FEBS Letters 534 (2003) 156–160 FEBS 26883

DmsD is required for the biogenesis of DMSO reductase in *Escherichia coli* but not for the interaction of the DmsA signal peptide with the Tat apparatus

Nicola Ray[a], Joanne Oates[a], Raymond J. Turner[b], Colin Robinson[a,*]

[a] *Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK*
[b] *Department of Biological Sciences, University of Calgary, Calgary, AB, Canada T2N 1N4*

Received 22 November 2002; revised 9 December 2002; accepted 9 December 2002

First published online 18 December 2002

Edited by Gunnar von Heijne

Abstract The DmsD protein is essential for the biogenesis of DMSO reductase in *Escherichia coli*, and binds the signal peptide of the DmsA subunit, a Tat substrate. This suggests a role as a guidance factor to target pre-DmsA to the translocase. Here, we have analysed the export of fusion proteins in which the DmsA and TorA signal peptides are fused to green fluorescent protein. Both chimeras are efficiently exported to the periplasm in wild-type *E. coli* cells and we show that their export efficiencies are essentially identical in a mutant lacking DmsD. An authentic Tat substrate, TMAO reductase, is also efficiently exported in the *dmsD* mutant. The data indicate that DmsD carries out a critical role in DMSO reductase biogenesis/assembly but is not required for the functioning of the DmsA signal peptide.

Key words: Twin-arginine translocase (Tat) pathway; Dimethyl sulfoxide reductase; DmsD; Protein targeting; *Escherichia coli*

1. Introduction

In *Escherichia coli* the majority of exo-cytoplasmic proteins are transported from their site of synthesis to their subcellular destination via the Sec pathway. These proteins, which are made as precursors with an amino-terminal signal peptide, are threaded through an integral membrane protein complex in an energy-dependent, unfolded state [1]. Recently, another protein translocation system has been discovered in the cytoplasmic membrane of *E. coli* and many other bacteria (for review see [2]). This complementary pathway was denoted the Twin-arginine translocase (Tat) pathway, as substrates transported via this route possess an essential twin-arginine motif in their signal peptides. In contrast to the well-characterised Sec pathway, which transports only unfolded proteins, the Tat system uses an entirely different mechanism and has the unique ability to transport fully folded proteins across coupled membranes [3,4].

Prime examples of Tat substrates in *E. coli* are a range of proteins that acquire redox cofactors, such as iron–sulphur and molybdopterin centres, in the cytoplasm prior to transport [5–7]. One such protein is the molybdopterin cofactor-containing DmsA, which is the catalytic subunit of the enzyme dimethyl sulfoxide (DMSO) reductase. Additional subunits in the membrane-associated enzyme are DmsB, an iron–sulphur protein and DmsC, which acts as a membrane anchor. DMSO reductase is a terminal electron transfer enzyme which enables anaerobic respiratory growth of *E. coli* on media containing an electron donor/carbon source, such as glycerol, and the electron acceptor DMSO [8].

The *E. coli* Tat system consists of four *tat* genes, three of which (*tatABC*) form an operon while the fourth gene (*tatE*) is monocistronic. Knockout studies have shown that Tat-dependent export requires minimally TatB [7,9], TatC [9] and either TatA or TatE [10,11]. TatA, B and E contain a single transmembrane span with a small C-terminal domain exposed on the *cis* face of the membrane (i.e. the cytoplasm in bacteria, or stroma in chloroplasts [12]), whereas TatC contains four transmembrane regions with the N- and C-termini localised in the cytoplasm [13].

No additional membrane-bound Tat subunits have been identified to date and a complex of 600 kDa comprising only TatA, B and C has been purified from solubilised *E. coli* membranes [14]. However, Oresnik et al. [15] recently tested whether other components are involved in the secretion of proteins containing a twin-arginine (RR) signal peptide. Using the DmsA twin-arginine leader as a probe they identified two proteins that interacted with this protein in *E. coli*: DmsD and DnaK. The DmsD protein contains 204 amino acids, and in vitro characterisation of this protein showed that it interacts with the precursor form of DmsA. DmsD also interacts with the precursor form of a second Tat substrate, TorA (trimethyl amine *N*-oxide (TMAO) reductase). These data point to an important role for DmsD, possibly as a guidance factor for RR-signal peptides that may ensure delivery to the translocase. In this work we have further characterised the role of DmsD in the transport of proteins via the Tat pathway. We show that the DmsA signal peptide can function efficiently in the absence of DmsD and that a related Tat substrate, TMAO reductase, is efficiently exported in the DmsD mutant strain. The data point to an essential role for DmsD in the activity or assembly of DMSO reductase, but not in the functioning of the DmsA signal peptide.

*Corresponding author. Fax: (44)-2476-523701.
E-mail address: crobinson@bio.warwick.ac.uk (C. Robinson).

0014-5793/02/$22.00
doi:10.1016/S0014-5793(02)03839-5

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

E. coli strains used in this work were TG1 [16], a wild-type strain, TALB01, a DmsD null mutant [14] and Δ*tatABCDE* which has been described before [17]. *E. coli* was aerobically grown at 37°C in modified low salt Luria broth (lsLB) [18], or anaerobically in lsLB-GT medium, consisting of lsLB supplemented with glycerol (0.5%), TMAO (0.4%), and ammonium molybdate (1 μM), or in minimal TMAO/glycerol medium or minimal DMSO/glycerol medium [19]. Medium supplements were used at the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; arabinose 100 μM.

To generate a vector encoding the DmsA pre-sequence fused to green fluorescent protein (GFP), the GFP gene sequence was amplified from the plasmid pJDT1 [18] using primers GFP1F (ccggaattcatgtctaaaggagaagaactt) and GFP1R (ctctcaagcttatttgtatagttcatccatgc). These primers introduced a unique *Eco*RI site upstream of the GFP start codon and a unique *Hin*dIII site downstream of the stop codon; these sites are underlined. The resulting PCR product was cloned into the *Eco*RI–*Hin*dIII-digested pBAD24 vector [20], resulting in the plasmid pBAD-GFP. The DmsA pre-sequence was amplified from total chromosomal DNA with primers dmsaF (gcaagcttgaattcccattatg) and dmsaR (ggaatggcgaattcgacagcgtg), with *Eco*RI restriction sites underlined. The resulting PCR product was digested with *Eco*RI and cloned into the *Eco*RI-digested pBAD-GFP plasmid, generating pBAD-DmsAGFP, in which the DmsA pre-sequence is fused to GFP.

2.2. Cell fractionations

Cells were grown aerobically (in lsLB) or anaerobically (in lsLB-GT) medium in the presence or absence of arabinose (as stated in the text). Periplasm and spheroplasts were prepared by the EDTA/lysozyme/cold osmotic shock procedure [21]. Spheroplasts were lysed by sonication, and intact cells and cellular debris were removed by centrifugation (5 min at 10 000 × *g*). Membranes were separated from the cytoplasmic fraction by centrifugation (30 min at 250 000 × *g*). Protein concentration was determined using a BCA-linked assay (Pierce). Protein fractions were separated on a 10% non-denaturing polyacrylamide gel, allowing detection of TMAO reductase activity via a gel-based methyl viologen-linked assay [22], or were separated by SDS–PAGE and immunoblotted with specific antibodies to GFP (Clontech) and horseradish peroxidase anti-rabbit IgG conjugates, using the ECL detection system (Amersham Pharmacia Biotech).

2.3. Microscopy

E. coli strains containing pJDT1 or pBAD-DmsAGFP were grown aerobically at 37°C in lsLB containing 100 μM arabinose. After 2 h incubation, cells were pelleted, washed with lsLB, and resuspended in fresh lsLB. After a further 1 h incubation, 500 μl of cells was collected by centrifugation and washed with lsLB. These cells were mounted onto poly-L-lysine-treated microscope slides (Sigma) and examined by phase contrast and fluorescence (fluorescein isothiocyanate filter set) microscopy. Images were taken with a Zeiss Axiovert 200 microscope equipped with a Quantix camera containing a 1536 × 1024 pixel and 9 μm pitch chip (Digital Pixel Advanced Imaging Systems). The digital images were processed using METAMORPH version 4.6.4 (Universal Imaging Corp.) and the files were exported to Microsoft Powerpoint for printing.

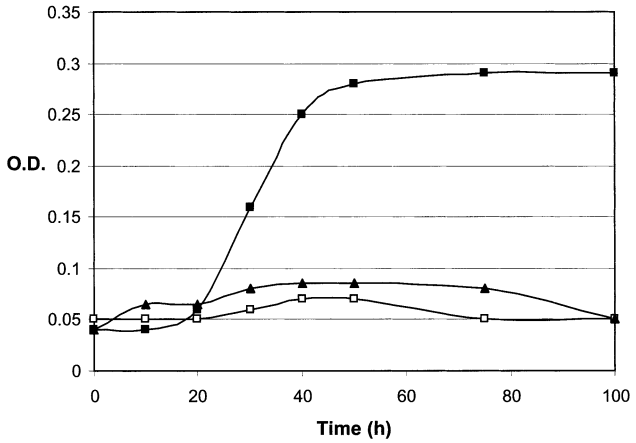

Fig. 1. Anaerobic growth of TG1, Δ*tatABCDE* and Δ*dmsD*. *E. coli* strains Δ*tatABCDE* (□), wild-type (■) and Δ*dmsD* (▲) were grown anaerobically on minimal glycerol medium in the presence of DMSO and growth was monitored using OD_{600} measurements over a 4-day period.

3. Results and discussion

3.1. DmsD is required for anaerobic growth using DMSO

To further investigate the role DmsD plays in protein targeting to the Tat complex, the DmsD-minus mutant, TALB01, was grown anaerobically on minimal glycerol medium in the presence of DMSO and growth was monitored over a 4-day period. Fig. 1 shows a typical growth curve for the wild-type TG1 strain, whereas the Δ*tatABCDE* strain fails to grow under these conditions, as expected since targeting of the DmsA subunit is blocked. Fig. 1 shows that the *dmsD* mutant also fails to grow, indicating that DmsD is required for DMSO reductase function, as found previously. The *dmsD* mutant is able to grow on TMAO, although growth is slightly reduced for unknown reasons (data not shown), which further suggests that the defect may be specific for DMSO reductase.

3.2. TMAO reductase localisation is not affected by DmsD deletion

To investigate more directly the consequences of the DmsD deletion, we next analysed the distribution of a known Tat substrate, TorA, in the *dmsD* mutant. This was achieved using native gels in which the TorA activity is visualised directly in the polyacrylamide gel, using a methyl viologen-linked assay. Fig. 2 shows that the majority of active TorA is localised in the periplasm in wild-type cells, as expected and shown previously [6]. In contrast, in a strain lacking all known Tat

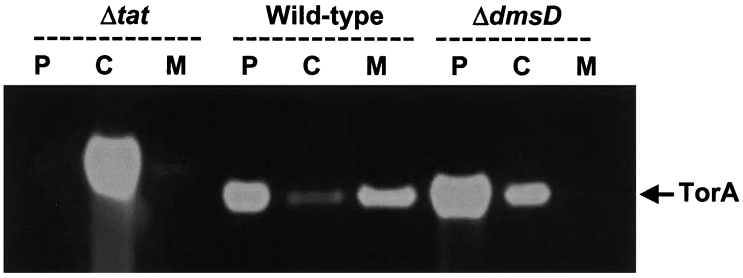

Fig. 2. Localisation of TMAO reductase activity in the Δ*dmsD* mutant. *E. coli* strains Δ*tatABCDE* (Δ*tat*), wild-type and Δ*dmsD* were grown anaerobically and fractionated into periplasmic (P), cytoplasmic (C) or membrane (M) samples. The samples were analysed by native gel electrophoresis and TMAO reductase activity was visualised in the gel, as detailed in Section 2.

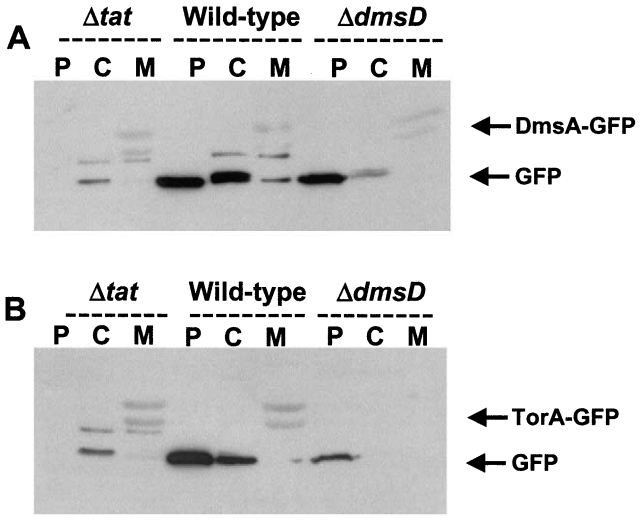

Fig. 3. DmsA–GFP and TorA–GFP are efficiently exported in the Δ*dmsD* mutant. *E. coli* strains Δ*tatABCDE* (Δ*tat*), wild-type and Δ*dmsD* containing either pBAD-DmsAGFP (expressing pre-DmsA–GFP; panel A), or pJDT1 (expressing TorA–GFP; panel B), were grown for 2 h in the presence of 100 μM L-arabinose. Cells were fractionated to yield periplasmic (P), cytoplasmic (C) and membrane (M) samples and immunoblotted using antibodies to GFP. Mature-size GFP and the GFP precursor proteins are indicated.

components (Δ*tatABCDE*), all of the active TorA is present in the cytoplasm, showing the complete block of TorA export (Δ*tat* panel). The results obtained with the *dmsD* mutant strain show that the vast majority of active TorA is localised in the periplasm. These results show that the DmsD protein is not absolutely required for correct localisation of TMAO reductase.

3.3. *The DmsA RR-signal peptide targets GFP to the periplasm in the dmsD mutant*

The above data show that the Tat system is functioning in the *dmsD* mutant, at least for TorA export, and so the important question is whether DmsD is specifically required for the targeting of DmsA through an interaction with the DmsA signal peptide. It should be emphasised that the final destination of DmsA is a matter of debate [7,9]. However, targeting of the DmsAB subunits into the mature DMSO reductase enzyme complex requires a functional Tat system and processing of the DmsA leader peptide [7,23]. While DmsC is widely agreed to be located in the membrane, the subcellular location of DmsA in particular is controversial. It has been proposed that DmsA and B are located on the cytoplasmic face of the inner bacterial membrane, based on a combination of techniques including DmsA– and DmsB–PhoA fusions [7,24,25]. However, in the absence of the membrane anchor DmsC, DMSO reductase activity was predominantly located in the periplasmic fraction. DmsB was also located primarily in the periplasm and, because this subunit is not synthesised with a signal peptide, it was suggested that it may be targeted and exported by virtue of its interaction with DmsA [26].

DMSO activity is absent in the *dmsD* mutant [15], suggesting that DmsD may indeed be required for targeting of DmsA/DmsB to the Tat system. However, it is important to establish whether DmsD is a true 'guidance factor', which simply recognizes the DmsA signal and initiates targeting to the translocase, or whether it plays a distinct role in the overall biogenesis of this enzyme. We addressed these questions by generating a chimeric protein in which GFP is linked to the DmsA signal peptide. Previous studies [18,27] have shown that the TorA signal peptide is able to direct the export of GFP exclusively by the Tat pathway, and studies on this TorA–GFP chimera were also carried out to determine whether the lack of DmsD affects export efficiency; this GFP-based export assay is generally more quantitative than the TorA native gel assay shown in Fig. 2.

In order to examine its export, synthesis of DmsA–GFP was induced with 100 μM arabinose and cells were fractionated. The localisation of the GFP was monitored by immuno-

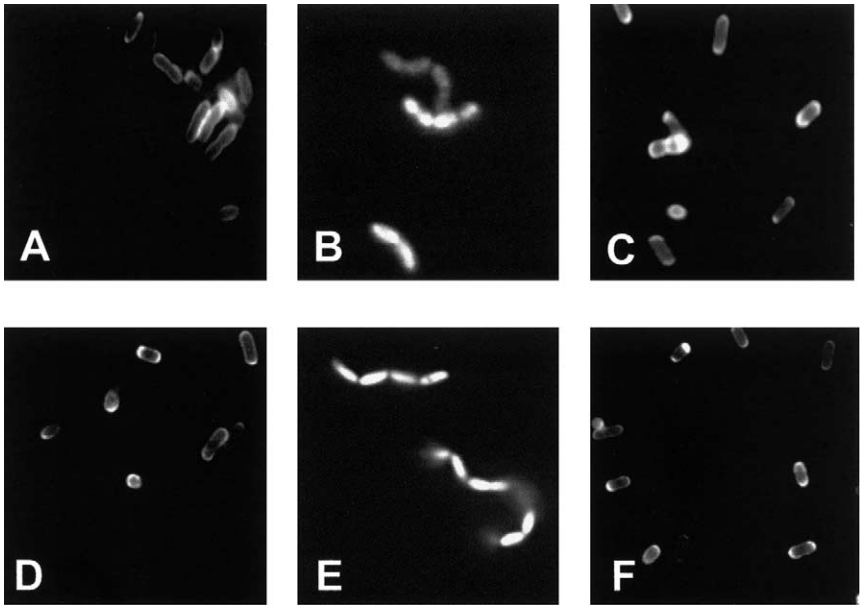

Fig. 4. Localisation of GFP by fluorescence microscopy. *E. coli* wild-type, Δ*tatABCDE* and Δ*dmsD* cells expressing TorA–GFP (A, B and C respectively) or DmsA–GFP (D, E and F respectively) were grown aerobically for 2 h in the presence of 100 μM L-arabinose, followed by 1 h incubation after the removal of arabinose, after which they were examined using fluorescence microscopy as detailed in Section 2.

blotting and Fig. 3A shows that in wild-type TG1 cells the majority of the GFP is found in the periplasmic fraction (lane P) as the mature form. Some mature-size GFP is also found in the cytoplasmic fraction (lane C), apparently from proteolysis of the signal peptide [18]. In the Δ*tatABCDE* strain (Δ*tat* panel) there is no export of DmsA–GFP to the periplasm, as expected, and precursor/degradation forms are found in the cytoplasm (C) and membrane (M) fractions. The final panel shows that DmsA–GFP is efficiently exported to the periplasm in the *dmsD* mutant lacking DmsD (Δ*dmsD* panel), and repeat experiments (not shown) have shown the export efficiency to be consistently as high as in wild-type cells. The total amount of GFP is slightly lower in the *dmsD* cells, but this is not always the case and the significance of this point is therefore unclear.

The export of TorA–GFP in *dmsD* mutant cells is shown in Fig. 3B. The data show that the export of TorA–GFP in the *dmsD* strain is again comparable with that observed in wild-type cells, with the majority of protein found as mature GFP in the periplasm in both cases. No export is observed in the Δ*tatABCDE* control panel. Thus, the combined data in Fig. 3 indicate that DmsD is not required for the efficient export of a protein bearing either the DmsA or TorA signal peptides, suggesting that DmsD is required for DMSO reductase activity or assembly, rather than the targeting of DmsA to the bacterial inner membrane.

3.4. Imaging of GFP

The results presented above show that GFP can be exported to the periplasm of *E. coli* cells when GFP is fused to either of two Tat-substrate signal peptides. To test the localisation of GFP more directly, cells over-expressing TorA–GFP or DmsA–GFP were examined by fluorescence microscopy and the data are shown in Fig. 4. Wild-type cells (A and D) and the *dmsD* mutant strain (C and F) showed prominent halos of GFP localised in the periplasm with both GFP constructs. In contrast, and as expected, the Δ*tatABCDE* strain (B and E) showed a clear cytoplasmic location of GFP and the typical chain-like phenotype of this mutant.

3.5. Conclusions

The DmsD protein carries out an essential function in the biogenesis of DMSO reductase and has the ability to bind to the DmsA signal peptide in vitro [15]. These properties suggested a role as a guidance factor functioning in the targeting of this subunit (and perhaps the associated DmsB protein) to the Tat apparatus. In this report we have analysed the role of DmsD using chimeric proteins, and the data show clearly that DmsD is not required for the functioning of the DmsA signal peptide under the conditions studied here. In the absence of DmsD, DmsA– and TorA–GFP fusions are exported with high efficiency, as is TMAO reductase, indicating that these signal peptides are fully operational. These data effectively rule out a role as a guidance factor and point to a very different role for the DmsD protein.

One possibility is that DmsD is required for cofactor insertion during DMSO reductase assembly. Sequence comparisons show that DmsD is related to, if not a member of, the TorD family of proteins (R.J. Turner and F. Sargent, unpublished observations). TorD has been proposed to be a chaperone protein specific for TorA, involved in the insertion of the cofactor into the apoprotein [28]. TorA and DmsA are homologous proteins, each binding a molybdopterin cofactor, and it therefore seems likely that the DmsD and TorD proteins may have similar functions. It is also possible that a chaperone function of DmsD may involve its binding to the signal peptide of DmsA; while we have shown that DmsA–GFP can be exported in the absence of DmsD, it is certainly possible that the binding of DmsD to pre-DmsA may influence its assembly. Binding may also inhibit export to some extent (although a substantial inhibition or block is ruled out since our data show that this signal peptide functions correctly in *dmsD* mutant cells). This would be an efficient means of preventing export of the protein prior to assembly, although there is evidence that the homologous TorD protein binds to the mature region of TorA [29]. In summary, our data point to an essential function of DmsD in the assembly of DMSO reductase but not in the functioning of the DmsA or TorA signal peptides.

Acknowledgements: This work was supported by the award of a Biotechnology and Biological Sciences Research Council Professorial Fellowship to C.R., and by Grant P15253 to C.R. We thank Claire Barrett for help in the development of the GFP export assay.

References

[1] Manting, E.H. and Driessen, A.J. (2000) Mol. Microbiol. 37, 226–238.
[2] Robinson, C. and Bolhuis, A. (2001) Nature Rev. Mol. Cell Biol. 2, 350–356.
[3] Clark, S.A. and Theg, S.M. (1997) Mol. Biol. Cell 8, 923–934.
[4] Hynds, P.J., Robinson, D. and Robinson, C. (1998) J. Biol. Chem. 273, 34868–34874.
[5] Berks, B.C. (1996) Mol. Microbiol. 22, 393–404.
[6] Santini, C.-L., Ize, B., Chanal, A., Müller, M., Giordano, G. and Wu, L.-F. (1998) EMBO J. 17, 101–112.
[7] Weiner, J.H., Bilous, P.T., Shaw, G.M., Lubitz, S.P., Frost, L., Thomas, G.H., Cole, J.A. and Turner, R.J. (1998) Cell 93, 93–101.
[8] Weiner, J.H., Rothery, R.A., Sambasivarao, D. and Trieber, C.A. (1992) Biochim. Biophys. Acta 1102, 1–18.
[9] Sargent, F., Bogsch, E.G., Stanley, N.R., Wexler, M., Robinson, C., Berks, B.C. and Palmer, T. (1998) EMBO J. 17, 3640–3650.
[10] Bogsch, E.G., Sargent, F., Stanley, N.R., Berks, B.C., Robinson, C. and Palmer, T. (1998) J. Biol. Chem. 273, 18003–18006.
[11] Sargent, F., Stanley, N.R., Berks, B.C. and Palmer, T. (1999) J. Biol. Chem. 274, 36073–36082.
[12] Settles, M.A., Yonetani, A., Baron, A., Bush, D.R., Cline, K. and Martienssen, R. (1997) Science 278, 1467–1470.
[13] Gouffi, K., Santini, C.L. and Wu, L.F. (2002) FEBS Lett. 525, 65–70.
[14] Bolhuis, A., Mathers, J.E., Thomas, J.D., Barrett, C. and Robinson, C. (2001) J. Biol. Chem. 276, 20213–20219.
[15] Oresnik, I.J., Ladner, C.L. and Turner, R.J. (2001) Mol. Microbiol. 40, 323–331.
[16] Sambrook, J., Fitsch, E.F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[17] Wexler, M., Sargent, F., Jack, R.L., Stanley, N.R., Bogsch, E.G., Robinson, C., Berks, B.C. and Palmer, T. (2000) J. Biol. Chem. 275, 16717–16722.
[18] Thomas, J.D., Daniel, R.A., Errington, J. and Robinson, C. (2001) Mol. Microbiol. 39, 47–52.
[19] Bolhuis, A., Bogsch, E.G. and Robinson, C. (2000) FEBS Lett. 472, 88–92.
[20] Guzman, L.-M., Belin, D., Carson, M.J. and Beckwith, J. (1995) J. Bacteriol. 177, 4121–4130.
[21] Randall, L.L. and Hardy, S.L.S. (1986) Cell 46, 921–928.
[22] Silvestro, A., Pommier, J., Pascal, M.C. and Giordano, G. (1989) Biochim. Biophys. Acta 994, 208–216.
[23] Sambasivarao, D., Turner, R.J., Simala-Grant, J.L., Shaw, G., Hu, J. and Weiner, J.H. (2000) J. Biol. Chem. 275, 22526–22531.

[24] Sambasivarao, D., Scraba, D.G., Trieber, C. and Weiner, J.H. (1990) J. Bacteriol. 172, 5938–5948.
[25] Weiner, J.H., Shaw, G., Turner, R.J. and Trieber, C. (1993) J. Biol. Chem. 268, 3238–3244.
[26] Stanley, N.R., Sargent, F., Buchanan, G., Shi, J., Stewart, V., Palmer, T. and Berks, B.C. (2002) Mol. Microbiol. 43, 1005–1021.
[27] Santini, C.-L., Bernadac, A., Zhang, M., Chanal, A., Ize, B., Blanco, C. and Wu, L.-F. (2001) J. Biol. Chem. 276, 8159–8164.
[28] Sargent, F., Berks, B. and Palmer, T. (2002) Arch. Microbiol. 178, 77–84.
[29] Pommier, J., Mejean, V., Giordano, G. and Iobbi-Nivoli, C. (1998) J. Biol. Chem. 273, 16615–16620.