Protein phosphorylation in *Escherichia coli* L-form NC-7

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Wall-less L-forms of *Escherichia coli* constitute an interesting, and relatively underused, model system for numerous studies of bacterial physiology including the cell cycle, intracellular structure and protein phosphorylation. Total extracts of the L-form revealed a pattern of protein phosphorylation similar to that of an enteropathogenic strain but very different from its parental K-12 strain. In particular, the L-form extract revealed phosphorylation on tyrosine of a protein important in pathogenesis, TypA, and calcium-specific phosphorylation of a 40 kDa protein. Two new phosphoproteins were identified in the L-form as the DNA-binding protein Dps, and YfiD, a protein of 14 kDa with homology to pyruvate formate-lyase and a region containing a tRNA cluster in bacteriophage T5.

Keywords: Dps, YfiD, TypA, tyrosine phosphorylation, calcium

INTRODUCTION

L-forms are bacteria that have lost their ability to construct cell walls and, bounded only by a cytoplasmic membrane, are particularly fragile. There appear to be two classes of L-forms. Unstable L-forms regain the ability to construct their walls and revert readily to a bacterium resembling the parental strain whilst stable L-forms have almost lost this ability and revert much less readily. Both stable and unstable L-forms have been obtained by independent workers from many bacteria including *Escherichia coli* (Gumpert, 1992; Lederberg & St Clair, 1958; Onoda & Oshima, 1988; Paton, 1987; Waterhouse *et al.*, 1996). *E. coli* L-forms should be particularly interesting for studies of bacterial physiology since they combine the advantages of, firstly, the *E. coli* model system with its well-explored genetics, secondly, a structure in which the peptidoglycan sacculus is absent and the cytoplasmic membrane is exposed, and, thirdly, an extreme fragility which allows the rapid extraction of labile enzymes.

L-forms remain intact, grow, segregate their chromosomes and divide because they possess an intracellular structure that can serve as an alternative to peptidoglycan in the particular conditions in which they grow. In normal cells, intracellular structure may be conferred by self-associating enzymes (Mendes *et al.*, 1995; Sreer,

1987) and a 'solid-state' metabolism (Ingber, 1993) that may exist as an 'enzoskeleton' (Norris *et al.*, 1996). In L-forms, the elements of such an enzoskeleton might be expected to be up-regulated and evident candidates include FtsZ, a protein with some similarity to tubulin that assembles into a 'contractile ring' at the start of cell division (Bi & Lutkenhaus, 1991; Ma *et al.*, 1996), Dps, a principal component of 'actomyosin' preparations of *E. coli* (Foster, 1993) which is induced to protect DNA from oxidative damage (Almiron *et al.*, 1992), and elongation factor EF-Tu, which has actin-like properties (Weiser *et al.*, 1982; Guerrero-Barrera *et al.*, 1996).

It is conceivable that a bacterial enzoskeleton would be regulated, like its eukaryotic equivalent, by eukaryotic-like kinase activities that phosphorylate proteins on serine, threonine and tyrosine residues (STY phosphorylation) or by the 'two-component' kinases that phosphorylate on histidine (Saier, 1993). In *E. coli*, STY-phosphorylated proteins make up the majority of the 130 proteins phosphorylated *in vivo* (Cortay *et al.*, 1986a). A number of these proteins have now been identified (for references see Kennelly & Potts, 1996) and include elongation factor Tu (Lippmann *et al.*, 1993), heat-shock protein DnaK (Cegielska & Georgopoulos, 1989; McCarthy & Walker, 1991; Sherman & Goldberg, 1993), a Ras-like protein, Era (Sood *et al.*, 1994), the Universal Stress Protein, UspA (Freestone *et al.*, 1997), and possibly the cell division protein FtsA (Sanchez *et al.*, 1994). Using an *in vitro* assay (Norris *et*

Abbreviation: TPA, 12-tetradecanoylphorbol 13-acetate.

al., 1991), we have identified several other phosphoproteins including ribosomal protein S1, succinyl-CoA synthetase and TypA (Freestone *et al.*, 1995; Sweeney *et al.*, 1995), which appears to be a new class of virulence regulator (Farris *et al.*, 1998) and which interacts with global regulators such as H-NS and UspA (Freestone *et al.*, 1998). In these studies, difficulties were often encountered due to the lability of the kinase activities and the multiplicity of interacting factors. Here, we study protein phosphorylation in L-form extracts and identify phosphoproteins that may be involved in L-form structure.

METHODS

Bacteria and growth conditions. L-form NC-7, parental strain 3301 (Onoda *et al.*, 1987) and enteropathogenic strain MAR001 (Baldini *et al.*, 1983) were used in this study. The L-form and strain 3301 were grown without agitation in 200 ml of a 1 l solution containing 10 g peptone, 5 g yeast extract, 2 g glucose and 19.8 g NaCl at 30 °C for 2 d (to OD₆₀₀ 0.5–0.7 for the L-form and to OD₆₀₀ 2.4 for strain 3301; measured with a Philips PU 8720 UV/VIS Scanning spectrophotometer), as described previously (Onoda *et al.*, 1992). Strain 3301 and other bacteria were also grown aerobically at 37 °C in the same medium to an OD₆₀₀ of 1 (Freestone *et al.*, 1995).

Preparation of extracts and Mono Q 5/5 fractionation. L-forms were harvested by centrifugation at 2000 g, washed in 20 mM Tris/HCl, 1 mM EDTA, 1 mM DTT (TED buffer) containing 0.34 M NaCl, and centrifuged again. The L-form cell pellet was resuspended in 10 ml TED buffer containing protease inhibitors (chymostatin, leupeptin, pepstatin A and aprotinin at 1 µg ml⁻¹) and sonicated for 5–10 s. Extracts from other bacteria were prepared as previously described (Freestone *et al.*, 1995). Aliquots (25 mg) of whole extract were filtered (0.2 µm) and loaded onto a Pharmacia Mono Q 5/5 column equilibrated in TED buffer as described previously (Freestone *et al.*, 1995). Proteins were typically eluted at 1 ml min⁻¹ in 1 ml fractions with a 25 ml linear gradient of 0–600 mM NaCl. Protease inhibitors were added to all fractions (see above); these were stored at –20 °C.

Kinase assays. Assays of total cell extracts and Mono Q fractions (which contain autophosphorylating enzymes as well as co-eluting kinases and their substrates) were performed in 25 µl volumes containing 20 mM Tris/HCl, pH 7.5, 5 mM MgCl₂ and 0.185 MBq [γ -³²P]ATP (specific activity 1.10 × 10⁸ MBq mmol⁻¹) in 1 µM cold ATP (minimal assay medium). Additions were made, where appropriate, of 1 mM EGTA, 5 mM CaCl₂, 2.5 mM MnSO₄, 2.5 mM ZnSO₄, 0.10 µM TPA (12-tetradecanoylphorbol 13-acetate) (in DMSO) and 40 µg L- α -phosphatidylserine ml⁻¹. After 30 min incubation at 37 °C, the reactions were stopped by the addition of 25 µl SDS-PAGE sample buffer, containing 10 mM DTT. The samples were heated at 95 °C for 5 min, followed by SDS-PAGE on appropriate percentage minigels. After electrophoresis, proteins were blotted onto PVDF membranes (Immobilon P; Millipore) at 0–4 °C in 25 mM Tris, 192 mM glycine, 0.037% (w/v) SDS and 10% (v/v) methanol. Blotted proteins were visualized with 0.1% Coomassie blue 250 in 50% methanol, followed by destaining with 50% methanol and 10% acetic acid. Autoradiography of the PVDF blots was carried out at –70 °C using Dupont Cronex X-ray film.

N-terminal sequencing. Chromatographically purified proteins (concentrated by lyophilization where necessary)

were phosphorylated, separated by SDS-PAGE and electroblotted onto PVDF membranes, as described above. ³²P-labelled protein bands were located by autoradiography, excised and the radioactive bands sequenced using an Applied Biosystems 470A gas-phase sequencer. In each case, the N-terminal sequence obtained corresponded to only one protein.

Residue stability analysis. PVDF membranes containing the ³²P-labelled phosphoprotein were heated to either 90 °C for 90 min in 16% TCA, to test for acid stability, or 60 °C for 120 min in 1 M KOH, to test for base stability. Membranes were neutralized, dried and reautoradiographed as described above.

Immunoblotting. For phosphotyrosine detection, proteins were separated on 10% SDS-polyacrylamide gels, transferred to PVDF membranes and probed with a 1:1000 dilution of anti-phosphotyrosine monoclonal antibodies (Sigma). Antibody recognition was detected using horseradish-peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham).

RESULTS

Phosphorylation in total cell extracts

The L-form and its parental strain are so different in morphology and rates of growth that comparisons, whilst informative, are not easy. Initially, both L-form

Fig. 1. ATP-dependent kinase activities in total extracts of (a) L-form and (b) parent. Extracts of L-form and parental strain 3301, growing exponentially and harvested at OD₆₀₀ 0.7 and 1, respectively, were assayed (approximately 100 µg protein) in minimal assay medium plus 1 mM EGTA, as described in Methods, with the following additions. Lanes: 1, no addition; 2, 5 mM CaCl₂; 3, 2.5 mM MnCl₂; 4, 2.5 mM ZnCl₂; 5, 40 µg L- α -phosphatidylserine ml⁻¹; 6, DMSO control (10%); 7, 0.1 µM TPA in DMSO (10%); 8, 5 mM CaCl₂ + 0.1 µM TPA in DMSO (10%); 9, 2.5 mM ZnCl₂ + 0.1 µM TPA in DMSO (10%). Proteins were separated by SDS-PAGE on 12% gels and transferred onto PVDF followed by autoradiography.

and parent were harvested at similar optical densities whilst growing exponentially (and, in the latter case, aerobically) although we cannot exclude the possibility that 'exponential growth' of the L-form actually resembles stationary phase growth of the parent. After breaking cells carefully and rapidly to preserve possibly labile kinase activities, comparison of protein kinase activity in total extracts of the L-form and the parental strain revealed considerable differences (Fig. 1). In particular, proteins of 80 and 28 kDa were strongly phosphorylated in the L-form but not in its parent whilst a calcium-specific phosphorylation of a 40 kDa protein was also observed in the L-form but not in the parent (Fig. 1, lanes 2 and 8). Although addition of zinc inhibited the phosphorylation of several proteins in the L-form, including those of 52, 48 and 28 kDa, it stimulated specific phosphorylation of proteins of 52 and 48 kDa in the parent (Fig. 1, lane 4). Finally, TPA had little effect on phosphorylation in the L-form but stimulated phosphorylation of proteins of 50 and 45 kDa in the parent (Fig. 1, lanes 7 and 8).

Phosphorylation in fractions

To identify proteins phosphorylated in the L-form, anion-exchange chromatography (Mono Q) was used to generate fractions from the L-form. To facilitate comparisons between the L-form and its parent, the protein extracts used for fractionation were made from cells grown in L-form medium to similar optical densities (Fig. 2a, b) and from the parent grown as a stagnant culture for 2 d (Fig. 2c). As observed previously (Freestone *et al.*, 1995), most proteins were eluted in the 100–500 mM salt range. Although the pattern of phosphorylation in the L-form (using ATP as phosphate donor) varied with the age of the culture and with different experiments (data not shown), six prominent phosphoproteins were reproducibly observed of 80, 70, 65, 36, 32 and 17 kDa (Fig. 2a). With the exception of the 17 kDa protein, these almost certainly correspond to those obtained from an enteropathogenic strain which include TypA (formerly $\alpha 591$, see below), DnaK, S1 and SucD (Freestone *et al.*, 1995). This pattern of phosphorylation is different from that observed in fractions of laboratory strains such as the parental strain of the L-form (compare Fig. 2a with b and c). For example, the phosphoproteins of 80 and 17 kDa were absent from the fractions of the laboratory strains whilst a protein of 95 kDa was present (Fig. 2b). The N-terminal sequence obtained for the 95 kDa protein was SERFPNDVDPIE, which corresponds to residues 2–13 of the E1 component of the pyruvate dehydrogenase complex, a known phosphoprotein (Freestone *et al.*, 1995). Synthesis of this enzyme is repressed in anaerobic growth and, not surprisingly, its phosphorylation was not detected in either the L-form (Fig. 2a) or the parental strain grown as a stagnant culture (Fig. 2c). The Universal Stress Protein, which is phosphorylated *in vivo* and *in vitro* (Freestone *et al.*, 1997), was identified as a phosphoprotein (on the basis of antibody reactivity and phosphorylation characteristics) in the parent

Fig. 2. ATP-dependent kinase activities in Mono Q fractions of (a) L-form grown for 2 d as a stagnant culture to OD_{600} 0.7, (b) parent grown aerobically to OD_{600} 0.7 and (c) parent grown for 2 d as a stagnant culture to OD_{600} 2.4. Extracts of L-form and parental strain 3301 were fractionated using Mono Q anion-exchange chromatography, as described in Methods. Samples (20 μ l) of fractions from the 100 mM to 500 mM region of the NaCl gradient were assayed in minimal assay medium plus 5 mM $CaCl_2$ and 2.5 mM $MnCl_2$ and phosphorylated proteins were analysed as in Fig. 1. P, Pyruvate dehydrogenase E1 subunit; M, TypA; D, Dps ($\bar{D}$, Dps absent); U, UspA; Y, YfiD.

grown as a stagnant culture (Fig. 2c) but could not be readily detected in the L-form. Several of the L-form phosphoproteins were characterized further.

80 kDa – TypA

The 80 kDa phosphoprotein (protein M, Fig. 2a) corresponds to TypA (Tyrosine Protein A), the product of the $\alpha 591$ gene (Freestone *et al.*, 1995, 1998), on the

Fig. 3. Cross-reaction with anti-phosphotyrosine antibodies. *In vivo* phosphorylation of TypA in total cell extracts of *E. coli* probed with anti-phosphotyrosine antibodies. Lanes: 1, K-12 strain 3301; 2, MAR001; 3, L-form NC-7.

basis of molecular mass, cross-reactivity with antibodies to TypA, stability to acid and base hydrolysis, elution characteristics and stimulation of phosphorylation by divalent metal ions and hydrophobic compounds (Fig. 1, lanes 2–6, and data not shown). TypA is one of the very few proteins in *E. coli* phosphorylated on tyrosine residues (Freestone *et al.*, 1995) and neither we nor others have observed its phosphorylation in laboratory strains under a wide range of conditions including anaerobic growth (Freestone *et al.*, 1998; Farris *et al.*, 1998). Probing with anti-phosphotyrosine antibodies confirmed that TypA is phosphorylated on tyrosine in the L-form *in vivo* but not in the parental or other K-12 strains (Fig. 3).

19 kDa – Dps

A particularly labile kinase activity responsible for phosphorylation of a 19 kDa protein – but distinct from it on the basis of elution characteristics – was observed in a fraction eluted by 150 mM NaCl (protein D, Fig. 2a). This activity required only magnesium: no stimulation was observed with calcium, manganese or lipid, while zinc was slightly inhibitory (data not shown). Phosphorylation of the 19 kDa protein was stable to acid but not base hydrolysis consistent with phosphorylation on serine or threonine residues; only a small proportion of the protein was phosphorylated in our *in vitro* assay (data not shown). N-terminal sequencing of this protein gave MSTAKLVKSKA, which corresponds to the first 11 amino acids of the Dps protein (Almiron *et al.*, 1992). Phosphorylation of Dps was not observed under either of the conditions in which the parental strain was grown (Fig. 2b, c).

14 kDa – YfiD

The kinase activity responsible for the phosphorylation of a 14 kDa protein (protein Y in Fig. 2a) in a fraction adjacent to that containing the Dps protein was also

YfiD: MITGIQITKA	ANDDLLNSFW	LLDSEKGEAR
ecpfl: ANPMHGRDQK	GAVASLTSVA	KLPFAYAKDG
YfiD: CIVAKAGYAE	DEVVAVSKLG	DIEYREVPVE
ecpfl: ISYTFSIVPN	ALGKDDEVK	TNLAGLMDGY
YfiD: VKPEVRVEGG	QHLNVNVLRR	ETLEDAVKHP
ecpfl: FHHEASIEGG	QHLNVNVMNR	EMLDAMENP
YfiD: EKYPQLTIRV	SGYAVRFNSL	TPEQQRDVIA
ecpfl: EKYPQLTIRV	SGYAVRFNSL	TKEQQQDVIT
YfiD: RTFTESL		
ecpfl: RTFTQSM		

Fig. 4. Comparison of the entire YfiD sequence with residues 633–759 of pyruvate formate-lyase. Identical residues are shown in bold.

very labile (data not shown). Phosphorylation of the 14 kDa protein was only dependent upon magnesium; this phosphorylation was neither stimulated by manganese, calcium and lipid nor inhibited by zinc (data not shown). The phosphoresidue(s) of this protein was unstable to acid hydrolysis and partially stable to hot alkaline hydrolysis consistent with phosphorylation on histidine or lysine residues (data not shown). N-terminal sequencing of the 14 kDa phosphoprotein gave MTITGIQITKA. This matches nine out of the first ten residues, MITGIQITKA (protein accession no. p33633.swiss and gene no. yfiD_ecoli), of a 127-residue product of predicted molecular mass 14.3 kDa in the 3' *ung* region at 56 min, which encodes uracil DNA glycosylase (Green & Baldwin, 1997). The 14 kDa protein showed a 95% homology over its entire length to the possible product of a gene of unknown function in *Serratia liquefaciens* and a 77% homology over 64 residues (positions 65–129) to the C-terminus of *E. coli* pyruvate formate-lyase (Rodel *et al.*, 1988), which is encoded by a gene in the 20 min region of the genome (Fig. 4). Essentially the same region (positions 41–109) has a 68% homology over 68 residues to a sequence in bacteriophage T5 located in a region containing a tRNA cluster (data not shown). Although the 14 kDa product of *yfiD* was one of the most abundant proteins in extracts of the L-form as judged by Coomassie blue staining, only a small proportion of the protein was phosphorylated in our *in vitro* assay (data not shown). Under native conditions, YfiD exists as a particularly heat-stable, monomeric protein which, when separated by two-dimensional electrophoresis, makes at least two isoforms (data not shown). Neither YfiD protein nor YfiD phosphorylation was detected in the L-form parent when growing aerobically (Fig. 2b) and, although YfiD can be produced in cells that are grown anaerobically (Green & Baldwin, 1997), phosphorylation of YfiD in the L-form parent could not be detected following growth as a stagnant culture (Fig. 2c).

DISCUSSION

Studies of protein phosphorylation in L-forms may benefit from the ease and rapidity of extracting proteins, which helps preserve labile activities, and the nature of L-form growth, which may up-regulate these activities. Although *in vitro* phosphorylation studies are subject to a number of artefacts, most of the proteins we have identified as phosphorylated *in vitro* are also phosphorylated *in vivo* (Freestone *et al.*, 1995, 1997, 1998; Sweeney *et al.*, 1995). Phosphorylation of TypA on tyrosine is intriguing. Although phosphorylation on tyrosine in prokaryotes has been subject to controversy (Cortay *et al.*, 1986b; Foster *et al.*, 1989), phosphorylation on this residue has now been observed in several species of bacteria (for example, Duclos *et al.*, 1996; Freestone *et al.*, 1995; Ostrovsky & Maloy, 1995; Smith *et al.*, 1997). TypA, encoded by the *o591* sequence and having a significant homology to ribosome-binding elongation factors G (Plunkett *et al.*, 1993), is phosphorylated *in vitro* and *in vivo* on tyrosine residue(s) in clinical isolates of *E. coli* but not in laboratory strains (Freestone *et al.*, 1995, 1998; Farris *et al.*, 1998). TypA, also known as BipA, has recently been implicated in the formation of actin-rich pedestals in epithelial cells during enteropathogenic *E. coli* infections and in resistance to the cationic host defence peptide BPI (Farris *et al.*, 1998). The BipA homologue in *Salmonella typhimurium* is considered a global regulator involved in bacterial survival during the invasion of the host (Qi *et al.*, 1995). In *E. coli*, TypA is an autophosphorylating protein that affects the phosphorylation of UspA, a protein that is induced by a variety of stresses and that determines bacterial survival during growth arrest (Freestone *et al.*, 1997). A role for TypA as a global regulator would be consistent with our findings that in a *typA* mutant the synthesis and/or post-translational modification of 12 other proteins are affected in exponential phase and still more in stationary phase (Freestone *et al.*, 1998). It is far from clear how L-forms maintain their structural integrity and it is conceivable that TypA promotes both rearrangements of the cytoskeleton in host cells and reinforcement of an 'enzoskeleton' in L-forms (Norris *et al.*, 1996). The fact that phosphorylation of TypA occurs in the NC-7 L-form, which is derived from a K-12 laboratory strain, means that the factors responsible for stimulating its phosphorylation might in principle be identified by comparative studies of the L-form, its parental strain and clinical isolates.

Phosphorylation of the Dps protein was observed. This protein is produced in starvation conditions to protect DNA against oxidative damage (Almiron *et al.*, 1992). The characteristics of the stability of the phospholabelling of Dps to acid and base hydrolysis are indicative of phosphorylation on serine or threonine residues. When purified, Dps forms highly ordered arrays in the presence of DNA (Almiron *et al.*, 1992) and is a principal component of 'actomyosin' preparations of *E. coli* (Foster, 1993). The reason for the phosphorylation of Dps in L-forms is unclear since this

phosphorylation was not detected in the parental strain when grown in similar conditions (including growth as a stagnant culture) or, indeed, in any other conditions; it is conceivable that Dps contributes to the structural stability of the L-forms and Dps phosphorylation is part of the regulation of the putative enzoskeleton (Norris *et al.*, 1996). The kinase activity responsible for Dps phosphorylation has not been reported previously. This may be because it is both very labile, requiring the rapid extraction that L-forms permit, and quite elusive, being absent under many growth conditions.

A similarly labile phosphorylation was also observed of the 14 kDa product of the *yfiD* gene at 56 min on the genome. The speculation that the same kinase activity is responsible for the phosphorylation of both YfiD and Dps would be consistent with the distribution of these proteins and their phosphorylated isoforms. However, zinc inhibits Dps but not YfiD phosphorylation and this argues against a common kinase. The characteristics of the stability of the phosphoresidue(s) of the proteins to acid and base hydrolysis are also different, with those of YfiD indicative of phosphorylation on either histidine or lysine residues (or both). *In vivo*, YfiD exists as two isoforms consistent with a substantial proportion of the protein being modified by, for example, phosphorylation. *yfiD* has a class 1 promoter with FNR boxes to which FNR, the anaerobic transcription regulator of *E. coli*, binds (Green & Baldwin, 1997). *yfiD* is induced by the synthesis in anaerobically growing (but not aerobically growing) *E. coli* of HlyX, a homologue of FNR found in *Actinobacillus pleuropneumoniae* (Green & Baldwin, 1997). YfiD is one of four proteins in *E. coli* with homology to pyruvate formate-lyase (G. Sawers & J. Knappe, personal communication). In particular, YfiD has a remarkable homology to the C-terminus of pyruvate formate-lyase which plays a role in the catalysis by pyruvate formate-lyase of the non-oxidative conversion of pyruvate to acetyl-CoA in anaerobically growing *E. coli* (Rodel *et al.*, 1988). A free radical, assigned to glycine-734 in the C-terminus of pyruvate formate-lyase (Wagner *et al.*, 1992), is involved in the generation of the catalytically active thiyl radical that initiates homolytic cleavage of the pyruvate C-C bond (Knappe *et al.*, 1993). The equivalent of glycine-734 is conserved in YfiD (Fig. 4). A related function for YfiD and its phosphorylation in metabolism is conceivable. However, this would not explain why YfiD is one of the most abundant proteins in the L-form under conditions (nearly 2 d growth without agitation) in which it is undetectable in the parental strain.

Phosphorylation of a 40 kDa protein was stimulated by addition of calcium to total cell extracts. However, a phosphorylation with similar characteristics was not observed in fractions. One obvious explanation for this is that kinase and substrate are separated by anion-exchange chromatography. In view of the potential importance of calcium-stimulated kinase activities in the control of cellular structure and the cell cycle (Norris, 1989; Yu & Margolin, 1997), the identification of this phosphoprotein must be a priority for future research.

Although L-forms have been shown to prevent the development of disease in plants due to pathogenic attack (Waterhouse *et al.*, 1996), they remain a relatively neglected tool for studies of fundamental bacterial processes. These studies include those *in vitro* where labile enzymes must be purified and those *in vivo* where the outer membrane and peptidoglycan sacculus interfere with export, import or the action of various compounds (Klessen *et al.*, 1989), and, as we and others have argued (for example, Gumpert, 1992; Waterhouse *et al.*, 1994), they may also include intracellular structure, signal transduction, cell cycle regulation and even the origin of life. Indeed, the ability of L-forms to survive and grow suggests that these wall-less bacteria manage to co-opt enzymic pathways and the force-generating processes of transcription and translation to maintain their structural integrity as perhaps protocells did early in evolution (Norris *et al.*, 1998).

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