Protein complexes are an intrinsic aspect of life in the membrane. Knowing which proteins are assembled in these complexes is therefore essential to understanding protein function(s). Unfortunately, recent high throughput protein interaction studies have failed to deliver any significant information on proteins embedded in the membrane, and many membrane protein complexes remain ill defined. In this study, we have optimized the blue native-PAGE technique for the study of membrane protein complexes in the inner and outer membranes of *Escherichia coli*. In combination with second dimension SDS-PAGE and mass spectrometry, we have been able to identify 43 distinct protein complexes. In addition to a number of well characterized complexes, we have identified known and orphan proteins in novel oligomeric states. For two orphan proteins, YhcB and YjdB, our findings enable a tentative functional assignment. We propose that YhcB is a hitherto unidentified additional subunit of the cytochrome *bd* oxidase and that YjdB, which co-localizes with the ZipA protein, is involved in cell division. Our reference two-dimensional blue native-SDS-polyacrylamide gels will facilitate future studies of the assembly and composition of *E. coli* membrane protein complexes during different growth conditions and in different mutant backgrounds.

It has been suggested that nearly all biochemical processes are performed by protein complexes (1). This is particularly true in cellular membranes, where many well characterized proteins assemble into complexes that carry out important tasks in energy generation, protein trafficking, and small molecule transport. Many uncharacterized proteins (“orphans”) are also predicted to be localized in cell membranes (2, 3), and it is probable that they also often assemble into complexes. Identifying the interacting partners of these proteins is critical to understanding their function.

Unfortunately, our knowledge of protein complexes in cellular membranes is poor, because membrane proteins are incompatible with commonly used protein interaction assays. High throughput studies on model systems (4–11) have therefore consistently disregarded membrane proteins (12). Although genetic tools specific for membrane proteins are available (12), the ubiquity of their roles is not known, and little is known about the assembly process. Robust and effective experimental assays are required to tackle the question of membrane protein assembly.

Blue native (BN)-PAGE (16, 17) offers an attractive proteomic solution for the analysis of membrane protein complexes. It has been successfully applied to respiratory complexes in mitochondria and *Paracoccus denitrificans* (18–24) and photosynthetic complexes of chloroplasts and *Synechocystis* (25, 26). BN-PAGE is also an attractive proteomic solution for basic expression profiling and can be used to complement traditional two-dimensional gel electrophoresis, since it does not discriminate against membrane proteins.

In this study, we have optimized the BN-PAGE methodology for the analysis of protein complexes in the *Escherichia coli* cell envelope. The *E. coli* cell envelope is an ideal model system for membrane protein studies as it contains a diverse array of biochemical functions, many of which are analogous to those found in more specialized membranes in eukaryotic organisms and pathogenic prokaryotes. However, despite being extensively studied as a model system, 36% of α-helical proteins in the inner membrane and many β-barrel proteins of the outer membrane remain orphans (3, 27). We report the identification of a large number of protein complexes, suggest oligomeric state and possible functions for a number of orphan proteins and provide reference two-dimensional BN/SDS-PAGE maps for both the outer and inner membranes.

**MATERIALS AND METHODS**

**Preparation of Membrane Vesicles**—The *E. coli* strain BL21 (DE3) pLysS (F-*ompT hsdS*<sub>B</sub> (r<sub>B</sub> m<sub>B</sub>)) gal dcm (DE3) pLysS) was grown at 37 °C in Luria Bertani broth. Cells were harvested at late log phase by centrifugation at 9,300 × *g*. Inner and outer membrane vesicles (IMVs and OMVs) were separated as described previously (28) with minor modifications. Following the removal of cell debris, the membrane fraction was layered on a two-step sucrose gradient (8.8:55% (w/v) sucrose) and centrifuged at 210,000 × *g* for 2.5 h. The membrane fraction was removed, and IMVs and OMVs were separated on a six-step sucrose gradient (37 °C) with 8.5% (w/v) sucrose and centrifuged at 210,000 × *g* for 2.5 h. The membrane fraction was removed, and IMVs and OMVs were separated on a six-step sucrose gradient as described previously (28). Membrane vesicles were resuspended in ACA750 buffer (750 mM *n*-aminocaproic acid, 50 mM Bis-Tris, 0.5 mM Na<sub>2</sub>EDTA, pH 7.0) and stored at −80 °C. Protein content was determined using the BCA assay (Pierce).

A related and elusive aspect of membrane biology pertains to how proteins are assembled into complexes following their insertion into the membrane. Although some folding chaperones have been identified for model substrates, the ubiquity of their roles is not known, and little is known about the assembly process. Robust and effective experimental assays are required to tackle the question of membrane protein assembly.

For two orphan proteins in novel oligomeric states. For two orphan proteins, YhcB and YjdB, our findings enable a tentative functional assignment. We propose that YhcB is a hitherto unidentified additional subunit of the cytochrome *bd* oxidase and that YjdB, which co-localizes with the ZipA protein, is involved in cell division. Our reference two-dimensional blue native-SDS-polyacrylamide gels will facilitate future studies of the assembly and composition of *E. coli* membrane protein complexes during different growth conditions and in different mutant backgrounds.

It has been suggested that nearly all biochemical processes are performed by protein complexes (1). This is particularly true in cellular membranes, where many well characterized proteins assemble into complexes that carry out important tasks in energy generation, protein trafficking, and small molecule transport. Many uncharacterized proteins (“orphans”) are also predicted to be localized in cell membranes (2, 3), and it is probable that they also often assemble into complexes. Identifying the interacting partners of these proteins is critical to understanding their function.

Unfortunately, our knowledge of protein complexes in cellular membranes is poor, because membrane proteins are incompatible with commonly used protein interaction assays. High throughput studies on model systems (4–11) have therefore consistently disregarded membrane proteins (12). Although genetic tools specific for membrane proteins are available (12), the ubiquity of their roles is not known, and little is known about the assembly process. Robust and effective experimental assays are required to tackle the question of membrane protein assembly.

Blue native (BN)-PAGE (16, 17) offers an attractive proteomic solution for the analysis of membrane protein complexes. It has been successfully applied to respiratory complexes in mitochondria and *Paracoccus denitrificans* (18–24) and photosynthetic complexes of chloroplasts and *Synechocystis* (25, 26). BN-PAGE is also an attractive proteomic solution for basic expression profiling and can be used to complement traditional two-dimensional gel electrophoresis, since it does not discriminate against membrane proteins.

In this study, we have optimized the BN-PAGE methodology for the analysis of protein complexes in the *Escherichia coli* cell envelope. The *E. coli* cell envelope is an ideal model system for membrane protein studies as it contains a diverse array of biochemical functions, many of which are analogous to those found in more specialized membranes in eukaryotic organisms and pathogenic prokaryotes. However, despite being extensively studied as a model system, 36% of α-helical proteins in the inner membrane and many β-barrel proteins of the outer membrane remain orphans (3, 27). We report the identification of a large number of protein complexes, suggest oligomeric state and possible functions for a number of orphan proteins and provide reference two-dimensional BN/SDS-PAGE maps for both the outer and inner membranes.

**MATERIALS AND METHODS**

**Preparation of Membrane Vesicles**—The *E. coli* strain BL21 (DE3) pLysS (F-*ompT hsdS*<sub>B</sub> (r<sub>B</sub> m<sub>B</sub>)) gal dcm (DE3) pLysS) was grown at 37 °C in Luria Bertani broth. Cells were harvested at late log phase by centrifugation at 9,300 × *g*. Inner and outer membrane vesicles (IMVs and OMVs) were separated as described previously (28) with minor modifications. Following the removal of cell debris, the membrane fraction was layered on a two-step sucrose gradient (8.8:55% (w/v) sucrose) and centrifuged at 210,000 × *g* for 2.5 h. The membrane fraction was removed, and IMVs and OMVs were separated on a six-step sucrose gradient (37 °C) with 8.5% (w/v) sucrose and centrifuged at 210,000 × *g* for 2.5 h. The membrane fraction was removed, and IMVs and OMVs were separated on a six-step sucrose gradient as described previously (28). Membrane vesicles were resuspended in ACA750 buffer (750 mM *n*-aminocaproic acid, 50 mM Bis-Tris, 0.5 mM Na<sub>2</sub>EDTA, pH 7.0) and stored at −80 °C. Protein content was determined using the BCA assay (Pierce).
Protein Complexes of the E. coli Cell Envelope

One-dimensional BN-PAGE—Approximately 100 μg of protein was used for each lane of the BN-PAGE. Protein complexes were solubilized at 4 °C for 20 min, in ACA750 buffer containing varying amounts of detergent. We trialed Triton X-100, n-dodecyl β-D-maltoside (DDM), and digitonin at concentrations ranging from 0.25 to 1.5% (w/v). Solubilization with 0.5% (w/v) DDM was determined to be the most effective, as evidenced by the number of complexes in the BN gel, their intensity, and their molecular mass range. Although solubilization with Triton X-100 and digitonin appeared to perform similarly for some complexes, the overall picture obtained was not as comprehensive as when DDM was used, and we decided not to optimize further with these detergents. Subsequent experiments were therefore performed using 0.5% (w/v) DDM. Following solubilization, samples were cleared by centrifugation at 264,000 × g for 30 min at 4 °C. The supernatant was added to 15 μl of G250 solution (5% (w/v) Coomassie G250 in ACA750 buffer) and loaded onto the gel. BN-PAGE was performed in a Hoefer SE-600 using a 4% stacking and a 5–15% separating gel (gel dimensions 14 cm × 16 cm × 1.5 mm). Buffers and gel compositions used were essentially prepared as described previously (16, 18), with a few modifications. The gel buffer contained 250 mM n-aminoacaproic acid, 25 mM Bis-Tris, pH 7.0; the cathode buffer contained 50 mM Tricine, 15 mM Bis-Tris, 0.05% (w/v) Coomassie G250, 0.03% (w/v) DDM, pH 7.0; and the anode buffer contained 50 mM Bis-Tris, pH 7.0. BN-polyacrylamide gels were destained (10% (v/v) acetic acid, 30% (v/v) methanol) and cut into lanes for use in the two-dimensional SDS-PAGE. High molecular mass markers were obtained from Amersham Biosciences (Sweden). The highest molecular mass band was corrected to be 880 kDa (29).

Two-dimensional SDS-PAGE—Gel strips obtained from the one-dimensional BN-PAGE were soaked for 20 min in equilibration buffer (2% (w/v) SDS, 250 mM Tris-HCl, pH 6.8). SDS-PAGE was performed in a Hoefer SE-600 using a 4% stacking and a 8–16% separating gel (gel dimensions 14 cm × 20 cm × 1.5 mm) according to standard protocols. Gels were stained with Coomassie (10% (v/v) acetic acid, 45% (v/v) methanol, 0.25% (w/v) Coomassie R250) or silver (30), and protein spots were detected using the PDQuest software (Bio-Rad).

Western Blotting—Prior to transfer, SDS gels were soaked for 40 min in transfer buffer (39 mM glycine, 48 mM Tris-HCl, pH 8.3, 0.037% (w/v) SDS, 20% (v/v) methanol). Proteins were transferred to Protran® Nitrocellulose membranes (Schleicher & Schuell) by electrophoresis at 15 V for 60 min using a Transfer-Blot Semidry Transfer cell (Bio-Rad). Detection was performed using a Fuji LAS 1000-Plus CCD camera and the ECL Advance Western blot developing kit (Amersham Biosciences).

Mass Spectrometry—Protein spots from SDS gels were excised manually. In-gel trypsin digestion was carried out according to Shevchenko et al. (31). Extracted peptides were mixed 1:1 with α-cyano-4-hydroxycinnamic acid (5 mg/ml) in 50% acetonitrile and 0.1% trifluoroacetic acid and left to air-dry after spotting on the MALDI target. MALDI-TOF analysis was performed on a Voyager-ED STR (Applied Biosystems), mass spectrometer. External calibration was done using the Sequazyme Peptide Mass Standards Kit (Applied Biosystems) and internal calibration based on trypsin autodigestion peaks when available (842.5094 and 2,211.1046 Da). Samples that could not be identified through fingerprinting were sequenced by tandem mass spectrometry (MS/MS) using the 4700 Proteomics analyzer equipped with TOF/TOF optics (Applied Biosystems). MS/MS analyses were carried out with air as collision gas using 1-kV collision energy, which is defined by the potential difference between the source acceleration voltage (8 kV) and the floating collision cell (7 kV). Mass spectra were analyzed using the Data Explorer and GPS Explorer software, and peptide masses were searched against the E. coli data base in SwissProt, using the Mascot search engine (available on the World Wide Web at www.matrixscience.com). Search parameters allowed for a mass accuracy of ±100 ppm, one missed cleavage of trypsin, oxidation of methionine, and carbamido-methylation of cysteine. All identified proteins were size-checked against the protein spot on the SDS gel. Aberrant migration caused by hydrophobicity (32) and predicted signal peptides was taken into account when considering molecular masses.

Results—Topography predictions were performed using the TMHMM predictor (available on the World Wide Web at www.cbs.dtu.dk/services/TMHMM/), and signal peptide predictions using SignalP 3.0 (available on the World Wide Web at www.cbs.dtu.dk/services/SignalP/).

RESULTS

Purification of Membrane Protein Complexes

In a first step, inner and outer membrane vesicles (IMVs and OMVs) were purified from E. coli using a six-step sucrose gradient (28). Immunodecoration with marker antibodies indicated that there was only minor cross-contamination between the different fractions (data not shown). Purification of protein complexes in an intact form (i.e. complete with all peripherally associated proteins) is largely dependent on the solubilization conditions used and can differ for various complexes. We sought to obtain a set of generic conditions that would be suitable for a large number of membrane protein complexes when resolved by BN-PAGE. By screening three commonly used detergents at different concentrations (see "Materials and Methods"), we were able to decide on a protocol using DDM, Fig. 1. This protocol resolved a large number of complexes from both the IMVs and OMVs in the molecular mass range from ~60 to >1,000 kDa.

To identify the individual proteins in each complex, the one-dimensional BN gels were analyzed in the second dimension by SDS-PAGE, Fig. 2. Complexes were consequently resolved into vertical “channels,” enabling visualization of the individual constituents. Proteins that had formed a complex in the BN gel, were identified by drawing a vertical line through the gel, and had a similar shape in the SDS gel (as a result of co-migration in the BN gel). The second dimension SDS-PAGE also enabled us to detect less abundant complexes that were hidden in the one-dimensional BN gel. Using the PDQuest two-dimensional analysis software on Coomassie- and silver-stained gels, we could detect 115 protein spots in the IMV gel and 62 protein spots in the OMV gel.

Protein Identification

We were able to identify 44 proteins from the IMVs: 35 by MALDI-TOF, an additional seven by MALDI-TOF/TOF, and two using available antibodies (Fig. 2A and TABLE ONE). 55% of the identified proteins were predicted by TMHMM (2) to be α-helical membrane proteins: 18 proteins with at least two transmembrane helices (TMHs) and six proteins with one TMH. All peptides identified were from the soluble domains of the membrane proteins. A further 34% of the identified proteins corresponded to soluble components of membrane protein complexes. In total, these proteins represent 34 distinct inner membrane protein complexes (see below). Five soluble proteins were also identified (11%), which may be peripherally associated with the membrane fractions or minor contamination.

From the OMVs, we were able to identify 12 proteins: 10 by MALDI-TOF and an additional two by MALDI-TOF/TOF (Fig. 2B and TABLE TWO). Of these, 67% were annotated as β-barrel type proteins, and 33% were annotated as lipoproteins associated with the inner leaflet of the outer membrane. These proteins represented nine different outer membrane protein complexes.
Protein Complexes in the Inner Membrane

Bioenergetic Complexes—The majority of the proteins solubilized from the IMVs were from complexes involved in bioenergetic processes. This is not surprising, since *E. coli* possesses a large, modular respiratory chain consisting of 15 primary dehydrogenases and 10 terminal reductases/oxidases (reviewed in Ref. 33). We were able to identify two terminal oxidases, three primary dehydrogenases, and the F$_{1}$-F$_{0}$-ATP synthase. All but one of these known complexes resolved intact in the gels and are described in detail below.

Succinate dehydrogenase is a known heterotetramer, SdhABCD, which trimerizes to form a functional complex with a predicted molecular mass of 355 kDa (34). From the SDS gel, we could identify the 66-kDa flavoprotein subunit (SdhA) and the 26-kDa iron-sulfur protein (SdhB) (Fig. 3A). Two other unidentified proteins that correspond in molecular mass to that predicted for SdhC (14 kDa) and SdhD (15 kDa) were also detected in the same channel. Based on the size of the succinate dehydrogenase complex in the BN gel (Fig. 1A) and the fact that all four constituent proteins were present in the SDS gel, we conclude that the (SdhABCD)$_{3}$ complex is intact.

The cytochrome bo$_{3}$ ubiquinol oxidase is also a known heterotetrameric complex, CyoABCD, with a predicted molecular mass of 145 kDa (35). In the SDS gel, we could identify CyoA (35 kDa) (Fig. 3B). Three other proteins in the same channel could not be identified, but their molecular masses correspond to that predicted for CyoB (apparent molecular mass of 45 kDa/predicted molecular mass 74 kDa), CyoC (22 kDa) and CyoD (12 kDa). Again, based on the size of the complex in the BN gel (Fig. 1A) and the fact that all four constituent proteins were present in the SDS gel, we conclude that the cytochrome bo$_{3}$ ubiquinol oxidase is intact.

Glucose dehydrogenase (Gdh) is a monomeric protein with five transmembrane segments (36). Although not in an oligomeric complex,
...we could identify the Gdh monomer at the predicted molecular mass of 85 kDa in both the BN and SDS gels (Fig. 2A).

Accurate molecular mass determination of membrane protein complexes using BN-PAGE is possible if the mass of extra Coomassie and detergent is taken into account (37). We used the SdhABCD, CyoABCD, and Gdh proteins to generate a standard curve that could be used to estimate the molecular mass of other membrane protein complexes. This standard curve more accurately reflected the predicted molecular mass of membrane protein complexes than commercial markers generated from soluble proteins (supplemental Fig. 1A).

In addition to the cytochrome \( b_03 \) ubiquinol oxidase, we also detected the cytochrome \( bd \) ubiquinol oxidase. This complex is reported to be a heterodimer, CydAB, with a molecular mass of \( \sim100 \) kDa (38). Unexpectedly, we identified CydA (58 kDa) and CydB (42 kDa) along with a third protein, YhcB (15 kDa), in a complex with an estimated molecular mass of \( \sim118 \) kDa (Fig. 3B). YhcB therefore appears to be a hitherto unidentified subunit of the cytochrome \( bd \) ubiquinol oxidase.

The NADH dehydrogenase was also identified but was unfortunately fragmented by our solubilization conditions. This is not surprising, since it has a pronounced detergent susceptibility and is known to fragment at \( pH > 6.5 \) when solubilized in DDM (23, 39). Since the BN-PAGE is performed at \( pH 7.0 \), the NADH dehydrogenase was resolved as two subcomplexes. A 404-kDa subcomplex contained the NuoCD protein, and a 135-kDa complex contained the 90-kDa NuoG subunit (Fig. 2A).

Based on the size of the subcomplexes in the BN-PAGE, other subunits are presumed to also be present. Attempts to run the gel at \( pH 6.5 \) were not successful (data not shown).

The \( F_1-F_0 \)-ATP synthase contains eight subunits arranged in two subcomplexes: \( F_1 (\alpha_x, \beta_y, \gamma, \delta, \epsilon) \) and \( F_0 (a, b_x, c_{10-14}) \) (reviewed in Ref. 40). We detected all eight constituent subunits in a complex with an...
estimated molecular mass of ~493 kDa. We were able to identify subunit c (11 kDa), subunit b (18 kDa), the α-subunit (54 kDa), the β-subunit (51 kDa), and the γ-subunit (29 kDa) (Fig. 3A). Other protein spots were detected that corresponded in molecular mass to subunit a (30 kDa), the δ-subunit (19 kDa), and the ε-subunit (15 kDa), and we conclude that the F1-F0-ATP synthase is intact. Two subcomplexes were also found at ~276 and ~230 kDa (Fig. 2A), which correspond to the F1 and F0 subcomplexes, respectively.

**Complexes Involved in the Biogenesis of the E. coli Inner Membrane**—A number of identified proteins formed complexes that play a role in the biogenesis of the inner membrane. Uroporphyrin-III C-methyltransferase (HemX) is a single spanning inner membrane protein that regulates the activity of NAD(P)H:glutamyl-tRNA reductase (HemA) in the tetrapyrrole biosynthesis pathway (41). HemX (43 kDa) was identified in a 208-kDa complex in the BN gel (Fig. 2A and supplemental Fig. 2). Since no additional proteins could be detected in the same channel, we conclude that HemX exists as a homo-oligomer, possibly a homopentamer.

The peptidyl-prolyl isomerase (PpiD) is another inner membrane protein with one predicted membrane-spanning region. It contains a large periplasmic domain that is postulated to facilitate the folding of outer membrane proteins (42). To date, there are no reports on the oligomeric state of PpiD. We resolved the 68-kDa PpiD protein in two higher molecular mass complexes with estimated molecular masses of 156 and 104 kDa (Fig. 2A and supplemental Fig. 2). Since neither of the PpiD complexes had any interacting partners in the SDS gel, we conclude that PpiD is present as both a trimer and a dimer.

The 36-kDa ZipA protein is an essential component of the septal ring structure, mediating cell division via an interaction with the Z ring (43). We identified ZipA in a channel with two other proteins (Fig. 4A). Although the 24-kDa interacting partner could not be identified, we could identify YjdB (61 kDa) as an interacting partner to ZipA. Although
Proteins identified by mass spectrometry from inner membrane vesicles

Proteins identified by mass spectrometry are assigned a molecular weight search (MOWSE) score, which is expressed as a protein score for a peptide mass fingerprint or as ion score in an MS/MS data base search. Total ion scores are calculated from weighted ion scores for individual peptides that are matched to a given protein. Scores greater than the mascot significance level (MSL) calculated for each search indicate that the protein identifications are considered statistically nonrandom at 95% confidence interval. A total ion score confidence interval calculation (C.I.) is calculated to allow comparison of searches with varying MSL. The closer the C.I. value is to 100%, the more likely the protein is correctly identified. The number of peptides used for each identification and their coverage of the total protein are indicated. The number of TMHs predicted by TMHMM for each protein is also indicated.

| Protein Complexes of the E. coli Cell Envelope | Protein | Swissprot no. | Gene name | Protein score | Total ion score | Total ion C.I. % | Predicted Mₚ | Peptides matched | Sequence coverage | TMHMM prediction |
|-----------------------------------------------|---------|--------------|-----------|--------------|----------------|----------------|-------------|-----------------|------------------|------------------|
| ATP synthase a-chain                          | P00822  | atpA         |           | 119          | 55,416         | 9             | 25          | 0               |                  |                  |
| ATP synthase δ-chain                          | P00824  | atpD         |           | 79           | 50,220         | 8             | 24          | 0               |                  |                  |
| ATP synthase γ-chain                          | P00837  | atpG         |           | 53           | 31,671         | 5             | 20          | 0               |                  |                  |
| ATP synthase F₈ sector, subunit b             | P00859  | atpE         |           | 66           | 17,310         | 4             | 25          | 1               |                  |                  |
| ATP synthase F₈ sector, subunit c             | P00859  | atpH         |           | 81           | 99,908         | 8,119         | 1           | 2               |                  |                  |
| NADH-quinone oxidoreductase chain C/D        | P33599  | nuoCD        |           | 89           | 68,859         | 8             | 17          | 0               |                  |                  |
| PTS system mannose-specific EIAB component   | P18186  | manX         |           | 132          | 34,894         | 9             | 34          | 0               |                  |                  |
| Succinate dehydrogenase flavoprotein subunit | P10444  | sudB         |           | 94           | 6,500          | 6             | 13          | 0               |                  |                  |
| Succinate dehydrogenase iron-sulfur protein  | P37014  | uisB         |           | 69           | 27,379         | 5             | 28          | 0               |                  |                  |
| Multidrug resistance protein, MdrF            | P17675  | yhiV         |           | 55           | 111,617        | 7             | 10          | 11              |                  |                  |
| Acriflavine resistance protein B, AcrB        | P31224  | acrB         |           | 96           | 113,615        | 14            | 13          | 11              |                  |                  |
| Mechanosensitive channel of small conductance, MacS | P11666 | yggB         |           | 101          | 30,877         | 6             | 34          | 3               |                  |                  |
| Polyadenylate phosphorylase                   | P02055  | pnp          |           | 101          | 77,110         | 14            | 17          | 0               |                  |                  |
| Uroporphyrinogen III methylase                | P04127  | hemX         |           | 112          | 42,937         | 11            | 35          | 1               |                  |                  |
| Peptidylproplyl-cis-trans-isomerase D         | P77241  | ppiD         |           | 175          | 68,108         | 14            | 33          | 1               |                  |                  |
| Maltose transport complex, ATP-binding subunit| P02914  | malK         |           | 67           | 41,136         | 5             | 22          | 0               |                  |                  |
| Cytochrome b₃ ubiquinol oxidase subunit II    | P18400  | cyoA         |           | 84           | 34,756         | 10            | 33          | 3               |                  |                  |
| Putative ATP-binding component of transport system | P31220 | yhiB         |           | 83           | 26,652         | 12            | 63          | 0               |                  |                  |
| NADH-dehydrogenase I subunit                | P35602  | nooG         |           | 229          | 101,078        | 18            | 29          | 0               |                  |                  |
| Protein export membrane protein, SecD         | P19675  | secD         |           | 202          | 66,648         | 13            | 30          | 6               |                  |                  |
| Galactosylpermease IC component              | P51789  | grnC         |           | 49           | 97,817         | 48            | 201         | 2               |                  |                  |
| Cytochrome of terminal oxidase, polyprotein subunit I | P1026 | cyoA         |           | 192          | 58,338         | 15            | 21          | 9               |                  |                  |
| Cytochrome of terminal oxidase subunit II    | P11027  | cyoB         |           | 95           | 42,293         | 11            | 27          | 8               |                  |                  |
| Hypothetical protein, YbcB                   | P39436  | ybcB         |           | 84           | 15,230         | 5             | 55          | 1               |                  |                  |
| Acriflavine resistance protein A precursor, AcrA | P31223 | acrA         |           | 115          | 42,228         | 11            | 29          | 0               |                  |                  |
| Multidrug resistance protein MdrE precursor  | P37636  | yhdI         |           | 78           | 41,310         | 7             | 32          | 0               |                  |                  |
| Hypothetical protein, YidC                   | P25714  | yidC         |           | 66           | 61,557         | 5             | 20          | 4               |                  |                  |
| Glutamine transport ATP-binding protein GlnQ | P10346  | glnQ         |           | 82           | 26,771         | 6             | 27          | 0               |                  |                  |
| Dihydrolipoamide dehydrogenase               | P00391  | ddiH         |           | 69           | 50,811         | 5             | 13          | 0               |                  |                  |
| Cation/acetate symporter, ActP                | P32705  | ykJG         |           | 29           | 98,451         | 59            | 82          | 13              |                  |                  |
| Sodium/proline symporter                     | P07117  | putP         |           | 49           | 99,957         | 54            | 176         | 2               |                  |                  |
| Hypothetical protein, YidB                   | P30845  | yidB         |           | 42           | 99,796         | 61            | 496         | 1               |                  |                  |
| Cell division protein, ZipA                  | P77173  | zipA         |           | 83           | 36,323         | 2             | 50          | 2               |                  |                  |
| Hypothetical protein, YidP                   | P32678  | yidP         |           | 151          | 66,625         | 15            | 25          | 5               |                  |                  |
| Hypothetical protein, YidG                   | P77804  | yidG         |           | 191          | 54,665         | 12            | 36          | 0               |                  |                  |
| Chain length determinant protein              | P74672  | wzzB         |           | 60           | 36,432         | 6             | 21          | 2               |                  |                  |
| Acridine protein glucose dehydrogenase       | P19877  | gad          |           | 132          | 87,091         | 15            | 19          | 0               |                  |                  |
| Chaperone protein, DnaK                      | P04475  | dnaK         |           | 97           | 68,998         | 9             | 18          | 0               |                  |                  |
| Chaperone protein, HspG                       | P10413  | hspG         |           | 60           | 73,378         | 6             | 10          | 0               |                  |                  |
| Glycine betaine/c-proline transport ATP-binding protein | P13756 | proV         |           | 58           | 44,192         | 7             | 20          | 0               |                  |                  |
| Hypothetical protein, YacC                   | P19677  | yacC         |           | 42           | 99,773         | 11            | 747         | 1               |                  |                  |
| Hypothetical protein, YagU                   | P72762  | yagU         |           | 69           | 23,123         | 5             | 26          | 3               |                  |                  |

**TABLE ONE**

Proteins identified by mass spectrometry from inner membrane vesicles
all three proteins clearly co-localized, the molecular mass of the complex was estimated at only ~91 kDa in the BN gel. However, both ZipA and YjdB also ran aberrantly in the SDS gel (see also Ref. 43), and we conclude that the 91-kDa complex contains ZipA-YjdB and an unidentified 24-kDa protein.

The chain length determinant protein (WzzB) is responsible for the degree of O-antigen polymerization during lipopolysaccharide biosynthesis. Cross-linking experiments indicate that WzzB is able to dimerize and subsequently form higher homo-oligomers (44). We identified the 36-kDa WzzB protein in a complex of ~70 kDa (Fig. 2A and supple-

![Figure 3](image-url)  
**FIGURE 3.** Bioenergetic complexes of the E. coli inner membrane resolved by BN- and SDS-PAGE and stained with Coomassie. A, succinate dehydrogenase and the $F_{1}$$F_{0}$-ATP synthase. B, cytochrome $b_6$ and $bd$ oxidases. Proteins identified by mass spectrometry are indicated, and proteins inferred from their position in the gel are marked with an asterisk. Molecular masses of complexes in the BN gel were calculated from an internal standard curve (see “Materials and Methods”) and are indicated at the top of the gel. Molecular mass markers for the SDS gel are indicated to the right.

![Figure 4](image-url)  
**FIGURE 4.** Protein complexes involved in the biogenesis of the E. coli inner membrane. A, “ZipA-YjdB-unknown protein” complex resolved by BN/SDS-PAGE and stained with Coomassie. B–D, complexes resolved by BN- and SDS-PAGE and probed with antibodies to YidC, SecA, and LepB. Molecular masses of specific complexes in the BN gel were calculated from an internal standard curve (see “Materials and Methods”) and are indicated at the top of the gel. Molecular mass markers for the SDS gel are indicated to the right.

| Protein | Swissprot no. | Gene name | Protein score | Total ion score | Total ion C.I.% | Predicted Mr | Peptides matched | Sequence coverage |
|---------|--------------|-----------|---------------|----------------|----------------|-------------|----------------|------------------|
| Unknown protein from two-dimensional-PAGE | P39170 | yaeT | 150 | 90,362 | 90% | 14 | 24 |
| Lipoprotein-34 | P21167 | nlpB | 64 | 36,687 | 80% | 6 | 8 |
| Hypothetical UPF0169 lipoprotein YfIO precursor | P77146 | yfIO | 92 | 27,680 | 80% | 7 | 29 |
| Outer membrane protein TolC precursor | P02930 | tolC | 74 | 53,574 | 80% | 8 | 21 |
| Maltoporin precursor | P02943 | lamB | 83 | 49,746 | 80% | 8 | 31 |
| Organic solvent tolerance protein precursor, OstA | P31554 | imp | 133 | 89,481 | 80% | 18 | 23 |
| General diffusion porin, OmpF | P02931 | ompF | 185 | 39,309 | 80% | 11 | 43 |
| Outer membrane protein Slp precursor | P37194 | slp | 136 | 100 | 94,121 | 20 | 23 |
| Major outer membrane lipoprotein precursor, Lpp | P02937 | null | 61 | 8,186 | 60% | 6 | 48 |
| Long-chain fatty acid transport protein precursor, FadL | P10384 | fadL | 63 | 48,607 | 60% | 6 | 19 |
| Outer membrane protein A, OmpA | P02934 | ompA | 146 | 37,292 | 60% | 10 | 34 |
| MltA-interacting protein precursor, MipA | P77486 | mipA | 27 | 94,121 | 60% | 27,831 | 3 |

### Table Two

Proteins identified by mass spectrometry from outer membrane vesicles

Proteins identified by mass spectrometry are assigned a MOWSE score, expressed as a protein score for a peptide mass fingerprint or as ion score in an MS/MS data base search. Total ion scores are calculated from weighted ion scores for individual peptides that are matched to a given protein. Scores greater than the MSL calculated for each search indicate that the protein identifications are considered statistically nonrandom at 95% confidence interval. A total ion score C.I.% is calculated to allow comparison of searches with varying MSL. The closer the C.I.% value is to 100%, the more likely the protein is correctly identified. The number of peptides used for each identification and their coverage of the total protein are indicated.
Protein Complexes of the E. coli Cell Envelope

mental Fig. 2). Since no interacting proteins could be detected, we conclude that WzzB is present as a dimer.

Protein translocation and insertion via the SecYEG translocon is facilitated by the heteromeric SecDF-YajC-YidC complex (45) and the SecA motor ATPase (reviewed in Refs. 46 and 47). Using antibodies, we could confirm the presence of YidC (61 kDa) in three different complexes (Fig. 4B). The highest molecular mass complex was estimated to be ∼141 kDa and localized to the same channel on the gel where we had previously identified the 66-kDa SecD (Fig. 2A). We found an abundant form of YajC (11 kDa) in a separate complex with an estimated molecular mass of 56 kDa (Fig. 2A and supplemental Fig. 2). Since we could not find any interacting proteins in this complex, we conclude that YajC is present as a homo-oligomer in excess to the other components of the translocon.

The oligomeric state of SecA is also controversial; both monomeric (48) and dimeric forms (49–51) have been suggested to be physiologically active. Using soluble molecular mass markers to estimate molecular mass, we observed that SecA resolved at ∼200 kDa in the BN gel (Fig. 4C), corresponding exactly to a dimer.

Many translocated proteins contain cleavable targeting signals that must be removed by the 35-kDa leader peptidase LepB (52). Although leader peptidase is assumed to be in close proximity to the SecYEG/DFYajC-YidC protein translocation machinery, there are no reports alluding to its existence in an oligomeric complex. We resolved LepB in a complex corresponding to ∼60 kDa in the BN gel (Fig. 4D). Since the LepB complex is not abundant, we could not identify interacting partners, but the size of the complex in the BN gel suggests that LepB may form a dimer in the inner membrane.

Membrane Transport Complexes—Data base annotations suggest that transport proteins represent the largest functional class of proteins in the E. coli inner membrane; 33% of predicted α-helical membrane proteins are annotated as influx transporters, and 7% are annotated as efflux transporters (3). We identified two phosphotransferase system transporters, three ATP binding cassette (ABC) transporters, two transporters of the major facilitator superfamily, and two multidrug efflux transporters.

The mannose phosphotransferase system transporter (EIIman) is composed of three subunits, ManX (IIABman), ManY (IICman), and ManZ (IIDman), which are reported to assemble in a 2:1:2 stoichiometry, corresponding to a molecular mass of 160 kDa (53). We identified ManX as a weakly stained protein spot from a complex with an estimated molecular mass of ∼370 kDa (Fig. 5). Since the complex was not abundant, we could not detect any interacting proteins; however, the size of the complex in the BN gel indicates that the proposed stoichiometry is not complete.

The galactitol phosphotransferase system transporter (EIIGat) is also composed of three subunits, GatA (IIAGat), GatB (IIBGat), and GatC (IICGat). These three subunits assemble in a 1:1:2 stoichiometry to form a complex with a predicted molecular mass of ∼122 kDa (54). We identified the 48-kDa GatC subunit in a complex with an estimated molecular mass of 127 kDa (Fig. 5). Based on the size of the complex in the BN gel, we conclude that the EIIGat complex is intact.

The maltose ABC transporter is composed of three subunits MalFGK2 and has a predicted molecular mass of 171 kDa (55). We identified the cytoplasmic ATP binding cassette MalK in a complex of ∼195 kDa (Fig. 5). Since the complex was not abundant, no protein spots corresponding to Mal or MalG were detected on the Coomassie-stained gel. Based on the size of the complex in the BN gel, we suggest that the maltose ABC transporter MalFGK2 is intact.

Glutamine uptake in the cell is facilitated by the glutamine ABC transporter, GlnP2Q2 (56). We identified the ATP binding cassette protein, GlnQ (27 kDa), in a complex with an estimated molecular mass of ∼113 kDa (Fig. 5). Another protein, corresponding in molecular mass to the integral membrane GlnP (24 kDa) was in the same complex but could not be identified. We conclude that the GlnP2Q2 complex was intact.

Glycine betaine uptake in the cell is undertaken by the ABC transporter, ProV2W2. We identified the ATP binding cassette protein ProV (27 kDa), in a complex with an estimated molecular mass of ∼65 kDa (Fig. 2A). Again, accurate molecular mass measurement is not possible in this molecular mass range, but it appears as though the dimeric ATP binding cassette ProV2 has detached from the membrane-embedded ProW dimer during solubilization.

Both the 54-kDa sodium/proline symporter (PutP) and the 59-kDa acetate permease (YicG/ActP) are major facilitator superfamily transporters. For neither protein are we aware of any reports alluding to their existence in an oligomeric complex. However, both proteins seemed to be dimeric (1) they resolved at higher molecular masses in the BN gel (80 and 93 kDa, respectively), and 2) no interacting partners could be detected in the SDS gel (Fig. 2A and supplemental Fig. 2).
AcrAB is the main multidrug efflux transporter in E. coli. In combination with the outer membrane protein TolC (see below), it forms a contiguous channel across the inner and outer membranes and can extrude a wide variety of toxic compounds. All three subunits (AcrA, AcrB, and TolC) have been shown to independently form trimers (57–59), which assemble during drug extrusion. We identified AcrB (113 kDa) in a complex of ~342 kDa and AcrA (42 kDa) in a separate complex of ~115 kDa, both corresponding to homotrimeric complexes (Fig. 5).

The MdtEF multidrug efflux transporter (formerly YhiUV) was previously identified by sequence homology to AcrAB and has also been shown to have broad substrate specificity (60). Like the AcrAB complexes, MdtE and MdtF were identified in two separate complexes of ~343 and ~114 kDa, corresponding to homotrimers (Fig. 5).

### Ion Channels

The mechanosensitive channel of small conductance MscS, is a homoheptameric ion channel that responds to membrane stretching and depolarization (61). We identified the 31-kDa MscS protein (encoded by yddB) in a complex of ~242 kDa (Fig. 2A and supplemental Fig. 2). No other proteins were detected in the channel, and we conclude that (MscS)7 was intact.

### Orphan Proteins

We identified six orphan proteins, for which no clear function has been proposed in the literature. Four of these proteins (YhcB, YjdB, YjIP, YagU) were predicted by TMHMM to be integral membrane proteins, one (YhbG) was annotated as a soluble component of a membrane protein complex, and one other (YdgA) was predicted to be soluble. As discussed above, YhcB co-localized with the cytochrome bd oxidase complex and YjdB with ZipA.

YjIP has been proposed to play a role in the pathogenesis of bacterial meningitis (62). It is a 67-kDa protein with five predicted TMHs and a 407-amino acid periplasmic C-tail. We identified YjIP along with an unidentified 36-kDa protein in a complex with an estimated molecular mass of ~81 kDa (Fig. 2A and supplemental Fig. 2).

YagU has three predicted TMHs and no known biochemical function. We identified YagU (22 kDa) at ~47 kDa in the BN (Fig. 2A and supplemental Fig. 2) and conclude that it is present as a dimer.

The 54-kDa YdgA protein is not predicted to be a membrane protein but has a potential signal sequence, suggesting that it functions in the periplasmic space. Its presence in our IMVs suggests that it may be tethered to the membrane. Since YdgA is a soluble protein, we used soluble molecular mass markers to estimate its molecular mass in the BN gel. YdgA resolved at ~106 kDa in the BN gel (Fig. 2A and supplemental Fig. 2), and we conclude that it is present as a dimer.

YhbG is a 27-kDa protein that is annotated in SwissProt as having homology to the ATP binding cassette of an ABC transporter. We identified YhbG in a complex of ~140 kDa (Fig. 2A and supplemental Fig. 2). YhbG stained weakly, and unfortunately no interacting partners could be detected.

### Protein Complexes in the Outer Membrane

By far the most abundant protein complex of the outer membrane is the trimeric general diffusion porin, OmpF3 (Fig. 2B). We identified the 37-kDa OmpF in a predominant complex that corresponds in molecular mass to a trimer in the BN gel (relative to the soluble markers), and we conclude that (OmpF)3 is intact. Other OmpF forms detected in the SDS gel are a result of smearing in the BN-gel or incomplete denaturation in the SDS gel.

Maltoporin (LamB) is another β-barrel protein that assembles into a trimeric complex (63). We identified the 47-kDa LamB protein in a complex that corresponds in molecular mass to a trimer (again relative to soluble markers) (Fig. 2B), and we conclude that LamB3 is intact. Drug extrusion through the cell envelope is facilitated by the TolC protein, which functions in conjunction with the multidrug efflux transporters of the inner membrane (see above). The TolC trimer spans both the outer membrane and the periplasmic space (57). We identified the 50-kDa TolC in a complex that corresponded in molecular mass to the trimer (again relative to soluble markers) (Fig. 2B), and we conclude that (TolC)3 is intact.

As for inner membrane proteins, accurate molecular mass estimation of the outer membrane β-barrel type proteins by BN-PAGE is difficult, due to the fact that the native conformations contain large pores that cause the complexes to have a larger apparent molecular mass. To more accurately predict the molecular mass of other β-barrel-containing protein complexes, we generated a standard curve using the apparent molecular masses of the OmpF, LamB, and TolC complexes. The molecular mass of other protein complexes were calculated using this standard curve (supplemental Fig. 1B).

Recently, Wu et al. (64) reported a 178-kDa complex containing a β-barrel protein (YaeT) and three outer membrane lipoproteins (Yfgl, NlpB, and YfoA) that is involved in the biogenesis of outer membrane proteins. We identified YaeT (85 kDa), NlpB (34 kDa), and YfoA (26 kDa), along with a fourth protein of 40 kDa, in a complex, corresponding to exactly 178 kDa in the BN gel (Fig. 2B). Based on its size in the SDS gel, we conclude that the unidentified 40-kDa protein is Yfgl, and that the complex is intact.

The Imp (increased membrane permeability) protein is also proposed to play a role in outer membrane biogenesis (65), and it has been postulated that it may interact with Yfgl (66). We resolved the 85-kDa Imp protein at 117 kDa in both the BN and SDS gels (Fig. 2B). The apparent molecular mass in the SDS gel is larger than expected (65) and suggests that the β-barrel has not been completely denatured by the SDS. Based on the size of the protein in the BN and SDS gels and the fact that no other interacting partners could be detected, we conclude that Imp is monomeric. Although it is still possible that Imp interacts with Yfgl, our observations indicate that it solubilizes as a separate entity in DDM.

Both the ubiquitous outer membrane protein A (OmpA) and the long chain fatty acid transporter (FadL) are reported to function as monomeric β-barrels (67, 68). We identified OmpA and FadL (Fig. 2B) at the anticipated molecular masses of 30 and 33 kDa in the SDS gel; however, in contrast to the literature, both were estimated to be dimers from their molecular mass in the BN gel (OmpA at 74 kDa, FadL at 77 kDa).

The stationary phase lipoprotein (Sip) is tethered to the outer membrane and induced during carbon starvation (69). We identified Sip as a smeared band in the SDS gel (Fig. 2B), indicating that it forms higher order oligomers.

The major outer membrane lipoprotein (Mul) is also a lipoprotein, which interacts with the peptidoglycan layer and contributes to cell envelope integrity. Previous reports indicate that Mul forms a homotrimer (70); however, our data indicate that it forms higher oligomers. We detected the 8-kDa Mul in a complex with an estimated molecular mass of 87 kDa (Fig. 2B). Other less abundant, higher molecular mass complexes were also detected.

### DISCUSSION

In Gram-negative bacteria, there is a clear distinction between integral membrane proteins in the cell envelope; α-helical type proteins are localized to the inner membrane, and β-barrel type proteins are local-
ized to the outer membrane. Bioinformatic prediction suggests that the α-helical proteins constitute 25–30% of the E. coli proteome (2) and β-barrel type proteins constitute 2–3% (71–73). Many soluble proteins are also tethered to the membranes through lipid moieties, hydrophobic patches, or charge interactions or in membrane protein complexes, so it is not unreasonable to suggest that 30–40% of all E. coli proteins may function in the membranes of the cell envelope. Understanding how these proteins assemble and interact is a fundamental question of membrane biology but unfortunately one that is difficult to address.

In this study, we report a first step toward proteome-wide characterization of membrane protein complexes in the cell envelope of E. coli. By optimizing the BN-PAGE technique for the study of purified inner and outer membranes, we have been able to identify and partially characterize 43 protein complexes and identify a number of novel protein/protein interactions. Although many of our findings have simply confirmed the existence of previously known complexes, the current analysis is a step forward in that it requires only two gels (one for each membrane). As reference maps, these gels will provide a much needed platform for assembly and expression profiling studies of membrane proteins, complementing traditional two-dimensional gel electrophoresis techniques (74, 75). Although other gel-based techniques have been developed for the study of membrane proteins (32, 76, 77), they do not resolve whole complexes, and we believe that the BN-PAGE system is currently the most suitable for the study of membrane protein complexes.

One goal of this study was to assign function to orphan proteins by identifying physical interactions with proteins of known function. The approach has so far been successful for two orphan membrane proteins, YhcB and YjdB. YhcB co-localizes with both CydA and CydB and can be tentatively assigned as a new subunit of the cytochrome bd oxidase. Its function as part of the cytochrome bd ubiquinol oxidase was not previously known and could not have been anticipated by genome organization; the yhcB gene exists as a single transcription unit in a disparate region of the genome from the cydAB operon.

YjdB is another orphan protein which we can now speculate on the function of. YjdB co-localizes with the ZipA protein, which tethers the FtsZ ring to the inner membrane at an early stage of cell division (43). As a result of the FtsZ-ZipA interaction, a number of other cell division proteins are recruited to the septal ring (78, 79). Our data therefore implicate YjdB in this process, although its exact role is not known. It has also recently been speculated that YjdB (renamed EptA) may play a role in lipopolysaccharide synthesis, although it has not been characterized enzymatically (80). Clearly, the ZipA-YjdB complex plays a very central role in cell division, coordinating both septal ring formation and lipopolysaccharide synthesis. Previously, all known interactions of ZipA were of a dynamic nature, occurring only during septal ring formation. Our observation is the first report that points to the existence of a stable complex. Unfortunately, the third protein in this complex could not be identified but will have to be considered when evaluating the functions of both ZipA and YjdB.

Although other orphan proteins were unidentified, we were unable to obtain enough information to assign a function. YjpG and YhbG were identified in higher molecular mass complexes, but unfortunately interacting proteins either could not be detected or could not be identified. For many membrane proteins with a biochemically characterized function, little is known about their oligomeric state in the membrane. We find that uroporphyrinogen-III C-methyltransferase (HemX), the peptidyl-prolyl isomerase (PpiD), and the stationary phase lipoprotein (Slp) all form homo-oligomeric complexes. The ubiquitous outer membrane protein (OmpA), the long chain fatty acid transporter (FadL), two orphan proteins (YagU and YdgA), and two major facilitator superfamily transporters (PutP and YjgC/ActP) form homodimers. For a few other proteins we suggest that the previously reported stoichiometry may be incomplete. The mannose phosphotransferase system transporter (Ell3), the leader peptidase (LepB), and the major outer membrane lipoprotein (Mull) all form complexes in the BN gel with higher molecular mass than anticipated. Unfortunately, the low abundance of these complexes in our gels precluded us from resolving the stoichiometry.

In conclusion, we present a first characterization of protein complexes from the E. coli cell envelope. Our reference inner and outer membrane panels will facilitate proteome level studies of complex formation and disassembly and the identification of new membrane protein complexes.

Acknowledgment—We thank Louise Baars (Stockholm University) for expert technical advice.

REFERENCES

1. Alberts, B. (1998) Cell 92, 291–294
2. Krogg, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. L. (2001) J. Mol. Biol. 310, 557–580
3. Dall, D. O. O., Rapp, M., Granath, E., Melen, K., Drew, D., and von Heijne, G. (2005) Science 308, 1321–1323
4. Utz, P., Giust, L., Cagnac, G., Mansfield, T. A., Judson, R. S., Narayan, V., Lockshon, D., Srivastava, M., Pochart, P., Qureshi-Eumi, A., Li, Y., Godwin, B., Conover, D., Kahlbesch, T., Vijayadamodar, G., Yang, M., Johnston, M., Fields, S., and Rothberg, J. M. (2000) Nature 403, 623–627
5. Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jensen, R., Bidlingmaier, S., Houfek, T., Mitchell, T., Miller, P., Dean, R. A., Gerstein, M., and Snyder, M. (2001) Science 293, 2101–2105
6. Rair, J. C., Selig, L., De Reuse, H., Iñestgila, V., Reverdy, C., Simon, S., Lenzen, G., Petel, F., Wojcik, J., Schacht, V., Chernaya, Y., Labigne, A., and Le grin, P. (2001) Nature 409, 211–215
7. Hsot, T., Chiba, T., Ozawa, R., Yoshiida, M., Hattori, M., and Sakaki, Y. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 4569–4574
8. Gavir, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., Bermon, M., Hofert, C., Scheder, M., Branevnic, M., Ruffner, H., Merino, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gna, V., Bausch, A., Bastack, S., Hulke, B., Leutwein, C., Heurtier, M. A., Copley, R. R., Eddelmann, A., Querfurth, E., Rybin, V., Drewees, G., Raim, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G., and Superti-Furga, G. (2002) Nature 415, 141–147
9. Ho, Y., Grulher, A., Heilbut, A., Badger, G. D. Moore, L., Adams, S.-L., Millar, A., Taylor, P., Bennett, K., Boutilier, K., Yang, L., Wolting, C., Donaldson, I., Schandoerff, S., Shevanniare, J., Vo, M., Taggart, J., Goudreau, M., Munkat, B., Alfarano, C., Dewar, D., Lin, M., Michalkicova, K., Willems, A. R., Sassi, H., Nielsen, P. A., Rasmusen, K. J., Andersen, J. R., Johansen, L. E., Hansen, L., Jespersen, H., Podtelejonikov, A., Nielsen, E., Crawford, J., Poulsen, V., Senorens, B. D., Matthiesen, J., Hendrickson, R. C., Gleeson, F., Lawson, T., Moram, M. F., Durocher, D., Mann, M., Hogue, C. W. V., Figers, D., and TYers, M. (2002) Nature 415, 180–183
10. Giust, L., Bader, J. S., Brouwer, C., Choudhuri, A., Kuang, B., Li, Y., Han, Y. L., Osei, E. C., Godwin, B., Vitols, E., Vijayadamodar, G., Pochart, P., Machineni, H., Welsh, M., Kong, Y., Zerhusen, B., Malcolm, R., Varrone, Z., Collins, A., Minto, M., Burgess, S., McDaniel, L., Stimpson, E., Spriggs, F., Williams, J., Neurath, K., Joine, N., Age, M., Voss, E., Furtak, K., Renzulli, R., Aanensen, C., Carolla, S., Bickelhuw, E., Lazovskly, V., DaSilva, A., Zhong, J., Stanyon, C. A., Finley, R., L., Jr., White, K. P., Braverman, M., Jarvie, T., Gold, S., Leach, M., Knight, J., Shimkets, R. A., McKenna, M. P., Chant, J., and Rothberg, J. M. (2003) Science 302, 1727–1733
11. Rutland, G., Peregrin-Alvarez, J. M., Li, J., Yang, W., Yang, X., Canadien, V., Staros, T., Richards, A., Beattie, B., Krogan, N., Davey, M., Parkinson, J., Greenblatt, J., and Emili, A. (2005) Nature 433, 531–537
12. Utz, P., and Finlay, R. F., Jr. (2005) FEBS Lett. 579, 1821–1827
13. Staglar, I., and Fields, S. (2002) Trends Biochem. Sci. 27, 559–563
14. Schneider, D., and Engelman, D. M. (2003) J. Biol. Chem. 278, 3105–3111
15. Ohridik, P., El-Bakkoury, M., Hamacher, M., Cappellaro, C., Vilarrino, C., Fleischer, C., Ellbrock, H., Kamuzunzi, R., Ledent, V., Blazude, D., Sanders, D., Revuelta, J. L., Boles, E., Andre, B., and Frommer, W. B. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 12242–12247
16. Schager, H., and von Jagow, G. (1991) Anal. Biochem. 199, 223–231
