were present in coimmunoprecipitates of the truncated BamE protein, at levels comparable to the wild-type complex (Fig. 3, lane 3), and protein identities were confirmed by mass spectrometry. Thus, functional impairment of the truncated BamE protein is likely to cause the chemical sensitivity phenotype, rather than an inability of BamEΔC to associate with other BAM proteins.

DISCUSSION

Genes encoding predicted lipoproteins of the BamE/OmlA family are found in sequenced genomes of alpha-, beta-

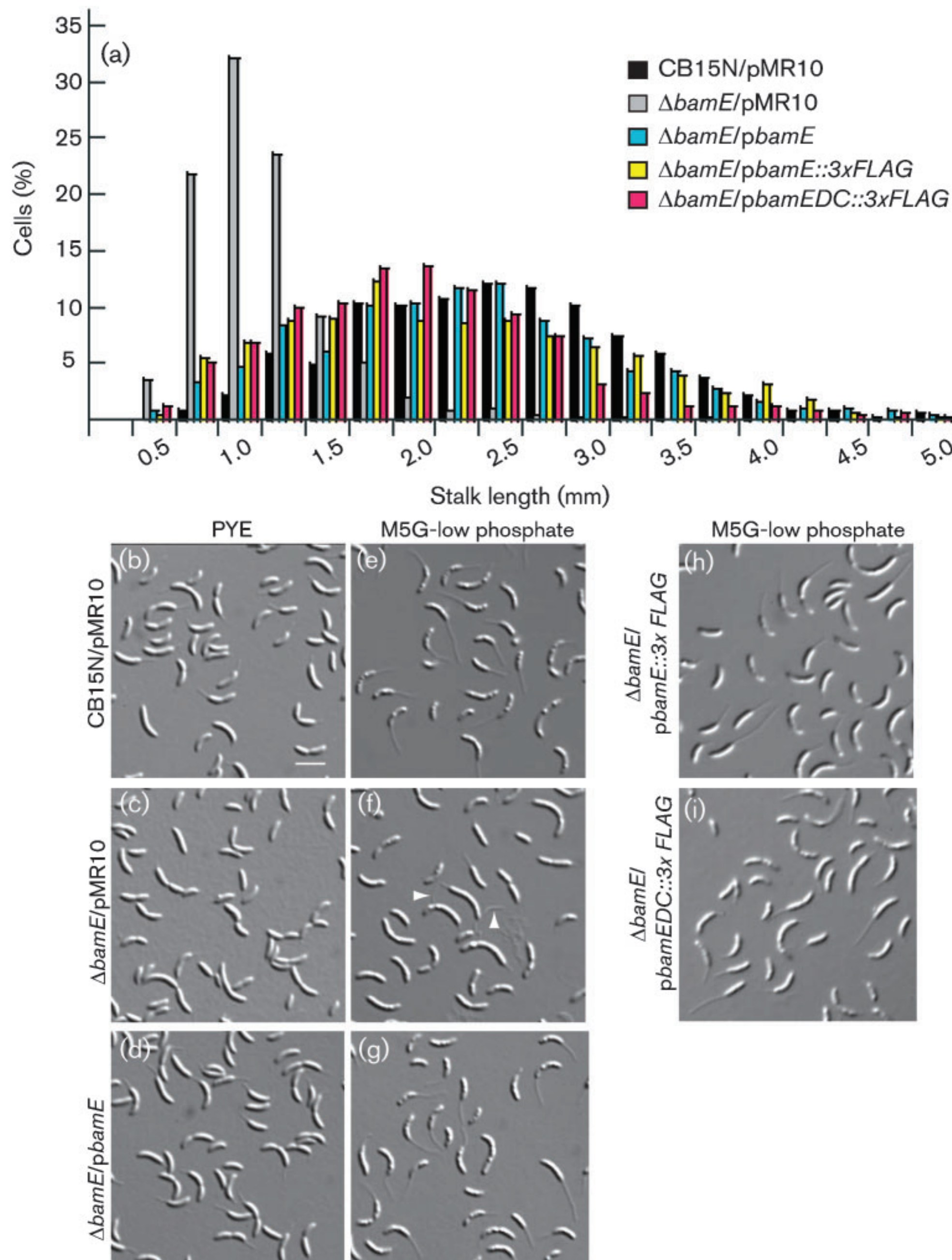

Fig. 6. Cells lacking BamE have short stalks. (a) Stalk lengths of the indicated cells grown in M5G-low phosphate medium were measured, and the distribution frequency is shown. (b–i) The indicated strains were grown in either rich medium (b–d) or minimal medium with limiting phosphate (e–i), immobilized on agarose pads and photographed. Bar [on (b)], 2.5 μm. White arrowheads denote debris resembling detached stalks.

and gammaproteobacteria, and *bamE* or *omlA* mutants have been characterized in *P. aeruginosa* (Ochsner *et al.*, 1999), *E. coli* (Sklar *et al.*, 2007), *S.* Typhimurium (Lewis *et al.*, 2008), *S.* Enteritidis (Fardini *et al.*, 2009), *X. campestris* (Fuangthong *et al.*, 2008) and *N. meningitidis* (Volokhina *et al.*, 2009). The phenotypes of these mutants include slow growth, reduced virulence and enhanced sensitivity to detergents, antibiotics and heat. The *E. coli bamE* mutant also contained reduced levels of OmpA and accumulated an unfolded form of LamB. Here, we report the first studies of a BamE/OmlA homologue in an alphaproteobacterium, *C. crescentus.* The Δ*bamE* mutant is hypersensitive to anionic detergents and some antibiotics, has reduced survival during heat exposure and grows slowly in rich

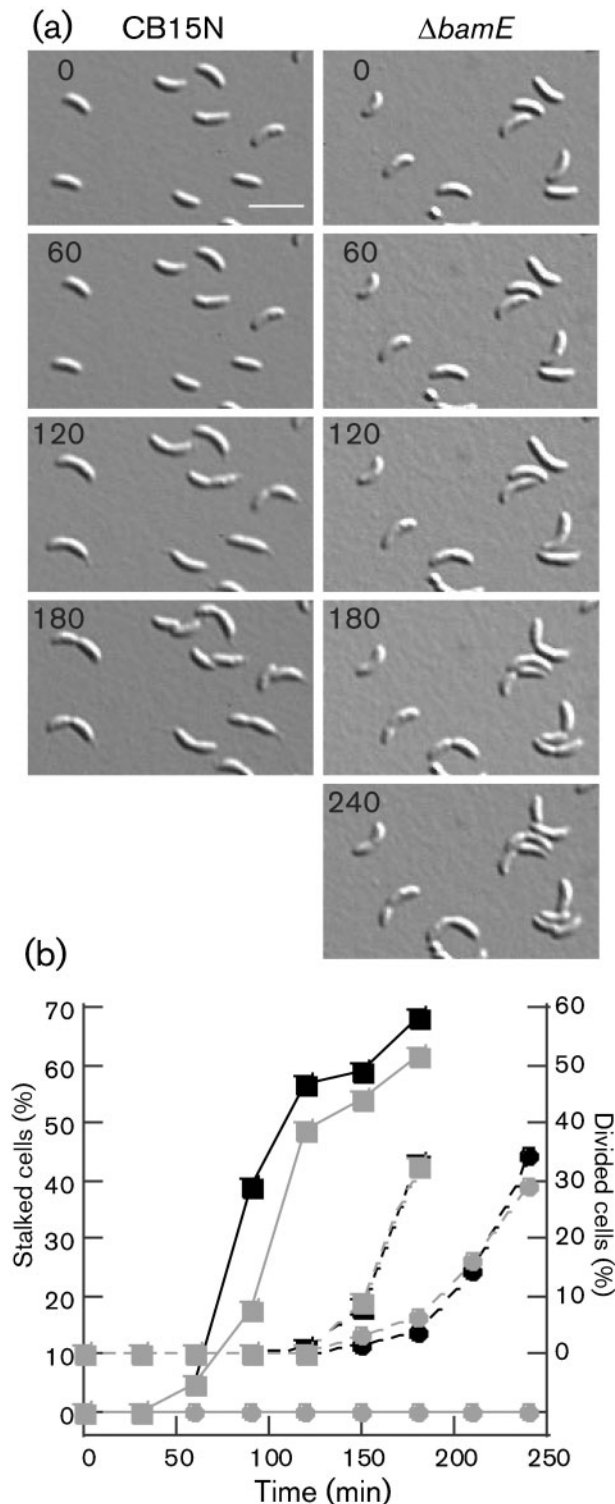

Fig. 7. BamE is required for normal stalk initiation during swarmer cell differentiation. (a) CB15N or Δ*bamE* swarmer cells were isolated, immobilized on M2G agarose pads and photographed at the indicated times (min) until divided cells were visible. Representative time points and fields for each strain are shown. Bar, 2.5 μm. (b) In time-lapse micrographs of CB15N (squares) and Δ*bamE* (circles), the percentage of cells that had grown a visible stalk (solid lines) or divided (dashed lines) was calculated at each time point. Black and grey symbols represent two independent experiments where >70 cells were observed.

medium. BamE is located in the *Caulobacter* outer membrane and coprecipitates with three other proteins whose homologues in *E. coli* are involved in OMP assembly. Finally, the membranes of Δ*bamE* cells are deficient in three TonB-dependent receptors and CpaC*, an aggregated form of the secretin CpaC. CpaC monomers are present in the *Caulobacter* membrane fraction in equal amounts in wild-type and Δ*bamE* cells, but the aggregated form CpaC* is specifically deficient in cells lacking BamE. Taken together, these results strongly suggest that BamE performs a function in *Caulobacter* similar to its role in other bacteria, namely to assemble outer membrane *β*-barrel proteins as part of the BAM complex. The pilus assembly protein CpaE is also required to accumulate CpaC* (Viollier *et al.*, 2002), so it is possible that BamE has an indirect effect upon CpaC via CpaE. However, CpaE itself is predicted to be located in the cytoplasm (Skerker & Shapiro, 2000; Viollier *et al.*, 2002), so a more likely hypothesis is that BamE affects CpaC downstream of the step requiring CpaE. A second caveat is that we have not yet examined the assembly kinetics of the missing TonB-dependent receptors in wild-type and Δ*bamE* cells. The synthesis of one or more of these receptors could be downregulated in the Δ*bamE* mutant, either due to the slower growth rate of Δ*bamE* cells or to a specific mechanism that reduces OMP expression during envelope stress (reviewed by Vogel & Papenfort, 2006).

Although they have generally similar phenotypes, *bamE* and *omlA* mutations cause phenotypes of different severity in different species. For example, the *E. coli bamE* mutant is not impaired in either growth rate or heat survival (Sklar *et al.*, 2007). The *P. aeruginosa omlA* mutant also grows at a normal rate and has no obvious changes in its OMP profile (Ochsner *et al.*, 1999). In these respects, the *Caulobacter* Δ*bamE* mutant seems to have a more severe phenotype, because its growth rate, its heat and chemical sensitivity, and its complement of OMPs are all affected.

The composition of the BAM complex differs in the three species where it has been determined. Compared with *E. coli*, the *Caulobacter* complex lacks the lipoprotein BamC (Gatsos *et al.*, 2008), while the *N. meningitidis* complex lacks the lipoprotein BamB and contains the additional protein RmpM (Volokhina *et al.*, 2009). The C terminus of RmpM is predicted to bind peptidoglycan and is similar to the C-terminal region of OmpA, but RmpM lacks the N-terminal *β*-barrel-forming region of OmpA (Grizot & Buchanan, 2004). Instead, RmpM contains an approximately 40 aa N-terminal domain followed by a proline-rich region of approximately 20 residues. Three proteins encoded in *Caulobacter* (CC3229, CC0201 and CC2845)

have features similar to RmpM (data not shown), but further studies will be necessary to determine if they associate with the *Caulobacter* BAM complex or are functionally similar to RmpM.

Differences in the composition of the BAM complex could render *Caulobacter* BamE more critical for membrane integrity than its counterparts in other bacteria. In *E. coli*, BamC, BamD and BamE interact with BamA as a group, while the BamA–BamB interaction is independent of the other lipoproteins (Malinverni *et al.*, 2006; Sklar *et al.*, 2007; Vuong *et al.*, 2008). In mutants lacking either BamC or BamE, BamD levels are normal but less BamD is associated with BamA. A recent report demonstrated that an *N. meningitidis bamE bamC* double mutant is inviable, supporting the idea that these two lipoproteins function in the same process (Volokhina *et al.*, 2009). Thus, because *Caulobacter* lacks a homologue of *bamC*, BamE may play a greater role in maintaining the interaction between BamA and BamD. We hypothesized that the C-terminal extension of *Caulobacter* BamE, beyond the conserved BamE/OmlA domain, might play an important role in stabilizing the BAM complex, particularly in the absence of a BamC homologue. When we truncated BamE, however, we found that the BAM complex was intact (Fig. 3). Thus, BamE itself may be more important for BAM complex stability in *Caulobacter*, but this is not the primary function of the BamE C-terminal segment.

Alternatively, the loss of BamE may cause a more severe phenotype in *Caulobacter* because this species contains a different complement of OMPs than *E. coli*, *P. aeruginosa*, *N. meningitidis* or *Salmonella* species. The *Caulobacter* outer membrane proteome is skewed toward OmpA homologues and TonB-dependent receptors (Phadke *et al.*, 2001). While there is evidence that BamA interacts directly with β-barrel protein substrates (Habib *et al.*, 2007; Knowles *et al.*, 2008; Robert *et al.*, 2006), different types of OMPs may be channelled to BamA via subsets of the accessory lipoproteins. If OmpA homologues or TonB-dependent receptors specifically require BamE for assembly, then bacteria whose outer membranes are rich in these proteins may be more susceptible to the loss of BamE.

Although Δ*bamE* cells have shorter stalks than wild-type cells in all media tested, the growth defect and chemical hypersensitivity of the mutant indicate that the function of BamE is not limited to stalk biogenesis. Consistent with these results, BamE and other members of the BAM complex are not enriched in the *Caulobacter* stalk proteome (Ireland *et al.*, 2002). However, stalk biogenesis in the Δ*bamE* mutant may be impaired more than membrane biogenesis overall. During synchronous growth of the Δ*bamE* mutant in time-lapse experiments, swarmer cells elongated and divided, but unlike wild-type cells, they did not synthesize visible stalks before cell division (Fig. 7). Because Δ*bamE* cells grown in liquid media possess stalks, we interpret these results to mean that stalk outgrowth is delayed in the mutant, and the loss of BamE affects stalk growth relatively more than growth and division of the cell body. Stalk length is not always reduced in mutants with a slower growth rate; for example, null mutants in *divJ* grow more slowly than wild-type cells (Sciochetti *et al.*, 2002), yet their stalks are as long as those of wild-type cells (Wheeler & Shapiro, 1999).

We propose that BamE, like its homologues in other species, participates in assembling a variety of β-barrel proteins into the outer membrane as part of the *Caulobacter* BAM complex. Without BamE, the complex may be inefficient at assembling many OMP substrates, causing a general loss of membrane integrity. To account for the delay in stalk outgrowth in Δ*bamE* cells, we also propose that the *Caulobacter* BAM complex assembles one or more substrates that specifically participate in stalk initiation or elongation. The assembly of these substrates may be particularly impaired in cells lacking BamE, causing cell division to occur before the first appearance of a stalk.

The lipoproteins of the *E. coli* BAM complex are transcriptionally regulated by σ^E (Dartigalongue *et al.*, 2001; Kabir *et al.*, 2005; Lewis *et al.*, 2008; Rezuchova *et al.*, 2003), an extracytoplasmic function (ECF) sigma factor that directs the transcription of chaperones, assembly factors and proteases to restore homeostasis after envelope stress (Ruiz & Silhavy, 2005). The *Caulobacter* genome encodes 13 ECF sigma factors (Nierman *et al.*, 2001), and it is currently unclear which of them perform functions analogous to *E. coli* σ^E (Alvarez-Martinez *et al.*, 2006, 2007). *Caulobacter* also contains an essential two-component system, composed of the histidine kinase CenK and the DNA-binding response regulator CenR (Skerker *et al.*, 2005). Depletion of either CenK or CenR leads to severe membrane blebbing and death, suggesting that the transcriptional targets of this system are intimately involved in cell envelope integrity. Future studies will determine how the components of the BAM complex are transcriptionally regulated in *Caulobacter*.

Our studies have identified the *Caulobacter* BAM complex and show that the BamE homologue CC1365 plays a role in *Caulobacter* membrane integrity, stalk outgrowth and establishing or maintaining normal levels of some OMPs. It will be of interest to determine if TonB-dependent receptors, which are abundant in *Caulobacter*, have a particular requirement for BamE in their assembly. Additional experiments are also needed to identify substrates of the BAM complex that promote stalk outgrowth.

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REFERENCES

Alley, M. R., Maddock, J. R. & Shapiro, L. (1992). Polar localization of a bacterial chemoreceptor. *Genes Dev* **6**, 825–836.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Alvarez-Martinez, C. E., Baldini, R. L. & Gomes, S. L. (2006). A *Caulobacter crescentus* extracytoplasmic function sigma factor mediating the response to oxidative stress in stationary phase. *J Bacteriol* **188**, 1835–1846.

Alvarez-Martinez, C. E., Lourenco, R. F., Baldini, R. L., Laub, M. T. & Gomes, S. L. (2007). The ECF sigma factor σ^T is involved in osmotic and oxidative stress responses in *Caulobacter crescentus*. *Mol Microbiol* **66**, 1240–1255.

Badger, J. H., Hoover, T. R., Brun, Y. V., Weiner, R. M., Laub, M. T., Alexandre, G., Mrázek, J., Ren, Q., Paulsen, I. T. & other authors (2006). Comparative genomic evidence for a close relationship between the dimorphic prosthecate bacteria *Hyphomonas neptunium* and *Caulobacter crescentus*. *J Bacteriol* **188**, 6841–6850.

Bassford, P. J., Jr & Kadner, R. J. (1977). Genetic analysis of components involved in vitamin B12 uptake in *Escherichia coli*. *J Bacteriol* **132**, 796–805.

Bayan, N., Guilvout, I. & Pugsley, A. P. (2006). Secretins take shape. *Mol Microbiol* **60**, 1–4.

Biondi, E. G., Skerker, J. M., Arif, M., Prasol, M. S., Perchuk, B. S. & Laub, M. T. (2006). A phosphorelay system controls stalk biogenesis during cell cycle progression in *Caulobacter crescentus*. *Mol Microbiol* **59**, 386–401.

Blanvillain, S., Meyer, D., Boulanger, A., Lautier, M., Guynet, C., Denance, N., Vasse, J., Lauber, E. & Arlat, M. (2007). Plant carbohydrate scavenging through TonB-dependent receptors: a feature shared by phytopathogenic and aquatic bacteria. *PLoS One* **2**, e224.

Bouvier, J., Pugsley, A. P. & Stragier, P. (1991). A gene for a new lipoprotein in the *dapA–purC* interval of the *Escherichia coli* chromosome. *J Bacteriol* **173**, 5523–5531.

Braun, V. & Endriss, F. (2007). Energy-coupled outer membrane transport proteins and regulatory proteins. *Biometals* **20**, 219–231.

Brun, Y. V. & Shapiro, L. (1992). A temporally controlled sigma-factor is required for polar morphogenesis and normal cell division in *Caulobacter*. *Genes Dev* **6**, 2395–2408.

Charlson, E. S., Werner, J. N. & Misra, R. (2006). Differential effects of *yfgL* mutation on *Escherichia coli* outer membrane proteins and lipopolysaccharide. *J Bacteriol* **188**, 7186–7194.

Clancy, M. J. & Newton, A. (1982). Localization of proteins in the inner and outer membranes of *Caulobacter crescentus*. *Biochim Biophys Acta* **686**, 160–169.

Collier, J. & Shapiro, L. (2007). Spatial complexity and control of a bacterial cell cycle. *Curr Opin Biotechnol* **18**, 333–340.

Collin, S., Guilvout, I., Chami, M. & Pugsley, A. P. (2007). YaeT-independent multimerization and outer membrane association of secretin PulD. *Mol Microbiol* **64**, 1350–1357.

Dartigalongue, C., Missiakas, D. & Raina, S. (2001). Characterization of the *Escherichia coli* sigma E regulon. *J Biol Chem* **276**, 20866–20875.

Delcour, A. H. (2002). Structure and function of pore-forming β-barrels from bacteria. *J Mol Microbiol Biotechnol* **4**, 1–10.

DelVecchio, V. G., Kapatral, V., Elzer, P., Patra, G. & Mujer, C. V. (2002). The genome of *Brucella melitensis*. *Vet Microbiol* **90**, 587–592.

Doerrler, W. T. & Raetz, C. R. (2005). Loss of outer membrane proteins without inhibition of lipid export in an *Escherichia coli* YaeT mutant. *J Biol Chem* **280**, 27679–27687.

Elias, J. E., Haas, W., Faherty, B. K. & Gygi, S. P. (2005). Comparative evaluation of mass spectrometry platforms used in large-scale proteomics investigations. *Nat Methods* **2**, 667–675.

Ely, B. (1991). Genetics of *Caulobacter crescentus*. *Methods Enzymol* **204**, 372–384.

Eng, J. K., McCormack, A. L. & Yates, J. R., III (1994). An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom* **5**, 976–989.

Evinger, M. & Agabian, N. (1977). Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. *J Bacteriol* **132**, 294–301.

Fardini, Y., Trotereau, J., Bottreau, E., Souchard, C., Velge, P. & Virlogeux-Payant, I. (2009). Investigation of the role of the BAM complex and SurA chaperone in outer-membrane protein biogenesis and type III secretion system expression in *Salmonella*. *Microbiology* **155**, 1613–1622.

Fuangthong, M., Sallabhan, R., Atichartpongkul, S., Rangkadilok, N., Sriprang, R., Satayavivad, J. & Mongkolsuk, S. (2008). The *omlA* gene is involved in multidrug resistance and its expression is inhibited by coumarins in *Xanthomonas campestris* pv. phaseoli. *Arch Microbiol* **189**, 211–218.

Gatsos, X., Perry, A. J., Anwari, K., Dolezal, P., Wolynec, P. P., Likic, V. A., Purcell, A. W., Buchanan, S. K. & Lithgow, T. (2008). Protein secretion and outer membrane assembly in *Alphaproteobacteria*. *FEMS Microbiol Rev* **32**, 995–1009.

Gonin, M., Quardokus, E. M., O'Donnol, D., Maddock, J. & Brun, Y. V. (2000). Regulation of stalk elongation by phosphate in *Caulobacter crescentus*. *J Bacteriol* **182**, 337–347.

Grizot, S. & Buchanan, S. K. (2004). Structure of the OmpA-like domain of RmpM from *Neisseria meningitidis*. *Mol Microbiol* **51**, 1027–1037.

Habib, S. J., Waizenegger, T., Niewienda, A., Paschen, S. A., Neupert, W. & Rapaport, D. (2007). The N-terminal domain of Tob55 has a receptor-like function in the biogenesis of mitochondrial β-barrel proteins. *J Cell Biol* **176**, 77–88.

House, B. L., Mortimer, M. W. & Kahn, M. L. (2004). New recombination methods for *Sinorhizobium meliloti* genetics. *Appl Environ Microbiol* **70**, 2806–2815.

Iba, H., Fukuda, A. & Okada, Y. (1977). Chromosome replication in *Caulobacter crescentus* growing in a nutrient broth. *J Bacteriol* **129**, 1192–1197.

Ireland, M. M. E., Karty, J. A., Quardokus, E. M., Reilly, J. P. & Brun, Y. V. (2002). Proteomic analysis of the *Caulobacter crescentus* stalk indicates competence for nutrient uptake. *Mol Microbiol* **45**, 1029–1041.

Jansen, C., Wiese, A., Reubsaet, L., Dekker, N., de Cock, H., Seydel, U. & Tommassen, J. (2000). Biochemical and biophysical characterization of *in vitro* folded outer membrane porin PorA of *Neisseria meningitidis*. *Biochim Biophys Acta* **1464**, 284–298.

Jimenez, C. R., Huang, L., Qiu, Y. & Burlingame, A. L. (1998). In-gel digestion of proteins for MALDI-MS fingerprint mapping. In *Current Protocols in Protein Science*, pp. 16.14.11–16.14.15. Edited by J. E. Coligan, B. M. Dunn, H. L. Ploegh, D. W. Speicher & P. T. Wingfield. New York: Wiley.

Kabir, M. S., Yamashita, D., Koyama, S., Oshima, T., Kurokawa, K., Maeda, M., Tsunedomi, R., Murata, M., Wada, C. & other authors (2005). Cell lysis directed by σ^E in early stationary phase and effect of induction of the *rpoE* gene on global gene expression in *Escherichia coli*. *Microbiology* **151**, 2721–2735.

Kainth, P. & Gupta, R. S. (2005). Signature proteins that are distinctive of alpha proteobacteria. *BMC Genomics* **6**, 94.

Knowles, T. J., Jeeves, M., Bobat, S., Dancea, F., McClelland, D., Palmer, T., Overduin, M. & Henderson, I. R. (2008). Fold and function of polypeptide transport-associated domains responsible for delivering unfolded proteins to membranes. *Mol Microbiol* **68**, 1216–1227.

Knowles, T. J., Scott-Tucker, A., Overduin, M. & Henderson, I. R. (2009). Membrane protein architects: the role of the BAM complex in outer membrane protein assembly. *Nat Rev Microbiol* **7**, 206–214.

Koronakis, V., Sharff, A., Koronakis, E., Luisi, B. & Hughes, C. (2000). Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* **405**, 914–919.

Koronakis, V., Eswaran, J. & Hughes, C. (2004). Structure and function of TolC: the bacterial exit duct for proteins and drugs. *Annu Rev Biochem* **73**, 467–489.

Lassmann, T. & Sonnhammer, E. L. L. (2005). Kalign – an accurate and fast multiple sequence alignment algorithm. *BMC Bioinformatics* **6**, 298.

Lewenza, S., Vidal-Ingigliardi, D. & Pugsley, A. P. (2006). Direct visualization of red fluorescent lipoproteins indicates conservation of the membrane sorting rules in the family *Enterobacteriaceae*. *J Bacteriol* **188**, 3516–3524.

Lewis, C., Skovierova, H., Rowley, G., Rezuchova, B., Homerova, D., Stevenson, A., Sherry, A., Kormanec, J. & Roberts, M. (2008). Small outer-membrane protein lipoprotein, SmpA, is regulated by σ^E and has a role in cell envelope integrity and virulence of *Salmonella enterica* serovar Typhimurium. *Microbiology* **154**, 979–988.

Malinverni, J. C., Werner, J., Kim, S., Sklar, J. G., Kahne, D., Misra, R. & Silhavy, T. J. (2006). YfiO stabilizes the YaeT complex and is essential for outer membrane protein assembly in *Escherichia coli*. *Mol Microbiol* **61**, 151–164.

McLeod, M., Craft, S. & Broach, J. R. (1986). Identification of the crossover site during FLP-mediated recombination in the *Saccharomyces cerevisiae* plasmid 2μ circle. *Mol Cell Biol* **6**, 3357–3367.

Molloy, M. P., Phadke, N. D., Maddock, J. R. & Andrews, P. C. (2001). Two-dimensional electrophoresis and peptide mass fingerprinting of bacterial outer membrane proteins. *Electrophoresis* **22**, 1686–1696.

Narita, S. & Tokuda, H. (2007). Amino acids at positions 3 and 4 determine the membrane specificity of *Pseudomonas aeruginosa* lipoproteins. *J Biol Chem* **282**, 13372–13378.

Narita, S., Matsuyama, S. & Tokuda, H. (2004). Lipoprotein trafficking in *Escherichia coli*. *Arch Microbiol* **182**, 1–6.

Neugebauer, H., Herrmann, C., Kammer, W., Schwartz, G., Nordheim, A. & Braun, V. (2005). ExbBD-dependent transport of maltodextrins through the novel MalA protein across the outer membrane of *Caulobacter crescentus*. *J Bacteriol* **187**, 8300–8311.

Nierman, W. C., Feldblyum, T. V., Laub, M. T., Paulsen, I. T., Nelson, K. E., Eisen, J. A., Heidelberg, J. F., Alley, M. R., Ohta, N. & other authors (2001). Complete genome sequence of *Caulobacter crescentus*. *Proc Natl Acad Sci U S A* **98**, 4136–4141.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* **67**, 593–656.

Ochsner, U. A., Vasil, A. I., Johnson, Z. & Vasil, M. I. (1999). *Pseudomonas aeruginosa fur* overlaps with a gene encoding a novel outer membrane lipoprotein, OmlA. *J Bacteriol* **181**, 1099–1109.

Phadke, N. D., Molloy, M. P., Steinhoff, S. A., Ulintz, P. J., Andrews, P. C. & Maddock, J. R. (2001). Analysis of the outer membrane proteome of *Caulobacter crescentus* by two-dimensional electrophoresis and mass spectrometry. *Proteomics* **1**, 705–720.

Poindexter, J. S. (1964). Biological properties and classification of the *Caulobacter* group. *Bacteriol Rev* **28**, 231–295.

Poindexter, J. L. S. & Cohen-Bazire, G. (1964). The fine structure of stalked bacteria belonging to the family *Caulobacteraceae*. *J Cell Biol* **23**, 587–607.

Postle, K. & Larsen, R. A. (2007). TonB-dependent energy transduction between outer and cytoplasmic membranes. *Biometals* **20**, 453–465.

Prinz, T. & Tommassen, J. (2000). Association of iron-regulated outer membrane proteins of *Neisseria meningitidis* with RmpM (class 4) protein. *FEMS Microbiol Lett* **183**, 49–53.

Pugsley, A. P. & Reeves, R. (1976). Iron uptake in colicin B-resistance mutants of *Escherichia coli* K-12. *J Bacteriol* **126**, 1052–1062.

Quon, K. C., Marczynski, G. T. & Shapiro, L. (1996). Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell* **84**, 83–93.

Raleigh, E. A., Elbing, K. & Brent, R. (2002). Selected topics from classical bacterial genetics. In *Current Protocols in Molecular Biology*, pp. 1.4.1–1.4.14. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Wiley.

Reisinger, S. J., Huntwork, S., Viollier, P. H. & Ryan, K. R. (2007). DivL performs critical cell cycle functions in *Caulobacter crescentus* independent of kinase activity. *J Bacteriol* **189**, 8308–8320.

Rezuchova, B., Miticka, H., Homerova, D., Roberts, M. & Kormanec, J. (2003). New members of the *Escherichia coli* σ^E regulon identified by a two-plasmid system. *FEMS Microbiol Lett* **225**, 1–7.

Robert, V., Volokhina, E. B., Senf, F., Bos, M. P., Van Gelder, P. & Tommassen, J. (2006). Assembly factor Omp85 recognizes its outer membrane protein substrates by a species-specific C-terminal motif. *PLoS Biol* **4**, e377.

Rolhion, N., Barnich, N., Claret, L. & Darfeuille-Michaud, A. (2005). Strong decrease in invasive ability and outer membrane vesicle release in Crohn's disease-associated invasive *Escherichia coli* strain Lf82 with the *yfgL* gene deleted. *J Bacteriol* **187**, 2286–2296.

Ruiz, N. & Silhavy, T. J. (2005). Sensing external stress: watchdogs of the *Escherichia coli* cell envelope. *Curr Opin Microbiol* **8**, 122–126.

Ruiz, N., Falcone, B., Kahn, D. & Silhavy, T. J. (2005). Chemical conditionality: a genetic strategy to probe organelle assembly. *Cell* **121**, 307–317.

Ruiz, N., Wu, T., Kahne, D. & Silhavy, T. J. (2006). Probing the barrier function of the outer membrane with chemical conditionality. *ACS Chem Biol* **1**, 385–395.

Sankaran, K., Gupta, S. D. & Wu, H. C. (1995). Modification of bacterial lipoproteins. *Methods Enzymol* **250**, 683–697.

Schmidt, J. M. & Stanier, R. Y. (1966). The development of cellular stalks in bacteria. *J Cell Biol* **28**, 423–436.

Sciochetti, S. A., Lane, T., Ohta, N. & Newton, A. (2002). Protein sequences and cellular factors required for polar localization of a histidine kinase in *Caulobacter crescentus*. *J Bacteriol* **184**, 6037–6049.

Seitz, L. C. & Brun, Y. V. (1998). Genetic analysis of mecillinam-resistant mutants of *Caulobacter crescentus* deficient in stalk biosynthesis. *J Bacteriol* **180**, 5235–5239.

Seydel, A., Gounon, P. & Pugsley, A. P. (1999). Testing the '+2 rule' for lipoprotein sorting in the *Escherichia coli* cell envelope with a new genetic selection. *Mol Microbiol* **34**, 810–821.

Skerker, J. M. & Shapiro, L. (2000). Identification and cell-cycle control of a novel pilus system in *Caulobacter crescentus*. *EMBO J* **19**, 3223–3234.

Skerker, J. M., Prasol, M. S., Perchuk, B. S., Biondi, E. G. & Laub, M. T. (2005). Two-component signal transduction pathways regulating growth and cell cycle progresion in a bacterium: A systems-level analysis. *PLoS Biol* **3**, e334.

Sklar, J. G., Wu, T., Gronenberg, L. S., Malinverni, J. C., Kahne, D. & Silhavy, T. J. (2007). Lipoprotein SmpA is a component of the YaeT complex that assembles outer membrane proteins in *Escherichia coli*. *Proc Natl Acad Sci U S A* **104**, 6400–6405.

Stove, J. L. & Stanier, R. Y. (1962). Cellular differentiation in stalked bacteria. *Nature* **196**, 1189–1192.

Tabb, D. L., McDonald, W. H. & Yates, J. R. (2002). DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. *J Proteome Res* **1**, 21–26.

Toporowski, M. C., Nomellini, J. F., Awram, P. & Smit, J. (2004). Two outer membrane proteins are required for maximal type I secretion of the *Caulobacter crescentus* S-layer protein. *J Bacteriol* **186**, 8000–8009.

Vanini, M. M. T., Spisini, A., Sforca, M. L., Pertinhez, T. A. & Benedetti, C. E. (2008). The solution structure of the outer membrane lipoprotein OmlA from *Xanthomonas axonopodis* pv. *cirtri* reveals a protein fold implicated in protein–protein interaction. *Proteins* **71**, 2051–2064.

Viollier, P. H., Sternheim, N. & Shapiro, L. (2002). A dynamically localized histidine kinase controls the asymmetric distribution of polar pili proteins. *EMBO J* **21**, 4420–4428.

Vogel, J. & Papenfort, K. (2006). Small non-coding RNAs and the bacterial outer membrane. *Curr Opin Microbiol* **9**, 605–611.

Volokhina, E. B., Beckers, F., Tomassen, J. & Bos, M. P. (2009). The *β*-barrel outer membrane protein assembly complex of *Neisseria meningitidis*. *J Bacteriol* **191**, 7074–7085.

Voulhoux, R., Bos, M. P., Geurtsen, J., Mols, M. & Tommassen, J. (2003). Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* **299**, 262–265.

Vuong, P., Bennion, D., Mantei, J., Frost, D. & Misra, R. (2008). Analysis of YfgL and YaeT interactions through bioinformatics, mutagenesis, and biochemistry. *J Bacteriol* **190**, 1507–1517.

Wagner, J. K., Galvani, C. D. & Brun, Y. V. (2005). *Caulobacter crescentus* requires RodA and MreB for stalk synthesis and prevention of ectopic pole formation. *J Bacteriol* **187**, 544–553.

Wagner, J. K., Setayeshgar, S., Sharon, L. A., Reilly, J. P. & Brun, Y. V. (2006). A nutrient uptake role for bacterial envelope extensions. *Proc Natl Acad Sci U S A* **103**, 11772–11777.

Werner, J. & Misra, R. (2005). YaeT (Omp85) affects the assembly of lipid-dependent and lipid-independent proteins of *Escherichia coli*. *Mol Microbiol* **57**, 1450–1459.

Wheeler, R. T. & Shapiro, L. (1999). Differential localization of two histidine kinases controlling bacterial cell differentiation. *Mol Cell* **4**, 683–694.

Wu, T., Malinverni, J., Ruiz, N., Kim, S., Silhavy, T. J. & Kahne, D. (2005). Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. *Cell* **121**, 235–245.

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