Roles of AtpI and Two YidC-Type Proteins from Alkalophilic Bacillus pseudofirmus OF4 in ATP Synthase Assembly and Nonfermentative Growth

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AtpI, a membrane protein encoded by many bacterial atp operons, is reported to be necessary for c-ring oligomer formation during assembly of some ATP synthase complexes. We investigated chaperone functions of AtpI and compared them to those of AtpZ, a protein encoded by a gene upstream of atpI that has a role in magnesium acquisition at near-neutral pH, and of SpoIIIJ and YqjG, two YidC/OxaI/Alb3 family proteins, in alkalophilic Bacillus pseudofirmus OF4. A strain with a chromosomal deletion of atpI grew nonfermentatively, and its purified ATP synthase had a c-ring of normal size, indicating that AtpI is not absolutely required for ATP synthase function. However, deletion of atpI, but not atpZ, led to reduced stability of the ATP synthase rotor, reduced membrane association of the F$_1$ domain, reduced ATPase activity, and modestly reduced nonfermentative growth on malate at both pH 7.5 and 10.5. Both spoIIIJ and yqjG, but not atpI or atpZ, complemented a YidC-depleted Escherichia coli strain. Consistent with such overlapping functions, single deletions of spoIIIJ or yqjG in the alkaliphile did not affect membrane ATP synthase levels or activities, but functional specialization was indicated by YqjG and SpoIIIJ showing respectively greater roles in malate growth at pH 7.5 and 10.5. Expression of yqjG was elevated at pH 7.5 relative to that at pH 10.5 and in ΔspoIIIJ strains, but it was lower than constitutive spoIIIJ expression. Deletion of atpZ caused the largest increase among the mutants in magnesium concentrations needed for pH 7.5 growth. The basis for this phenotype is not yet resolved.

The F$_1$F$_{\text{c}}$ ATP synthase is a large membrane complex in bacteria, mitochondria, and chloroplasts which synthesizes ATP by a rotary mechanism that is energized by a transmembrane proton-or sodium-motive force (1–3). The bacterial enzyme consists of a soluble F$_1$ domain (subunits α$_3$β$_3$γεδφ) and a membrane-embedded F$_{\text{c}}$ domain (subunits αβ$_2$ε$_{10,15}$) (4–6). In most bacteria, the atp operon contains nine open reading frames: eight structural genes (atpBEFHAGDC) of ATP synthase preceded by a ninth atpI gene, which encodes a membrane protein and is not a structural gene (7–9). When atpI, originally called uncI, was first reported (10), it was suggested that it encoded a pilot protein with a role in assembly of the membrane sector of the synthase, although AtpI clearly did not have an essential role, since deletion of the atpI gene from Escherichia coli only slightly reduced the growth yield and ATPase activity (11). Similarly, we recently showed that atpI, as well as the atpZ gene that is found upstream of atpI in many low %GC bacteria, could be deleted from the chromosome of alkalophilic Bacillus pseudofirmus OF4 without apparent loss of the capacity to grow nonfermentatively (8, 12).

More recently, these earlier findings have been challenged by the studies of Yoshida and colleagues (13), who have shown that AtpI plays a necessary and sufficient chaperone-like role in assembly of a hybrid Na$^+$-coupled ATP synthase containing all eight subunits and an intact c-ring rotor. The hybrid ATP synthase was expressed from a plasmid in an E. coli host deleted in its structural atp genes, atpBEFHAGDC, but not E. coli atpI. The enzyme encoded by the plasmid was a construct with the F$_1$ domain genes atpBEF of Propionigenium modestum and the atpF$^\text{H}$ HAGDC genes from thermophilic Bacillus sp. strain PS3; the b-subunit gene, atpF, was a hybrid of the P. modestum and Bacillus PS3 genes (13). The purified ATP synthase complexes expressed from this plasmid were found to contain a c-ring only when the P. modestum atpI was also included in the hybrid plasmid construct or was expressed separately on a second plasmid. Moreover, ATP synthase function, as measured by ion-pumping and ATP synthesis activities, was retained only with the coexpression of atpI. In that study, formation of a c-rotor ring, which is a homo-oligomeric ring of c-subunits, was observed with plasmids containing only the P. modestum atpE and atpI genes without other ATP synthase genes; the c-ring copurified with a His-tagged form of AtpI during affinity purification. In a second study (14), this group used an in vitro expression system to obtain membrane incorporation of the P. modestum ATP synthase rotor and showed the dependence of c-ring formation on coexpression of atpI with atpE. These observations were striking not only because of the required role for AtpI but also because of the lack of a requirement for a member of the YidC/OxaI/Alb3 protein family, none of which was included in the in vitro system (14). Cell-free in vitro synthesis and assembly of the H$^+$-coupled ATP synthase from Caldalkalibacillus thermarum TA2-A1 was also achieved (15) in the absence of both AtpI and a YidC homologue, but the presence of AtpI increased the enzyme yield. These two sets of in vitro experiments diverge from observations indicating that in vivo incorporation of c-subunits and the a-subunit of the membrane-embedded F$_{\text{c}}$ sector of the ATP synthase depends upon a protein from the YidC/OxaI/Alb3 family (16–19). The single YidC found in E. coli can be depleted but

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deletion of yidC is lethal (20, 21), and *Bacillus subtilis*, which has two yidC homologues, yqjG and spoIIIJ, can spare one of them but not both without losing viability (22, 23). It is possible that AtpI plays a more indispensable role in assembly of Na⁺-coupled than H⁻-coupled ATP synthases; a requirement for AtpI similar to that of a Na⁺-coupled *Acetobacterium woodii* operon may artificially bypass the need for additional proteins that play critical roles in *B. pseudofirmus* (24). Further, a plausible explanation for dispensability of YidC homologues for c-rotor or ATP synthase synthesis in *B. pseudofirmus* is that the use of a multicopy, inducible plasmid to express *atpI* and the subunit c-encoding *atpE* gene or the whole operon may be by-passed due to other proteins that play critical roles in assembly of a stable, completely functional ATP synthase. Although the two yidC-like genes exhibit functional overlap, phenotypes of the two deletion mutants and of double mutants of *atpI* and each yidC homologue revealed distinct contributions of YqjG and SpoIIIJ from AtpI and from each other.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *Table 1* lists the bacterial strains and plasmids used. The WT (wild-type) strain is alkaliphilic *B. pseudofirmus* OF4 (12). The Δ*atpZ*, Δ*atpI*, Δ*atpZI*, and Δ*Fₐ* strains are from laboratory stocks (12, 31). In order to facilitate purification of the ATP synthase from Δ*atpZ*, Δ*atpI*, and Δ*atpZI* strains, six histidines were placed in the β subunit of the ATP synthase as described previously (28). Δ*yqjG*, Δ*spoIIIJ*, Δ*atpI* Δ*yqjG*, and Δ*atpI* Δ*yqjG* strains were constructed as described previously (12); details are provided in the supplemental material. In order to express yqjG, spoIIIJ, atpZ, and atpI in *E. coli* FTL10, these genes were amplified from *Bacillus pseudofirmus* OF4 811M genomic DNA; the primers used are listed in Table S1 in the supplemental material. PCR products were digested with appropriate restriction enzymes and ligated into the corresponding sites of pTrc99A. The resulting constructs were pTrc-Bp-YqjG, pTrc-Bp-SpoIIIJ, pTrc-Bp-AtpZ, and pTrc-Bp-AtpI. As a positive control, the yidC gene was amplified from *E. coli* and ligated with pTrc99A to get the pTrc-Ec-YidC construct. Some of the primers used are listed in Table S1. In order to express yqjG, spoIIIJ, atpZ, and atpI in *B. pseudofirmus* OF4, the primers listed in Table S1. The amplified PCR product was ligated with pET3a (Novagen). The resulting construct

**TABLE 1  Bacterial strains and plasmids used in this study**

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<tr>
<th>Strain or plasmid</th>
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<tr>
<td><strong>Strains</strong></td>
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<td><em>B. pseudofirmus</em></td>
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<tr>
<td>Wild type (811M)</td>
<td>Methionine auxotroph of <em>B. pseudofirmus</em> OF4</td>
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<td>Strain with deletion of <em>atpZ</em></td>
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<td>Strain with deletion of <em>atpI</em></td>
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<tr>
<td>pET-Bp-AtpI</td>
<td>pET20b (Novagen) containing <em>B. pseudofirmus</em> 811M <em>atpI</em> with hexahistidine tag at 3’end</td>
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was designated pET-Bp-AtpE. In order to detect the expression of c-subunit, a Strep tag containing 8 amino acids (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) was added at the C-terminal end of atpE gene. The resulting construct was named pET-Bp-AtpE-Strep. The atpE gene was ligated with pET20b (Novagen) in the similar way with addition of 6 histidines at the 3’ end. The resulting construct was designated pET-Bp-AtpE.

Growth experiments. Growth curve experiments comparing the WT and mutant strains were carried out in semidefinned malate medium (MYE) at pH 7.5 or 10.5 (31). The precultures, grown in glucose-containing medium at pH 7.5, were washed once with either pH 7.5 MYE or pH 10.5 MYE medium prior to inoculation. Cultures (200 µl) were grown in 96-well plates (Greiner Bio-One, Germany). Plates were incubated with shaking at 30°C in a PowerWave XS2 microplate spectrophotometer (BioTek Instruments, Inc., USA), and the turbidity was monitored by the A600 hourly. The growth curves were conducted in two independent experiments with triplicate repeats. A possible limitation of the growth curves is an underestimation of the turbidity at high culture densities. Therefore, in some cases, additional growth experiments were carried out in MYE medium in tube cultures that were shaken for 16 h as described before (29), which allowed for appropriate dilutions to be made, such that the A600 was ca. 0.4 as read with a Shimadzu UV-1601 spectrophotometer. In order to check the effect of MgSO4 on the growth of deletion strains of B. pseudofirmus OF4, a defined QA medium at pH 7.5 was used for tube growth experiments with 50 mM malate as the carbon source (12). The precultures were grown in malate-containing medium at pH 10.5. Growth was monitored by the A600 after shaking for 21 h at 30°C. All tube growth experiments were conducted in duplicate in three independent experiments. Assessment of sporulation capacity by B. pseudofirmus OF4 WT, ΔspoIII, and ΔyqjG strains was conducted by phase microscopic examination of cultures grown in Difco sporulation medium for 48 h (34).

Molar growth yields. The wild-type strain and four single-deletion mutant strains were grown on semidefined medium (containing 0.1% yeast extract) with or without 5 mM L-malate at both pH 7.5 and 10.5. The growth on semidefined medium containing 0.1% yeast extract was subtracted as a background. When the cells reached maximal growth, a small portion of cells was collected and centrifuged. The cell-free supernatants were measured for malate consumption. The malate concentrations were determined using a malate assay kit (ab83391; Abcam Inc.).

For determinations of the dry weight, a standard curve relating the optical density at 600 nm (A600) to dry weight was established by weighing the wild-type cultures grown in pH 10.5 MYE medium from various A600 values. One thousand ml of wild-type cells at different A600 measurements were harvested and washed with a solution of 50 mM Tris-HCl, pH 8. The cells were dried at 80°C to constant weight on consecutive days. The standard curve yielded a linear relationship between A600 and dry weight and was used to calculate the dry weight of strains grown on 5 mM L-malate. Molar growth yield (Ymoleal) was expressed as mg (dry weight)/mmol malate consumed. The values from Table S2 in the supplemental material were calculated from two independent experiments.

Complementation analyses. pTrc99A recombinant constructs, including pTrc-Bp-YidC, pTrc-Bp-YiqG, pTrc-Bp-SpoIII, pTrc-Bp-AtpZ, and pTrc-Bp-AtpE and empty vector pTrc99A, were transformed into E. coli FTL10, in which the yidC gene was expressed under the control of the arabBAD operator/promoter (32). The precultures, grown in LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) with 0.2% arabinose and 100 µg/ml ampicillin, were washed once with LB medium prior to inoculation, after which the A600 of each culture was adjusted to 2 before 10-fold serial dilutions were made with LB medium. Three µl of each dilution was spotted on LB plates containing 100 µg/ml ampicillin with addition of either 0.2% arabinose or 0.2% glucose or 0.2% glucose with 0.5 mM IPTG (isopropyl β-D-thiogalactopyranoside). The plates were incubated at 37°C for 16 h.

Purification of His-tagged ATP synthase. His-tagged ATP synthase was purified as described previously (29). Three independent preparations were carried out for each strain. Where indicated, samples were precipitated with 10% TCA (trichloroacetic acid).

LC-MS/MS. Two relatively minor bands were observed in ATP synthase preparations from ΔatpI and ΔatpZI strains migrating between the γ and δ subunits at approximately 25 and 27 kDa (Fig. 1). As noted in Results, these bands are routinely observed in B. pseudofirmus OF4 ATP synthase preparations from the WT and different atp mutants but appeared to be much more intense in the ΔatpI and ΔatpZI strains. To determine the identity of the bands, two lanes containing 20 µg of F, F2, each were resolved on SDS 11% gels. The lanes were stained with colloidal Coomassie brilliant G (National Diagnostics, Atlanta, GA). The bands were carefully excised with razor blades. The gel fragments were reduced with 20 mM dithiothreitol (DTT) at 55°C for 30 min, followed by alkylation with 50 mM iodoacetamide for 30 min at room temperature in the dark. The bands were subsequently digested with 0.2 µg of trypsin overnight at 37°C. The peptides were extracted and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS; Thermo Electron, Bremen, Germany). The mass spectra were searched using Mascot (version 2.3; London, United Kingdom) using the Swiss-Prot database.

Everted membrane vesicle preparation, OG-stimulated ATPase assays, β subunit distribution between membrane and cytoplasm, and ATP-driven proton pumping assays. The WT and mutant strains were grown on malate-containing medium (MYE) at pH 10.5, while the ΔF55 strain was grown on glucose-containing medium at pH 10.5. Everted membrane vesicles were prepared as described previously (30). Protein concentration for this and other experiments was determined by the method of Lowry et al. (35). Octyl-glucoside (OG)-stimulated ATPase activities were conducted as described previously (30). In order to assess the β subunit in the cytoplasm, 2 ml of supernatant (cytoplasmic fraction) for each strain was collected while preparing the everted membrane vesicles. Membrane vesicles or cytoplasmic fractions were loaded on 11% SDS-PAGE gels (36). Western blot analyses of the β subunit were conducted as described previously (30).

ATP-driven proton pumping activity was assayed by monitoring the quenching of the fluorescence of ACMA (9-amino-6-chloro-2-methoxycridine) measured in an RF-5301PC spectrofluorophotometer (Shimadzu, Japan) with excitation and emission at 410 and 490 nm, respectively. The 2-ml assay solution contained 10 mM Tris-HCl, pH 8, 100 mM KCl, 5 mM MgCl2, 1 µM ACMA, and 0.8 mg everted membrane vesicle protein. One mM ATP was added to initiate the reaction, and 10 mM NH4Cl was added to the mixture in the end. The
percent fluorescence quenching was calculated using the following calculation:

\[
\text{percent fluorescence quenching} = \left(\frac{I_{0} - I}{I_{0}}\right) \times 100\%
\]

where \(I_{0}\) is the initial fluorescence and \(I\) is the remaining fluorescence after ATP addition. The magnitude of fluorescence quenching was taken as the proton-pumping activity. All of the above-described assays were conducted from at least three independent preparations.

**RNA isolation and relative and absolute qPCR.** The WT and mutant strains were grown in malate-containing medium (MYE) at pH 7.5 or 10.5. RNA was prepared when the \(A_{500}\) reached 0.4. Detailed information for RNA isolation, reverse transcription, and relative and absolute quantitative real-time PCR (qPCR) are provided in the supplemental material. For the relative qPCR, three independent RNA isolations were conducted on three independent samples of each strain at each growth condition. The relative fold changes in gene expression were calculated according to the delta delta threshold cycle method (37) after normalization using recA, gyrB, or rpoB as the reference gene. Similar results were obtained for the three different reference genes, and recA was used as the reference gene in the results. Absolute qPCR was used to analyze the transcript copies of the \(yqjG\) and \(spolIIJ\) genes at pH 7.5 or 10.5. The results were calculated from qPCR experiments conducted on 4 independent RNA preparations. As a comparison, the level of the recA transcript was monitored as the internal standard.

**In vitro protein synthesis assay.** AtpI and/or \(c\)-subunit were synthesized in vitro with a PURExpress in vitro protein synthesis kit (New England BioLabs). Four hundred nanograms of plasmid DNA was added in a 25-\(\mu\)L reaction mixture of the PURE system. The procedures were carried out according to reference 14 and included 0.6 mg/ml liposomes made from soybean phosphatidylcholine (Type II-S, Sigma) by sonication and freeze-thaw. After the reaction, the synthesized proteins were directly analyzed by SDS-PAGE or treated with proteinase K and RNase in order to remove ribosomes and all of the soluble proteins and then precipitated with 50 mM MgCl\(_2\) before SDS-PAGE analysis (14).

**RESULTS**

AtpI is not required for \(c\)-ring assembly, but it supports optimal ATP synthase assembly and stability. The earlier observation of nonfermentative growth by a \(B.\ pseudofirmus\) OF4 mutant with a deletion in \(atpI\) and/or \(atpZ\) indicated that an active ATP synthase, capable of supporting oxidative phosphorylation, was present in the mutants (12). However, no direct analyses of the ATP synthase were undertaken in those strains to definitively demonstrate its activity or examine the \(c\)-ring and individual subunit content of the mutant enzymes compared to that of the WT. We therefore constructed WT, \(\Delta\atpZ\), \(\DeltaatpI\), and \(\Delta\atpZI\) strains in which the \(b\) subunit of the \(F_{1}\), \(F_{o}\) ATP synthase contained a 6-histidine tag after the N-terminal methionine (28). We were able to purify the His-tagged enzymes from each of the strains; three preparations were made for each of the strains. The specific activities of the different preparations averaged between 39 and 49 \(\mu\)mol inorganic phosphate released min\(^{-1}\) mg protein\(^{-1}\) (U/mg) and were not significantly different from the activities routinely found in this laboratory for WT preparations, although it was noted that the \(\DeltaatpI\) strain preparations were at the lower end of the usual range, averaging 39 U/mg. While the WT and \(\DeltaatpZ\) strains gave the same yields of 1 mg \(F_{1}, F_{o}\)/liter, the \(\DeltaatpI\) and \(\Delta\atpZI\) strain yields were distinctly lower, 0.7 and 0.8 mg/liter, respectively. As will be discussed below, the yield of enzyme from the \(\DeltaatpI\) strain was consistent with a lower membrane \(F_{o}\) content. The enzyme complexes, analyzed by SDS-PAGE (Fig. 1), showed eight bands. The bands were those of the five \(F_{1}\) subunits, the \(a\)- and \(b\)-subunits of the \(F_{o}\) strain, and the \(c\)-ring, which contains 13 \(c\)-subunits in the rotor ring (6). The preparations from the \(\Delta\atpZ\) mutant exhibited bands of size and density comparable to those of the WT. The complexes from the \(\DeltaatpI\) and \(\Delta\atpZI\) mutants also showed a \(c\)-ring band with a size comparable to that of the WT band, which dissociated into a \(c\)-monomer band, as did the WT \(c\)-ring, upon treatment with trichloroacetic acid (TCA) (Fig. 1, right). These observations were consistent with the conclusion that neither AtpI nor AtpZ is required for synthesis of an intact \(F_{1}\), \(F_{o}\) ATP synthase. Unlike the enzymes from the WT or single \(atpZ\) mutant, preparations from mutants with an \(atpI\) deletion exhibited free \(c\)-monomer in the absence of TCA treatment (Fig. 1), indicating instability of the \(c\)-ring in the enzyme that was assembled without AtpI. In addition, the total amount of \(c\)-ring that was released upon complete dissociation by TCA treatment was slightly smaller for preparations from the \(\DeltaatpI\) and \(\Delta\atpZI\) mutants than that released from the WT and \(\Delta\atpZ\) mutant preparations. This probably reflects modest dissociation that occurred during extraction or purification before the enzyme was loaded on the gels.

The \(\delta\), \(a\)-, \(b\)-, and \(\epsilon\) subunit band intensities were somewhat lower in the \(atpI\) and \(atpZI\) preparations (Fig. 1). This suggested instability manifested during purification in both the \(F_{1}\) and \(F_{o}\) sectors.

In addition to the bands of the structural components of the ATP synthase seen in SDS-PAGE, there were two additional minor bands of approximately 25 to 27 kDa that were noticeable in the \(\DeltaatpI\) and \(\Delta\atpZI\) mutant preparations. Depending on the preparation and the amount of protein loaded on SDS-PAGE, these minor bands are more or less routinely observed in alkaliphilic ATP synthase preparations but were consistently more obvious in those from these two mutants. We gel purified these two bands and subjected them to LC-MS/MS (see Materials and Methods). Both bands were found to be breakdown products of the \(\beta\) subunit. The larger amount of these bands was further evidence of an increased lability evident in these preparations from \(\DeltaatpI\) mutants.

If the absence of a functional \(atpI\) gene leads to a defect in the assembly or folding of the \(F_{o}\) domain, e.g., because the \(c\)-ring is defective, an expected consequence would be a secondary defect in attachment of the \(F_{1}\) domain to its \(F_{o}\) partner at the membrane surface. In turn, such a defect would be expected to lead to increased levels of \(F_{1}\) in the cytoplasm. As shown in Fig. 2A and B, deletion of \(atpI\) resulted in a 34% reduction, relative to the WT, in ATP synthase \(\beta\) subunit content that is associated with the alkaliphilic membrane fraction; this was accompanied by a 2.7-fold increase in the amount of \(F_{1}\) sector in the cytoplasm compared to that in the WT, also assayed by detection of the \(\beta\) subunit. By comparison, assays of a control in which cell fractions came from a mutant with a complete deletion of the \(F_{o}\) sector (\(\DeltaatpB-F\)) showed that such a deletion resulted in a 10-fold increase in the amount of \(F_{1}\) sector in the cytoplasm relative to that in the WT. Deletion of \(atpZ\) did not result in an increase of \(F_{o}\) sector in the cytoplasm (Fig. 2A and Table 2). The enzyme from the \(\Delta\atpZ\) strain also had a proton-pumping activity comparable to that of the WT, while the \(\DeltaatpI\) strain showed more than a 50% reduction in ATP-driven proton-pumping activity relative to that of the WT (Fig. 2C and Table 2), a more severe reduction than the 34% reduction in membrane-associated \(\beta\) subunit content. Assays of ATPase activity in the presence of octyl-glucoside (OG), which activates the hydrolytic (ATPase) activity independently from proton pumping, again revealed no deficit for the \(\Delta\atpZ\) mutant enzyme, while the \(\DeltaatpI\) strain exhibited a 30% reduction in ATPase activity relative to the WT (Table 2). In sum, the observa-
The only statistically significant defect found was that the growth, molar growth yields on malate were determined for the subunit and B). The manifested as a longer lag than that of the WT at pH 10.5 (Fig. 3A); a small deficit was observed at both pH values and was mostly large growth defect on malate relative to the WT at either pH 7.5 or deficit in ATP synthase function, the subunit of F1Fo was detected in the membrane (A) or cytoplasm (B). The mutants compared to the WT strain fractions and properties of ATP synthase from different deletion strains except the Fo strain as the background. Those lipids, a crude soybean phosphatidylcholine preparation (Type II-S; Sigma), had been used previously to reconstitute the alkaliphilic ATP synthase in an active form (38). We successfully expressed AtpI and/or the c-subunit monomer. As shown in Fig. S2A in the supplemental material, the silver-stained gel after in vitro expression has numerous bands, which are the components of the PURE system, including one band whose migration overlaps with the band of the purified c-ring. Removal of the PURE component proteins by a protease, RNase, and a magnesium precipitation protocol revealed the c-subunit monomer band but no detectable c-ring band (AtpI was not observable by silver staining but was clearly seen by Western blotting; see Fig. S2B and C in the supplemental material). We noticed that coexpression of the two alkaliphilic genes lowered the amount of c-monomer compared to that of atpE expression alone. It was possible this was due to some c-ring formation in the presence of atpI. As one approach to increase the probability of detecting trace amounts of c-ring, the c-subunit was Strep tagged so that it could be detected by Western blotting. Again, the presence of AtpI did not result in the formation of any c-ring band detectable by Western blotting. A limitation to this approach is the uncertainty of whether the Strep tag prevents c-ring formation, although we note that a Strep tag on the c-subunit of the E. coli ATP synthase did not prevent c-ring assembly (39).

B. pseudofirmus OF4 yqjG and spoIIIJ complement an E. coli strain depleted in YidC, and deletion phenotypes indicate overlapping capacities as well as distinct functional profiles from each other. Two yidC homologues of B. pseudofirmus OF4 genes determined if the P. modestum c-subunit was assembled into a ring only in the presence of AtpI. They found a silver-stained c-ring band, which was rather intense, only when they coexpressed atpE and atpI. Upon TCA treatment, that band collapsed to a c-monomer band. We carried out similar experiments using the alkaliphilic c-subunit gene (atpE) without or with the alkaliphilic atpI gene. We used the same protein expression preparation, the PURE system, as well as the same phospholipids used by Osaki et al. (14) to make liposomes that these hydrophobic proteins could insert into. Those lipids, a crude soybean phosphatidylcholine preparation (Type II-S; Sigma), had been used previously to reconstitute the alkaliphilic ATP synthase in an active form (38). We successfully expressed AtpI and/or the c-subunit monomer. As shown in Fig. S2A in the supplemental material, the silver-stained gel after in vitro expression has numerous bands, which are the components of the PURE system, including one band whose migration overlaps with the band of the purified c-ring. Removal of the PURE component proteins by a protease, RNase, and a magnesium precipitation protocol revealed the c-subunit monomer band but no detectable c-ring band (AtpI was not observable by silver staining but was clearly seen by Western blotting; see Fig. S2B and C in the supplemental material). We noticed that coexpression of the two alkaliphilic genes lowered the amount of c-monomer compared to that of atpE expression alone. It was possible this was due to some c-ring formation in the presence of atpI. As one approach to increase the probability of detecting trace amounts of c-ring, the c-subunit was Strep tagged so that it could be detected by Western blotting. Again, the presence of AtpI did not result in the formation of any c-ring band detectable by Western blotting. A limitation to this approach is the uncertainty of whether the Strep tag prevents c-ring formation, although we note that a Strep tag on the c-subunit of the E. coli ATP synthase did not prevent c-ring assembly (39).

B. pseudofirmus OF4 yqjG and spoIIIJ complement an E. coli strain depleted in YidC, and deletion phenotypes indicate overlapping capacities as well as distinct functional profiles from each other. Two yidC homologues of B. pseudofirmus OF4 genes

![Image](https://example.com/image.png)

**FIG 2** Effects of deletion of the atpI gene on distribution of β subunit in the membrane or cytoplasmic fractions and ATP synthase activities. The β subunit of F1Fo was detected in the membrane (A) or cytoplasm (B). For purposes of quantitation, image analysis was performed using Image J 1.40 software (rsbweb.nih.gov/ij/), and the WT was designated 100%. The values are the averages ± standard deviations of determinations from at least three independent experiments. (C) ATP-driven proton-pumping activities in membrane vesicles from WT and mutant strains were monitored by the quenching of the fluorescence of ACMA. Arrows indicate the addition of ATP (1 mM) and NH4Cl (10 mM). Traces are representative of three independent preparations. AU, arbitrary units of fluorescence quenching.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Membrane ATPase (%)</th>
<th>Cytoplasm ATPase (%)</th>
<th>Membrane Proton-pumping (%)</th>
<th>Cytoplasm Proton-pumping (%)</th>
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</thead>
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<tr>
<td>WT</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ΔatpZ</td>
<td>96 ± 13</td>
<td>78 ± 9</td>
<td>113 ± 10</td>
<td>111 ± 5</td>
</tr>
<tr>
<td>ΔatpI</td>
<td>66 ± 7</td>
<td>266 ± 48</td>
<td>67 ± 6</td>
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</tr>
<tr>
<td>ΔyqjG</td>
<td>83 ± 6</td>
<td>100 ± 31</td>
<td>75 ± 2</td>
<td>86 ± 1</td>
</tr>
<tr>
<td>ΔspoIIIJ</td>
<td>100 ± 9</td>
<td>102 ± 33</td>
<td>109 ± 5</td>
<td>101 ± 7</td>
</tr>
<tr>
<td>ΔatpI ΔyqjG</td>
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<td>211 ± 34</td>
<td>67 ± 11</td>
<td>38 ± 4</td>
</tr>
<tr>
<td>ΔatpI ΔspoIIIJ</td>
<td>75 ± 7</td>
<td>242 ± 42</td>
<td>72 ± 9</td>
<td>35 ± 4</td>
</tr>
</tbody>
</table>

The values are averages ± standard deviations from at least 3 independent preparations. All strains except the ΔFo strain were grown on malate-containing medium at pH 10.5, while the ΔFo strain was grown on glucose-containing medium at pH 10.5. The ΔFo strain had 10% ± 2% and 1,011% ± 170% of the β subunit content of the WT in the membrane and cytoplasmic fractions, respectively. The OG-stimulated ATPase activity of the WT was 1.22 ± 0.12 U (mg of membrane protein)^−1 min^−1. The WT strain exhibited a proton-pumping activity of 35.8% ± 2% fluorescence quenching of ACMA. In the ATPase and proton-pumping assays, the data were corrected by subtracting the values of the ΔFo strain as the background.
were identified in the recently completed genome sequence, with locus tags of BpOF4_11410 and BpOF4_07940 (27). They were designated \textit{yqjG} and \textit{spoIIIJ}, respectively, as in \textit{B. subtilis} (22). The \textit{spoIIIJ} gene together with a downstream \textit{jagA} gene are in a context between \textit{rnpA} and \textit{trmE} in which many other bacterial \textit{yidC} homologues are found (40), while the \textit{yqjG} gene is in a genome region that encodes a sodium efflux system and a two-component system with a predicted role in secretion stress. The \textit{yqjG} gene is upstream of a small hypothetical gene and directly downstream of a gene identified as \textit{mifM} (see Fig. S3 and S4 in the supplemental material), whose homologue in \textit{B. subtilis} has been shown to sense \textit{SpoIIIJ} activity and allow increased \textit{YqjG} translation when \textit{SpoIIIJ} levels are low (41). \textit{YqjG} has a high sequence similarity with \textit{SpoIIIJ} (58% identity). Both \textit{YqjG} and \textit{SpoIIIJ} from \textit{B. pseudofirmus} \textit{OF4} show sequence similarity to \textit{Oxa1p} from \textit{Saccharomyces cerevisiae} (26% identity), \textit{YidC} from \textit{E. coli} (~ 40% identity), and \textit{B. subtilis} \textit{YqjG} (~ 37% identity), and they show 55 and 61% identity, respectively, to \textit{B. subtilis} \textit{SpoIIIJ} (see Table S3).

To test whether \textit{yqjG} and \textit{spoIIIJ} of \textit{B. pseudofirmus} \textit{OF4} are functional \textit{in vivo} and have overlapping functions, a plasmid expressing each of the genes was transformed into an \textit{E. coli} strain, FTL10. In this strain, the native single \textit{yidC} gene is under the control of the \textit{araBAD} promoter, so growth on LB–0.2% arabinose is robust but depletion of \textit{YidC} completely impairs growth on LB–0.2% glucose medium in the absence of arabinose (32). The capacity for growth on LB–0.2% glucose, i.e., complementation of the \textit{YidC}-depleted phenotype, was assessed after transformation of the strain with one of the following pTrc plasmids: empty pTrc99A transformant (negative control) and pTrc transformants expressing \textit{E. coli yidC} (positive control) or alkaliphilic \textit{yqjG}, \textit{spoIIIJ}, \textit{atpZ}, or \textit{atpI}. As shown in Fig. 4, all of the transformants grew in the presence of arabinose, which induced the chromosomal \textit{yidC} in the \textit{E. coli FTL10} host. In the absence of arabinose, strains with plasmids expressing \textit{E. coli yidC} or alkaliphilic \textit{yqjG} grew without IPTG induction of increased expression over a basal level and appeared to be toxic when induced by IPTG. Strains bearing plasmids expressing \textit{E. coli yidC} or alkaliphilic \textit{spoIIIJ} grew in the presence of added IPTG, which was required for complementation by \textit{SpoIIIJ}. The two alkaliphilic \textit{YidC} proteins had overlapping capacities to complement the \textit{E. coli YidC}-depleted strain. However, neither alkaliphilic \textit{AtpZ} nor alkaliphilic \textit{AtpI} could replace \textit{E. coli} \textit{YidC} to support the growth of \textit{E. coli FTL10} with or without induction.

Single-deletion mutants of \textit{yqjG} and \textit{spoIIIJ} as well as double deletion mutants of each of the \textit{yidC}-like genes, together with an \textit{atpI} deletion, were then made in the alkaliphilic chromosome to assess their phenotypic effects. Spores and prespores were observed by phase microscopy in cells from cultures of the WT and \textit{ΔyqjG} mutant grown for 48 h but not in cells of the \textit{ΔspoIIIJ} mutant. We tried to construct a \textit{ΔyqjG ΔspoIIIJ} double mutant
strain by introducing an spoIIIJ deletion into a ΔyqjG strain; these attempts were unsuccessful. This was consistent with the expectation that a double ΔyqjG ΔspoIIIJ deletion leads to a lethal phenotype, whereas the functional overlap of YqjG and SpoIIIJ allows either one to support viability. The ΔatpI ΔyqjG and ΔatpI ΔspoIIIJ double mutants exhibited comparable β subunit distribution relative to the WT, as observed in the single ΔatpI mutant. These double and single atpI-containing mutants also had similar patterns in the OG-stimulated ATPase and proton-pumping as-
sbution relative to the WT, as observed in the single ΔatpI mutant.

As discussed above, the phenotypes of ΔyqjG and ΔspoIIIJ strains indicated that YqjG plays a more important role at pH 7.5, and conversely SpoIIIJ is more critical at pH 10.5. It was of interest to examine whether differences in expression of atpI, spoIIIJ, and yqjG occurred in the WT as a function of growth pH and whether changes in the expression of one or more of these genes occurred in the mutant strains. First, the levels of expression of the two yidC homologues at pH 7.5 and 10.5 were determined by absolute quantification qPCR. As shown in Table 3, the transcript level of yqjG at pH 7.5 was a little less than half of the level of the spoIIIJ transcript. The level of spoIIIJ transcript was the same at pH 10.5 as at the lower pH, while the level of yqjG was significantly lower at pH 10.5 than at pH 7.5. qPCR then was used to assess pH-dependent changes in transcripts of atpI, spoIIIJ, and yqjG in the WT. As shown in Fig. 5A, the WT strain exhibited 3-fold greater expression of yqjG at pH 7.5 than at pH 10.5, while atpI and spoIIIJ did not exhibit significant pH-dependent changes in expression. We also examined the effects of single and double deletions on the expression of remaining atpI, yqjG, and spoIIIJ genes at pH 7.5 and 10.5. At pH 7.5 (Fig. 5B), yqjG was modestly upregulated in the ΔspoIIIJ and ΔatpI ΔspoIIIJ strains. At pH 10.5 (Fig. 5C), yqjG was upregulated 3-fold in the ΔspoIIIJ and double ΔatpI ΔspoIIIJ mutants, while there was an increase of yqjG expression in the ΔatpI strain that was smaller than the increase of its expression in the ΔspoIIIJ strain but larger than that observed upon atpI deletion at pH 7.5.

**Magnesium acquisition phenotype.** We had shown earlier that the ΔatpZ and ΔatpI mutants were both dependent on the presence of higher magnesium concentrations than the WT at pH 7.5 but not at pH 10.5 (12). Given the pH dependence of this phenotype and the growth pH-dependent profile of YqjG as well

<table>
<thead>
<tr>
<th>pH</th>
<th>yqjG (copies/ng total RNA)</th>
<th>spoIIIJ (copies/ng total RNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>(1.21 ± 0.17) × 10^4</td>
<td>(3.05 ± 0.63) × 10^3</td>
</tr>
<tr>
<td>10.5</td>
<td>(0.44 ± 0.09) × 10^4</td>
<td>(3.48 ± 0.23) × 10^3</td>
</tr>
</tbody>
</table>

*The recA gene was used as an internal standard gene, and it had values of (2.02 ± 0.3) × 10^6 and (2.14 ± 0.18) × 10^6 copies/ng total RNA at pH 7.5 and 10.5, respectively.*
as its pH-related expression, we carried out assays to see if YqjG also showed this magnesium phenotype. The malate-QA medium was chosen for this set of experiments instead of the semidefined malate-containing MYE used for all of the others in order to minimize contaminating magnesium. As shown in Fig. 6, the ΔatpZ strain had the most severe phenotype of all of the mutants. Deletion of either atpI or yqjG resulted in a significant increase in dependence on higher levels of added magnesium. A further significant increase in magnesium dependence was observed in the double ΔatpI ΔyqjG mutant. Conversely, the ΔspoIIIJ mutant exhibited no change in growth in the range of magnesium tested, and neither did the double ΔatpI ΔspoIIIJ mutant. The absence of a defect in the double ΔatpI ΔspoIIIJ mutant seemed likely to reflect the elevated level of yqjG in that double mutant (Fig. 5C), which could have offset the detrimental effects of the atpI deletion in this context at pH 7.5 (Fig. 3A).

**DISCUSSION**

This study confirmed and extended earlier evidence (12) that functional AtpI is not essential for assembly of the ATP synthase in *B. pseudofirmus* OF4. Active holoenzyme could be purified from the ΔatpI mutant of *B. pseudofirmus* OF4, and all of the structural subunits of the synthase were identified (Fig. 1). As noted, such observations are in contrast to the finding that AtpI from *P. modestum* was indispensable for formation of a c-rotor ring in *E. coli* from a hybrid atp operon which had *P. modestum* Fo genes and for the formation of a c-rotor ring from c-subunits that were expressed in vitro (13, 14). Similar results were observed with the Na+/H+-coupled rotor of *Acetobacterium woodii* (24). The current observations were closer to those of Matthies et al. (15), who reported that the absence of AtpI reduced the yield, but did not preclude formation, of functional ATP synthase obtained from a cell-free expression system using a plasmid with atp genes from *Caldalkalibacillus thermarum* TA2.A1. Although AtpI similarly is not required for synthesis of ATP synthase in *B. pseudofirmus* OF4, a major finding of the current work was that deletion of atpI was accompanied by a significant deficit in assembly of ATP synthase, a lowered stability of the c-rotor, and discernable defects in the catalytic and growth functions supported by the ATP synthase in this alkaliphile (Table 2 and Fig. 1 and 3). Thus, AtpI plays an important role in assembly of the complex, presumably at least in part via direct effects on F$_{o}$ subunit c insertion and formation of a rotor ring. Loss of AtpI function negatively impacts proper assembly of the F$_{o}$ sector and leads to a deficit in F$_{i}$ sector.
attachment to form a holoenzyme that has a fully stable c-ring (Fig. 1 and 2).

A second finding of interest emerged from the effects of deletion mutants in each of two YidC homologues, SpoIIIJ and YqiG, both alone and together with a deletion in ATP1. The observation of pH-dependent distinctions in the roles of the two YidC-like proteins is a new example of specialization of YidC isoforms. The two YidC homologues found in Gram-positive species presumably arose from gene duplication and then diverged functionally in ways that may differ in different species (42). Both yqiG and spoIIIJ from B. pseudofirmus OF4 restored the ability of YidC-depleted E. coli to grow on plates (Fig. 4), and consistent with an overlap of their functions, either gene was sufficient to support viability of the alkaliphile over its pH range (Table 2 and Fig. 3). Alkaliphilic spoIIIJ, but not yqiG, required induction (Fig. 4), and their overlapping complementation of the E. coli mutant indicates that either gene restores critical functions to the YidC-depleted E. coli strain. This does not rule out the possibility, however, that the complementation was incomplete. Less than complete cross-complementation was observed when the SpoIIIJ and YqiG homologues from Streptococcus mutans were used to complement YidC-depleted E. coli (43). Other studies showed that only YqiG of S. mutans had a signal recognition particle-like capacity to support cotranslational protein insertion, while no specialized role for SpoIIIJ was found (42, 44). While both SpoIIIJ and YqiG of Bacillus subtilis play a role in membrane biogenesis and are largely functionally exchangeable (22, 23, 33), they also exhibit specialization. As also observed here in B. pseudofirmus OF4, only SpoIIIJ is essential for spore formation (45), while in B. subtilis YqiG was shown to play an exclusive and critical role in genetic competence development (46), a process that is absent from the alkaliphile. The data gathered here support the expected overlap in YqiG and SpoIIIJ functions but also revealed a new example of specialization of the two YidC-like proteins of B. pseudofirmus OF4, one that relates to the major challenge of carrying out robust nonfermentative growth over a pH range from pH 7.5 up to at least pH 11.4 (47, 48). The alkaliphilic YidC homologues appeared to have acquired different functional profiles at pH 7.5 versus pH 10.5, with YqiG playing a larger role at pH 7.5 and SpoIIIJ playing a larger role at pH 10.5. The experimental support for this specialization included the growth deficits of the ΔyqiG strain at pH 7.5 but not at pH 10.5 relative to that of the WT and the converse growth deficits shown by the ΔspoIIIJ mutant at pH 10.5 but not at pH 7.5 (Fig. 3). The roles of the YidC proteins in ATP synthase assembly are likely to be at least partially distinct from that of ATP1, since deletion of yqiG or spoIIIJ alone did not increase the amount of F1 sector found in the cytoplasm in the way that atp1 deletion did, and introduction of a deletion in yqiG or spoIIIJ into the Δatp1 background did not further increase cytoplasmic F1, over that found in the single Δatp1 mutant. Interestingly, though, there may be some overlap of YqiG function with that of ATP1 pH 7.5, since the Δatp1 ΔspoIIIJ double mutant, in which there is an increased level of yqiG expression (Fig. 5B), abolished the deficit conferred in the Δatp1 background alone (Fig. 3A). The mRNA expression level of spoIIIJ was stable at different pH values. In contrast, yqiG showed a 3-fold upward change at pH 7.5 relative to that at pH 10.5 (Fig. 5A), which is compatible with the conclusion that yqiG plays a dominant role at pH 7.5. Expression of yqiG was also significantly induced under conditions of spoIIIJ deletion, as described previously for other bacteria (23, 41, 46, 49). We saw a small increase of yqiG expression in the spoIIIJ deletion strain grown at pH 7.5 (Fig. 5B) and a much larger 3-fold increase in yqiG expression at high pH (Fig. 5C). The expression of more yqiG at high pH may reflect the use of more protein to achieve a rescue upon deletion of spoIIIJ at pH where the role of SpoIIIJ is greatest and YqiG’s efficacy is lowest.

Finally, the results showed that among the full panel of atp- and yidC-like genes studied here, the deletion of atpZ had the largest effect in increasing the concentration of added magnesium that was required for growth (Fig. 6). This suggests that AtpZ is more directly involved in this phenotype than other genes whose deletion had more modest effects. The mechanism for this phenotype, which is observed at pH 7.5 but not pH 10.5 (12), is not yet known. The magnesium phenotype is only observed at near neutral pH, where YqiG was apparently more functional than SpoIIIJ. The finding that the ΔyqiG strain exhibited the most severe phenotype apart from the ΔatpZ strain was consistent with its pH profile. Recently, Price et al. showed that CorA protein, a magnesium transporter protein, was identified as a possible substrate for the YidC-dependent insertion pathway (50). Therefore, deletion of the yqiG gene might reduce the insertion of a magnesium transporter and lead to an increased requirement of magnesium acquisition. As described before (12), the magnesium phenotype of the Δatp1 strain was not as severe as that of the ΔatpZ strain and also was not as severe as the phenotype of the ΔyqiG strain. The Δatp1 ΔyqiG double mutant also exhibited a large increase in its magnesium requirement. As expected from the limited role of SpoIIIJ at pH 7.5 in the earlier experiments, the single ΔspoIIIJ and double Δatp1 ΔspoIIIJ mutants exhibited no requirement for increased levels of magnesium at pH 7.5. Future studies will be directed at the mechanism of AtpZ involvement in this phenotype and whether effects of ATP1 and YqiG on the magnesium requirement are mediated via AtpZ or are independent, somewhat less effective pathways of impacting magnesium acquisition. Alkaliphilic B. pseudofirmus OF4 is hard wired for sudden and sustained encounters with extremely high pH. As a result, growth at near-neutral pH is sometimes less optimized and may occasionally require specific adaptations in order to function adequately (51). Magnesium acquisition is a process that would be expected to be a special challenge at high pH, at which bioavailability is low, but B. pseudofirmus OF4 is apparently well adapted for that challenge. Some of those adaptations may be responsible for the need to add features to ensure adequate magnesium acquisition at more conventional pH values in this extremophile (27, 51).

Acknowledgments
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