The transport of group 2 capsular polysaccharides across the periplasmic space in Escherichia coli

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The cell surface expression of group 2 capsular polysaccharides involves the translocation of the polysaccharide from its site of synthesis on the inner face of the cytoplasmic membrane onto the cell surface. The transport process is independent of the repeat structure of the polysaccharide, and translocation across the periplasm requires the cytoplasmic membrane-anchored protein KpsE and the periplasmic protein KpsD. In this paper, we establish the topology of the KpsE protein and demonstrate that the C terminus interacts with the periplasmic face of the cytoplasmic membrane. By chemical cross-linking we show that KpsE is likely to exist as a dimer and that dimerization is independent of the other Kps proteins or the synthesis of capsular polysaccharide. No interaction between KpsD and KpsE could be demonstrated by chemical cross-linking, although in the presence of both KpsE and Lpp, KpsD could be cross-linked to a 7-kDa protein of unknown identity. In addition, we demonstrate that KpsD is present not only within the periplasm but is also in both the cytoplasmic and outer membrane fractions and that the correct membrane association of KpsD was dependent on KpsE, Lpp, and the secreted polysaccharide molecule. Both KpsD and KpsE showed increased protease K sensitivity in the different mutant backgrounds, reflecting conformational changes in the KpsD and KpsE proteins as a result of the disruption of the transport process. Collectively the data suggest that the trans-periplasmic export involves KpsD acting as the link between the cytoplasmic membrane transporter and the outer membrane with KpsE acting to facilitate this transport process.

The export of capsular polysaccharides in Gram-negative bacteria from their site of synthesis on the inner face of the cytoplasmic membrane onto the bacterial surface presents a unique challenge to the micro-organism. It requires the translocation of a high molecular weight negatively charged macromolecule across two lipid bilayers. Understanding this process offers potential benefits in terms of engineering polysaccharides of biomedical importance in bacteria and in designing new antimicrobials that inhibit this process. In contrast to protein secretion, our understanding of how capsular polysaccharide transport is achieved is currently scant. The expression of capsular polysaccharides (or K antigens) in Escherichia coli offers an experimentally tractable system in which to try to understand the mechanisms of polysaccharide transport. E. coli can express over 80 chemically and serologically distinct capsular polysaccharides that have been divided into four groups according to a number of biochemical and genetic criteria (1). Group 2 capsules resemble those found on the surfaces of Neisseria meningitidis and Hemophilus influenzae and are often expressed by pathogenic E. coli isolates causing extraintestinal disease (2). The biochemistry and genetics of E. coli group 2 capsules, as typified by the K1 and K5 antigens, have been studied in most detail (reviewed in Refs. 1, 3, and 4). Group 2 capsules are synthesized by a hetero-oligomeric membrane-bound biosynthetic complex on the inner face of the cytoplasmic membrane by the sequential action of glycosyltransferases that elongate the polysaccharide at its nonreducing end (5). A common export pathway, irrespective of the repeat structure of the particular polysaccharide molecule, is then used to translocate group 2 capsules from their site of synthesis onto the cell surface (3, 6). Translocation across the cytoplasmic membrane is mediated by the KpsC, M, S, and T proteins, whereas translocation across the periplasm and outer membrane involves the KpsD and E proteins (1, 3, 4). The KpsC and S proteins are believed to attach phosphatidyl-Kdo to the reducing end of the nascent polysaccharide chain, and this substitution permits the entry of the polysaccharide molecule into the export pathway (1, 3, 7). However, the enzymology of this process still awaits elucidation. Translocation of the polysaccharide across the cytoplasmic membrane is achieved by the KpsM and T proteins that constitute an ABC-2 (ATP-binding cassette type 2) transporter (8), in which KpsM is the integral membrane protein and KpsT is the ATPase (9–12). Typically such ABC-2 transporters involved in polysaccharide export require two accessory proteins to complete the export process, a cytoplasmic membrane-periplasmic auxiliary protein (MPA), of which KpsE is an example, and an outer membrane auxiliary protein (8). Based on computer analysis, a predicted topology has been suggested for MPA proteins in which the

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1 The abbreviations used are: Kdo, 2-keto-3-deoxyoctonate; MPA, membrane-periplasmic auxiliary protein; DSP, dithiobis(succinimidyl propionate); PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PCR, polymerase chain reaction.
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Spheroplast Formation and Protease Digestion of Periplasmic Proteins—Spheroplasts were prepared using the method of Rosenow et al. (21). Proteinase K was added at a final concentration of 500 μg ml⁻¹, and spheroplasts were incubated at 37 °C for the appropriate time before the enzyme was inhibited by the addition of trichloroacetic acid to a final concentration of 20%. Carboxypeptidases A and B were added to final concentrations of 200 and 100 μg ml⁻¹, respectively, and samples were incubated at 37 °C for 2 h before the enzyme was inhibited by the addition of 20% trichloroacetic acid. After trichloroacetic acid precipitation, the pellet was washed with acetone, dissolved in the appropriate volume of SDS-PAGE sample buffer, and analyzed by SDS-PAGE followed by Western blotting.

Cell Fractionation—Cells were fractionated to give cytoplasmic membrane and outer membrane fractions using a modification of the method of Maier et al. (22). A 400-ml culture was grown to an A₆₀₀ nm of 0.8 and cooled on ice. Cells were washed with 10 mM Tris-HCl, pH 8.0, and resuspended in 25 ml of 10 mM Tris-HCl, pH 8.0, 0.2 mM DTT, and 20 mg of DNase I were added. Bacteria were disrupted by passing them three times through a French pressure cell at 20,000 pounds/square inch or by sonication (5-s bursts with 10-s intervals for 3 min) on ice, and unbroken cells and debris were removed by centrifugation (7,000 × g for 15 min). The supernatant was subjected to centrifugation (80,000 × g for 1 h at 4 °C), and the resultant supernatant containing the cytoplasmic and periplasmic fractions was then decanted and saved for further analysis. The pellet containing the membrane fraction was washed with 20 ml of 10 mM Tris-HCl, pH 8.0, 0.2 mM DTT. The membranes were resuspended in ~1 ml of 10 mM Tris-HCl, pH 8.0, 0.2 mM DTT and layered on top of a two-step sucrose gradient (7.5 ml of 54% (w/v) sucrose on a cushion of 3 ml of 70% (w/v) sucrose in 10 ml Tris-HCl, pH 8.0). The gradient was centrifuged at 100,000 × g for 16–18 h at 6 °C, and fractions were collected from the sides of the tube with 23-gauge needles connected to 1-ml syringes and analyzed by SDS-PAGE followed by Western blotting. Following trichloroacetic acid precipitation the activity of the periplasmic enzyme alkaline phosphatase and the cytoplasmic enzyme malate dehydrogenase were assayed according to the method of de Maagd and Lugtenberg (23) to check for contamination of the membrane fractions with cytoplasmic and periplasmic enzymes. β-NADH oxidase activity was measured in all the fractions from the gradient as a marker for the cytoplasmic membrane fractions (24), and the level of Kdo was used as a marker for the outer membrane (25). Membrane fractions were typically found to exhibit less than 2% total alkaline phosphatase and 3% total malate dehydrogenase activities indicating that they were essentially free of periplasmic and cytoplasmic contamination. Cytoplasmic plus periplasmic fractions were found to contain less than 4% of the total amount of Kdo and 5% total β-NADH oxidase activity. Cytoplasmic fractions produced from periplasmic and cytoplasmic fractions were found to contain less than 2% of total amount of Kdo, and outer membrane fractions contained no more than 3% total β-NADH oxidase activity, suggesting that these fractions were also predominantly free from contamination from other fractions of the gradient.

In Vivo Cross-linking with DSP—Cross-linking with was carried out as described previously (26).

RESULTS

Localization of the C Terminus of KpsE—Previous attempts using β-lactamase fusions as topology probes had established that KpsE was anchored to the cytoplasmic membrane via a C-terminal membrane-spanning domain located between residues 28 and 49 (21). However, these studies failed to assign an unequivocal location for the C terminus of KpsE and to establish whether the predicted C-terminal transmembrane domain located between residues 362 and 379 spanned the cytoplasmic membrane as predicted for MPA2 proteins (8). To determine whether the C terminus of KpsE was exposed in the periplasm, spheroplasts of the strain JM109[DE3] (pACYC184, pTH1), which expresses full-length KpsE protein (26), were incubated with either proteinase K or carboxypeptidases A and B. The digestion products were analyzed by Western blot using anti sera to chloramphenicol acetyltransferase, β-lactamase, KpsE, or EnvZ (Fig. 1). The periplasmic enzyme, β-lactamase, was fully degraded by proteinase K and extensively degraded by carboxypeptidases A and B (Fig. 1). The extra bands of decreasing molecular weight seen in the presence of carboxypeptidases A and B can be explained by the slow release by these enzymes

2 C. Arrecubieta, unpublished results.
of aspartic acid, glutamic acid, and glycine residues, all of which occur in the C-terminal 30 amino acids of \( \beta \)-lactamase. KpsE was also completely digested by proteinase K, confirming the presence of a large periplasmic loop (Fig. 1). The addition of carboxypeptidases A and B resulted in a limited digestion of KpsE, leading to a predominant truncated product 1.0 kDa smaller than wild type KpsE (Fig. 1) confirming that the C terminus of KpsE is exposed to the periplasm. No degradation of the cytoplasmic enzyme chloramphenicol acetyltransferase could be detected (Fig. 1), confirming the integrity of the cytoplasmic membrane. To confirm the carboxypeptidase results, Western blot analysis using antisera against EnvZ was performed. EnvZ is an integral cytoplasmic membrane protein with cytoplasmic N and C termini with an extended periplasmic domain (27). No degradation of EnvZ was detectable following carboxypeptidase treatment (Fig. 1) confirming that the limited degradation of KpsE is due to the action of carboxypeptidase.

To establish whether the hydrophobic C terminus of KpsE interacts with either the cytoplasmic or outer membrane, plasmid pTH56 was generated. This plasmid encodes the C-terminal 29 amino acids of KpsE fused in frame onto the C terminus of wild type \( \beta \)-lactamase. Cells of strain PA360(pTH56) were fractionated by a two-step sucrose gradient to give cytoplasmic and outer membrane fractions and subjected to Western blot analysis (Fig. 2). As predicted, wild type \( \beta \)-lactamase encoded by pBR322 was located in the soluble periplasmic fraction (Fig. 2), whereas in the case of pTH56, the presence of the C terminus of KpsE resulted in the localization of a significant amount of the \( \beta \)-lactamase fusion protein to the cytoplasmic membrane (Fig. 2). The exposure of this fusion protein in the periplasm was confirmed by protease K treatment of spheroplasts prior to Western blot analysis. In both cases the proteins were digested by protease K, whereas no degradation of cytoplasmic chloramphenicol acetyltransferase was observed indicating that the cytoplasmic membranes of the spheroplasts were intact throughout the experiment (data not shown). The observation that plasmid pTH56 conferred resistance to ampicillin at levels greater than 100 \( \mu \)g ml\(^{-1}\) and that the fusion was sensitive to carboxypeptidase digestion (data not shown) confirms that the \( \beta \)-lactamase fusion protein is functional and is anchored to the periplasmic face of the cytoplasmic membrane rather than being trapped in an unfolded state in the cytoplasmic membrane. Therefore, the C-terminal 29 amino acids of KpsE appear to be sufficient to localize \( \beta \)-lactamase, the archetypal soluble periplasmic protein, to the cytoplasmic membrane, suggesting an intimate interaction between the C-terminal part of KpsE and the cytoplasmic membrane. The lack of other Kps proteins in strain PA360 would indicate that this association is not dependent on the interaction between the C terminus of KpsE and other Kps proteins.
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| Strain          | Relevant genotype | Properties/Ref. |
|-----------------|-------------------|----------------|
| PA360           | serA1, rpsL       |                |
| MS101           | PA360, rpsL, K5+  | SerA\(^{+}\)P1 transductant of PA360 expressing a K5 capsule (42) |
| MSBB101         | MS101kpsS         | Deletion of kpsS (5) |
| MSCP101         | MSCP101ΔkfiC      | In-frame deletion of kfiC (43) |
| MSCA102         | MS101pp           | MS101 lacking Lpp (26) |
| MSFE101         | MS101kpsE         | In-frame deletion of kpsE (44) |
| MSTH101         | MSTH101kpsD       | In-frame deletion of kpsD (26) |
| BL21(DE3)omp8   | ompA, ompC, ompF, ompT, lamB |                |
| JM109(DE3)      | Δ(lac-proAB) [F traD36, proAB\(^{+}\), lacFZΔM15] (λcl 857, ind1, Sam7, nin5, lacUV5-T7 gene 1) |                |

**Chemical Cross-linking of KpsE and KpsD**—To determine the interactions between KpsE and other proteins involved in capsule transport, whole cells of strain MS101 (Table I) were treated with the chemical cross-linking agent DSP. Strains with specific defects either in capsule export or in the production of several major proteins in the bacterial envelope (see below) were also analyzed in this way. The cross-linked proteins were separated by SDS-PAGE followed by Western blot analysis using antisera specific to KpsE and KpsD.

In MS101 four major cross-linked complexes were detected after treatment with DSP when KpsE-specific antiserum was used (Fig. 3, panel A). The most predominant cross-linked product had a molecular weight consistent with that of a dimer of KpsE. The three other cross-linked products had molecular weights of 110, 130, and in excess of 200 kDa (Fig. 3, panel A). Continued incubation with DSP resulted in higher molecular weight complexes that were difficult to resolve by SDS-PAGE (data not shown). The same pattern of cross-linking was seen in mutants lacking either KpsD, KpsM, KpsT, KpsS, or outer membrane proteins Pal, Lpp, OmpT, LamB, OmpF, OmpA, and OmpC (data not shown) suggesting that these proteins were not being cross-linked to KpsE. Cross-linking using cells expressing kpsE as the only gene from the capsular cluster resulted in the same pattern of cross-linking to that observed in MS101 (data not shown) indicating that the probable dimerization of KpsE is not dependent upon the expression of the polysaccharide molecule or on the presence of other Kps proteins. In all cases the cross-linking could be reversed by incubation in the presence of 50 mM DTT (data not shown). The ability of the purified KpsE protein to dimerize in vitro (Fig. 3, panel B) would suggest that the cross-linked complex at 86 kDa is likely to be dimeric KpsE.

When KpsD-specific antiserum was used to analyze the cross-linked products, a complex of 67 kDa was detected in MS101 but not in MSFE101 or MSCA102 (Fig. 4). Strain MSCA102 is an isogenic lpp mutant of MS101 that lacks the 7-kDa major outer membrane lipoprotein Lpp. However, Western blot analysis using Lpp-specific antiserum failed to detect any cross-linking of Lpp to KpsD (data not shown). As such the 7-kDa protein that is cross-linked to KpsD cannot be Lpp, and its identity is as yet unknown.

**Proteinase K Sensitivity of KpsE and KpsD in the Presence and Absence of Other Proteins**—To demonstrate possible interactions between KpsE and KpsD, their proteinase K sensitivity in different mutant backgrounds was assessed by Western blot analysis following a time course proteolysis of E. coli spheroplasts. After 30 min of incubation with proteinase K, no proteolysis of KpsD was observed in spheroplasts from MS101 with limited proteolysis of KpsE after 30 min (Fig. 5, panels A and B). However, in MSFE101, where KpsE was absent from the bacterial envelope, KpsD was quickly degraded by proteinase K (Fig. 5, panel A). Likewise in strain MSTH101 (Table I) that lacks KpsD, KpsE was very sensitive to proteinase K (Fig. 5, panel B). In MSCA102, lacking Lpp, KpsD was reproducibly more sensitive to proteinase K digestion than in MS101 (Fig. 5, panel A), although compared with MSFE101, a significant proportion of KpsD was still resistant to proteolysis (Fig. 5, panel A). In contrast KpsE was rapidly degraded in MSCA102 (Fig. 5, panel B). In all cases there was no appreciable difference in the proteinase K sensitivity of periplasmic β-lactamase (data not shown) confirming that the spheroplasts of each strain were equally permeable to proteinase K.

**Detection of KpsD in the Bacterial Envelope**—In the E. coli Tol-PAL system the periplasmic protein TolB has been shown to interact with the main peptidoglycan-associated outer membrane proteins Pal, OmpA, and Lpp (28). This interaction results in the localization of TolB both in the periplasm and the outer membrane (29). To establish if KpsD also interacts with the cytoplasmic and outer membranes, strains MS101,
KpsE-specific antisera (panel A) or KpsD-specific antisera (panel B) following a time course digestion with proteinase K of spheroplasts. Samples were extracted at different time points during the digestion, precipitated with trichloroacetic acid, and subjected to SDS-PAGE and Western blot analysis.

MSFE101, MSCA102, and MSCP101 were fractionated by a two-step sucrose gradient to give cytoplasmic and outer membrane fractions, which together with the soluble periplasmic fraction were subjected to Western blot analysis (Fig. 6). In MS101, KpsD was detected in all three fractions with the majority of the protein in the periplasm (Fig. 6). In MSFE101 lacking KpsE, KpsD was detected exclusively as soluble protein in the periplasm with no protein detectable in either membrane fraction (Fig. 6). In MSCA102 KpsD was distributed evenly in both membrane fractions, with no soluble KpsD in the periplasmic fraction (Fig. 6). In strain MSCP101 (Table I), which has all of the polysaccharide export machinery but makes no polysaccharide, KpsD was detected exclusively in the outer membrane fraction (Fig. 6).

**DISCUSSION**

The sensitivity of KpsE to carboxypeptidase demonstrates that its C terminus must be exposed in the periplasm and not be located in the cytoplasm as suggested previously for the MPA family of proteins (8). In addition, the observation that the C-terminal 29 amino acids of KpsE appear to be sufficient to localize β-lactamase, the archetypal soluble periplasmic protein, to the cytoplasmic membrane suggests an intimate interaction between the C terminus of KpsE and the cytoplasmic membrane. The decreased sensitivity of KpsE to carboxypeptidase digestion when compared with β-lactamase could be a consequence of the close association of the C terminus of KpsE with the periplasmic face of the cytoplasmic membrane, which makes the protein less accessible to the carboxypeptidase enzymes. A similar situation has been observed with TolR, the C terminus of which interacts with the periplasmic face of the cytoplasmic membrane and which has reduced sensitivity to carboxypeptidase digestion (30).

A helical wheel plot (31) of the C-terminal 20 amino acids of KpsE demonstrates the presence of a putative amphipathic α-helix with a segregation of hydrophobic and hydrophilic residues on either side of the helix (data not shown). It has been demonstrated that the interactions between PBPs (32), PBP6 (33), and TolR (30, 34) and the periplasmic face of the cytoplasmic membrane are mediated by C-terminal amphipathic α-helices in which hydrophobic residues are buried in the membrane interior and the hydrophilic residues are located on the other side of the helix and interact with the aqueous environment (35). Therefore, it is likely that the interaction between the C terminus of KpsE and the periplasmic face of the cytoplasmic membrane may be also mediated by an amphipathic α-helix. Taken as a whole these data establish the topology of KpsE, the archetypal MPA protein, and remove any ambiguity about the location of the C termini of this family of proteins involved in the transport of capsular polysaccharides across the periplasmic space in Gram-negative bacteria.

The cross-linking data are consistent with KpsE functioning as a dimer, and the ability to dimerize was independent of the presence of other Kps proteins or the synthesis of capsular polysaccharide. The ability of KpsE to dimerize is likely to be mediated by a predicted coiled-coil structure present within KpsE (26). The cross-linking of KpsD to an unidentified 7-kDa protein is curious. The observation that this cross-linking was abolished in the absence of KpsE and Lpp suggested that KpsE and Lpp were required for this interaction. One must be cautious in assigning any specific role to Lpp in mediating this interaction, since mutations in lpp are known to have pleiotropic effects on the integrity of the periplasm (36), and this could explain the differences in the pattern of KpsD cross-linking in an lpp mutant. The failure to cross-link KpsE and KpsD to each other could reflect that these proteins interact very transiently and/or via the exported polysaccharide molecule, making it impossible to detect the interaction with DSP cross-linking. It has been demonstrated in type I protein secretion in Gram-negative bacteria that the interaction between components in the secretion pathway is mediated by the exported protein molecule (37, 38). Likewise, in the Tol system for the uptake of colicins, it has been suggested that the colicin itself may provide a bridge between its outer membrane receptor and periplasmic TolA protein (39). As such it is possible that a similar situation exists in the transport of group 2 capsular polysaccharides, whereby the exported polysaccharide molecule plays a key role in linking the different components of the transport pathway together. The alteration in the proteinase K sensitivity of KpsD and KpsE in the different mutant backgrounds probably reflects conformational changes in the KpsD and KpsE proteins as a result of the disruption of the transport process. This supports the notion that these proteins are likely to interact in the transport process.

Previously, KpsD has been assigned as a soluble periplasmic protein involved in group 2 polysaccharide transport (40). The identification of KpsD in both the cytoplasmic and outer mem-
brane fractions suggests that the trans-periplasmic export of group 2 capsular polysaccharides requires the interaction of KpsD with both membranes as well as being present in the periplasm. One interpretation is that KpsD may cycle between the membranes moving polymer across the periplasm in the process. In this model KpsD would engage with the polysaccharide as it is exported across the cytoplasmic membrane by the KpsMT transporter before docking with the outer membrane and transporting the polysaccharide across the periplasm. Alternatively, some form of trans-periplasmic export complex consisting of KpsD may be formed. Whichever model ever is correct it would appear that KpsE is vital to allow the interactions between KpsD and the respective membranes, and it is possible that KpsE functions to bring the membranes together to facilitate this process. The lack of soluble periplasmic KpsD in MSCA102 suggests that in the absence of Lpp the export process is disrupted such that either a trans-periplasmic complex cannot form or KpsD cannot cycle between the two membranes. Whether this reflects a specific role for Lpp in the transport process or is a consequence of pleiotropic effects of the lpp mutation on the integrity of the periplasm are as yet unresolved. The lack of detectable KpsD in either the periplasmic or cytoplasmic membrane fractions of strain MSCP101, which is unable to synthesize K5 polysaccharide, indicates that there is an absolute requirement for the polysaccharide molecule to be engaged in the translocation process for the trans-periplasmic export pathway to be operational. In the absence of polysaccharide the KpsD protein is detected in the outer membrane fraction. These results support the notion that the polysaccharide molecule itself may mediate interactions between proteins involved in the different stages of the transport process and extends our earlier observations that there is a direct coupling between polysaccharide biosynthesis and export (5).

The translocation of polysaccharide across the outer membrane is still an area of conjecture. Unlike group 1 polysaccharides, which have an outer membrane pore-forming protein (13), no such protein is encoded by group 2 capsule gene clusters. The observation that strain BL21(DE3)pomp(pPC6), which lacks OmpT, LamB, OmpF, OmpA and OmpC (41), is unable to synthesize K5 polysaccharide, indicates that there is a direct coupling between the KpsMT transporter before docking with the outer membrane and transporting the polysaccharide across the periplasm. Alternatively, some form of trans-periplasmic export complex consisting of KpsD may be formed. Whichever model ever is correct it would appear that KpsE is vital to allow the interactions between KpsD and the respective membranes, and it is possible that KpsE functions to bring the membranes together to facilitate this process. The lack of soluble periplasmic KpsD in MSCA102 suggests that in the absence of Lpp the export process is disrupted such that either a trans-periplasmic complex cannot form or KpsD cannot cycle between the two membranes. Whether this reflects a specific role for Lpp in the transport process or is a consequence of pleiotropic effects of the lpp mutation on the integrity of the periplasm are as yet unresolved. The lack of detectable KpsD in either the periplasmic or cytoplasmic membrane fractions of strain MSCP101, which is unable to synthesize K5 polysaccharide, indicates that there is an absolute requirement for the polysaccharide molecule to be engaged in the translocation process for the trans-periplasmic export pathway to be operational. In the absence of polysaccharide the KpsD protein is detected in the outer membrane fraction. These results support the notion that the polysaccharide molecule itself may mediate interactions between proteins involved in the different stages of the transport process and extends our earlier observations that there is a direct coupling between polysaccharide biosynthesis and export (5).

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The Transport of Group 2 Capsular Polysaccharides across the Periplasmic Space in Escherichia coli: ROLES FOR THE KpsE AND KpsD PROTEINS
Carlos Arrecubieta, Tansy C. Hammarton, Brendan Barrett, Sorujsiri Chareonsudjai, Nigel Hodson, David Rainey and Ian S. Roberts

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