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Discovery and Characterization of the Cryptic ψ Subunit of the Pseudomonad DNA Replicase

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We previously reconstituted a minimal DNA replicase from Pseudomonas aeruginosa consisting of α and ϵ (polymerase and editing nuclease), β (processivity factor), and the essential δ, δ′, and β′ components of the clamp loader complex (Jarvis, T., Beaudry, A., Bullard, J., Janic, N., and McHenry, C. (2005) J. Biol. Chem. 280, 7890–7900). In Escherichia coli DNA polymerase III holoenzyme, χ and ψ are tightly associated clamp loader accessory subunits. The addition of E. coli χψ to the minimal P. aeruginosa replicase stimulated its activity, suggesting the existence of χψ counterparts in P. aeruginosa. The P. aeruginosa χ subunit was recognizable from sequence similarity, but ψ was not. Here we report purification of an endogenous replication complex from P. aeruginosa. Identification of the components led to the discovery of the cryptic ψ subunit, encoded by holD. P. aeruginosa χ and ψ were co-expressed and purified as a 1:1 complex. P. aeruginosa ψ increased the specific activity of χψ and single-stranded DNA binding protein was observed. Sequence similarity to P. aeruginosa ψ allowed us to identify ψ subunits from several other Pseudomonads and to predict probable translational start sites for this protein family. This represents the first identification of a highly divergent branch of the ψ family and confirms the existence of ψ in several organisms in which ψ was not identifiable based on sequence similarity alone.

Pseudomonas aeruginosa (PA)* is a ubiquitous Gram-negative bacterium. Whereas exposure to PA normally poses no threat, PA can act as an opportunistic pathogen in susceptible populations. At particular risk for PA infection are immunocompromised patients, burn patients, and cystic fibrosis patients. PA is the most common Gram-negative pathogen, accounting for 10% of all infections in intensive care patients. The lungs of cystic fibrosis patients are commonly colonized by PA before 10 years of age, and chronic infection is the most significant cause of morbidity and mortality (1). Multidrug-resistant PA is becoming increasingly common in hospitalized patients (2), driving the need for new antipseudomonal agents. We are exploring the DNA replication apparatus of PA as a target for inhibitors that might prove therapeutically useful in treating PA infections.

Bacterial DNA replication has been extensively characterized in Escherichia coli. Of the five DNA polymerases that have been identified in E. coli, only DNA polymerase III holoenzyme (pol III holoenzyme) plays a major role in chromosomal replication. Most of the genes encoding holoenzyme subunits are essential (3–9), and the multisubunit pol III holoenzyme exhibits all of the properties required for accurate and efficient DNA synthesis: rapid elongation, high processivity, high fidelity, and the ability to function under physiological salt conditions (10–12). 3 Pol III holoenzyme contains three functional units: the catalytic core, the processivity factor or “sliding clamp,” and the ATP-driven “clamp loader.” The catalytic core consists of α (the polymerase catalytic subunit), ϵ (the proofreading exonuclease), and δ, a nonessential subunit that binds to χ (13–15). Processivity is enhanced by the action of β, a bracelet-shaped dimer that encircles the DNA template, forming a “sliding clamp” (16, 17). The clamp loader complex (also known as the DNAX complex) consists of seven subunits: three copies of the dnaX gene product and one copy of δ, δ′, χ, and ψ (18, 19). In E. coli, dnaX encodes both the full-length protein τ and the shorter γ, a C-terminally truncated protein produced by translational frameshifting (20–22).

The native E. coli clamp loader complex consists of a mixture of τ and ψ subunits, most likely τ, δδ′, χψ (as discussed in Ref. 11). A complex containing τ, δδ′, χψ (τ-complex), however, appears to be fully functional (23). Both τ and the shorter γ can hydrolyze ATP (in concert with δδ′ χψ), which opens the ring of the β sliding clamp and positions β on the primer terminus (23–32). The clamp loader complex plays a role in elongation as well; τ interacts with and facilitates dimerization of the α subunit (33, 34), δ and δ′ are also required for processive elongation (9).

The contributions of χ and ψ to E. coli pol III holoenzyme function are more subtle. χ binds tightly to ψ, forming a 1:1 heterodimer (35). DNA synthesis activity of pol III holoenzyme under physiological salt conditions is ∼3-fold higher when χψ is present, and this χψ-dependent salt resistance requires SSB (36). χ interacts with the extreme C terminus of SSB and enhances the binding of SSB to DNA (37, 38). χψ also increases the affinity of δ and δ′ for τ (35). χψ interacts with domain III of γ (39), a contact probably mediated through the conserved N-terminal region of ψ (40). ψ also stimulates both the ATPase and replication functions of γ (41). Deletion of holD, the gene encoding ψ, results in chronic induction of the SOS response and a temperature-sensitive phenotype (8).

We previously reconstituted a minimal DNA replicase from PA consisting of αε, β, and τδδ′ (42). Although the reconstituted replicase lacked the τ, χ, and ψ subunits found in E. coli pol III holoenzyme, it exhibited many of the expected characteristics of a replicative holoenzyme.
zyme when assayed under low salt conditions in the presence of SSB. However, its activity was drastically inhibited under more physiological salt conditions, indicating that perhaps one of the missing subunits was required for full activity. We considered $\theta$, but it is not an essential subunit in *E. coli* (13, 14), and PA $\theta$ could not be readily identified by amino acid sequence similarity. We then turned to $\chi$ and there were no obvious sequences in *P. aeruginosa* DNA that would cause a translational frame-shift to produce a $\gamma$ subunit. Finally, in considering $\chi$ and $\psi$, we noted that the addition of *E. coli* $\chi\psi$ markedly enhanced PA $\tau_{\delta\delta'}$ specific activity and permitted elongation by the PA minimal replicase at elevated salt conditions, which suggested the presence of $\chi\psi$ analogs in the PA system.

We could identify PA $\chi$ by sequence similarity to the *E. coli* subunit (encoded by *holC*), but not PA $\psi$. In fact, in about half the organisms in which $\chi$ can be identified, there is no observable corresponding $\psi$ subunit (40). It is conceivable that these organisms lack a direct $\psi$ counterpart and that the functions normally attributed to $\psi$ are encompassed by other helicenzyme constituents. Alternatively, lack of an identifiable $\psi$ subunit may simply reflect poor sequence conservation. The marked effects of *E. coli* $\chi\psi$ on the minimal PA replicase argue for the existence of a functional equivalent of $\chi\psi$ in PA. Furthermore, the addition of $\chi$ alone (either *E. coli* or PA $\chi$) showed no effect on DNA synthesis activity, indicating that the $\psi$ subunit was necessary to achieve the stimulatory effect. Thus, we were interested in identifying the $\psi$ subunit from PA. Here we describe the identification and characterization of this subunit and propose the existence of a *Pseudomonas* family.

**EXPERIMENTAL PROCEDURES**

**PA Cell Growth and Lysis**—*P. aeruginosa* ($\Delta\rhoSNO$-Gm$^+$) cells were grown in a 180-liter fermentor at 37 °C in broth (yeast extract (14 g/liter), tryptone (8 g/liter), KH$_2$PO$_4$ (12 g/liter), KH$_2$PO$_4$ (1.2 g/liter), and glyceral (1%)) in the presence of 50 µg/ml gentamycin. The pH was maintained at 7.2 by the addition of NH$_4$OH. Cells were harvested at midlog phase ($A_{600}$ of ~2.5) with simultaneous chilling to 14 °C in the harvest line. Cells were suspended in an equal volume of Tris-sucrose (50 mM Tris-HCl (pH 7.5), 10% sucrose) and frozen by liquid nitrogen. Lysis was accomplished via creation of spheroplasts by treatment of cells with lysozyme. Finally, in considering $\chi\psi$-subunit $\nu$ (encoded by *holC*), but not PA $\nu$ on the minimal PA replicase argued for the existence of a functional equivalent of $\chi\psi$ in PA. Furthermore, the addition of $\chi$ alone (either *E. coli* or PA $\chi$) showed no effect on DNA synthesis activity, indicating that the $\psi$ subunit was necessary to achieve the stimulatory effect. Thus, we were interested in identifying the $\psi$ subunit from PA. Here we describe the identification and characterization of this subunit and propose the existence of a *Pseudomonas* family.

**Buffers Used in Purifications**—Subscripts indicate millimolar concentrations of NaCl (i.e. A$_{20}$ is A$_{20}$ + 20 mM NaCl), BW is 20% glycerol, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM EGTA, 0.1 M KOAc (pH 8.0), 0.20 g/ml ammonium sulfate (i.e. 35% ammonium sulfate saturation). A$_{0}$ is 25 mM Tris-HCl (pH 7.5), 10% glycerol, 0.1 mM EDTA, and 0.1 mM EGTA. A$_{1}$ is 25 mM Na-HEPES (pH 8.0), 5% glycerol, 0.5 mM EDTA, and 0.1 mM EGTA. A$_{2}$ is 25 mM Na-HEPES (pH 8.0), 5% glycerol, 1 mM EDTA, 0.1 mM EGTA, 25% saturated ammonium sulfate. B is 25 mM Na-HEPES (pH 8.0), 5% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 0.1 M NaCl. C is 25 mM Na-HEPES (pH 8.0), 5% glycerol, 1 M NaCl. D is 25 mM HEPES (pH 8.0), 5% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 0.1 M NaCl, and 50 mM NaCl. E is 25 mM HEPES (pH 8.0), 5% glycerol, 0.5 mM EDTA, and 0.1 mM EGTA. E is 25 mM Tris-HCl (pH 7.5), 5% glycerol, 0.1 mM EDTA, 0.1 mM EGTA, and 50 mM NaCl. F is 10% glycerol, 20 mM potassium phosphate (pH 8.0), 0.3 M NaCl, 0.01 mM EDTA, 0.01 mM dithiothreitol, 20 mM imidazole-Cl (pH 8.0), 0.005% Tween 20. G is F buffer except with 50 mM imidazole. H is F buffer except with 250 mM imidazole.

**Purification of Endogenous Replicase from PA**—All purification steps were performed at 4 °C. Purification was monitored using a helicenzyme reconstitution assay. Measuring DNA synthesis in the presence of saturating levels of exogenous PA $\alpha$ and $\beta$ allowed us to detect the stimulatory activity of the endogenous clamp loader complex. To Fraction I, ammonium sulfate (0.226 g to each initial ml of Fraction I; i.e. 40% saturation) was added slowly. The precipitate was collected by centrifugation (16,000 × g, 30 min). The pellet was resuspended using a Dounce homogenizer in 200 ml of buffer BW. The remaining precipitate was collected by centrifugation (16,000 × g, 45 min). The pellet was resuspended in 50 ml of buffer $A_{20}$, dialyzed for 4 h against $A_{20}$ and then diluted to 240 ml to match the conductivity of $A_{20}$, forming Fraction II. Fraction II was applied at a flow rate of 6.4 ml/min to a 2.5 × 3-cm MacroPrep High S (Bio-Rad) column connected in series to a 5 × 12.7-cm heparin-Sepharose Fast Flow (Amersham Biosciences) column equilibrated in $A_{20}$. The columns were washed with 860 ml of buffer $A_{20}$. The species giving rise to stimulatory DNA synthesis activity passed through the S column, leaving behind contaminating proteins that were strong cation exchange binders, and bound to the heparin column. The S column was then disconnected. The heparin column was developed with a 2.0-liter linear gradient from 20 to 300 mM NaCl at a flow rate of 2.8 ml/min. DNA synthesis activity was detected in fractions 35–45, which were pooled to form Fraction III. Fraction III was applied without desalting at a flow rate of 1.3 ml/min to a 5–ml HiTrap Q-Sepharose HP column (Amersham Biosciences) equilibrated in buffer $A_{20}$. The column was washed with 20 ml of buffer $A_{20}$, followed by a 50–ml linear gradient from 20 to 1000 mM NaCl. The stimulatory activity was detected in fractions 45–53, which were pooled to form Fraction IV. Fraction IV (4.3 ml) was adjusted to pH 8.0 by the addition of 0.4 ml of 1 M Na-HEPES (pH 8.0). 3.3 ml of a 40% slurry of methyl HIC resin (Bio-Rad) equilibrated in buffer B were then added to Fraction IV. The protein/resin slurry was then adjusted to 25% ammonium sulfate saturation by the addition of 2.7 ml of saturated ammonium sulfate. This slurry was gently rocked for 30 min. Fresh methyl HIC resin was packed in a 1.5 × 4.0-cm column and equilibrated in buffer C. Following the 30-min incubation, the protein/resin slurry was layered on the top of the methyl column bed at a flow rate of 1 ml/min. The column was then washed with 20 ml of buffer C and developed with a 50–ml linear reverse salt gradient from buffer C to buffer D. DNA synthesis stimulatory activity was detected in fractions 30–40, which were pooled to form Fraction V. Fraction V was precipitated with 70% ammonium sulfate, divided into six aliquots, collected by centrifugation (16,000 × g, 30 min), and flash-frozen and stored at −80 °C. A Superose-6 HR 10/30 column (Amersham Biosciences) controlled by an AKTA fast protein liquid chromatograph (Amersham Biosciences) was equilibrated in buffer E. Half of the Fraction V pellets were resuspended in 0.55 ml of buffer E and applied to the Superose column at a flow rate of 0.3 ml/min. 0.25–ml fractions were collected, and fractions 22–26 were pooled to form Fraction VI.

**Activity Assays**—DNA synthesis activity was measured in a reconstituted PA pol III helicenzyme assay as described previously (42). Briefly, M13Gori single-stranded phage DNA was used as template (1.4 nm; circles). Initial studies (Figs. 1 and 2, TABLE ONE) used template preprimed with 1.0 µg/ml *E. coli* DnaG primase and coated with 21 µg/ml *E. coli* SSB. 25–µl reactions containing preprimed DNA/SSB plus PA $\alpha$, $\tau_{\delta\delta'}$, and $\beta$ were incubated for 5 min at 22 °C. Formation of double-stranded DNA was measured using PicoGreen (Molecular Probes, Inc., Eugene, OR), expressed as relative fluorescent units (RFU). 100 RFU equals ~15 pmol of nucleotide incorporated. In Fig. 4 and TABLE THREE, PA DnaG and SSB were substituted for *E. coli* DnaG and SSB in the prepriming step; comparable results were obtained with either priming system. Primer extension assays in Fig. 5 were performed for 5 min at 22 °C with M13Gori single-stranded DNA template (2 nm; circles) primed with a synthetic DNA oligonucleotide, as described previously (42) with the exception that double-stranded DNA product was measured by PicoGreen fluorescence. Specific activities during purifications were determined in reactions containing [3H]dTTP as described.
Purification of an endogenous PA replicase. Purification of endogenous clamp loader complex was performed as described under “Experimental Procedures.” A, heparin-Sepharose column profile. B, Q Sepharose-HP column profile. C, methyl HIC column profile. D, Superose-6 HR column profile. In A–C, curves indicate salt gradients (A), protein concentration (B), and DNA synthesis activity (C). Representative fraction numbers are indicated by the arrows. DNA synthesis activity was measured as described under “Experimental Procedures,” using saturating levels of PA αα (16 μg/ml) and β (12 μg/ml).

Table One

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity</th>
<th>Total volume</th>
<th>Total protein</th>
<th>Specific activity&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>units × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>ml</td>
<td>mg</td>
<td>units/mg × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
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<tr>
<td>Fraction I</td>
<td>1600</td>
<td>28,000</td>
<td>870</td>
<td>2.3</td>
</tr>
<tr>
<td>Fraction II 35% (NH₄)₂SO₄</td>
<td>20</td>
<td>220</td>
<td>860</td>
<td>1.7&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Fraction II S-heparin load</td>
<td>9.1</td>
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<td>130</td>
<td>7.0</td>
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<td>Fraction III heparin pool</td>
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<td>130</td>
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</tr>
<tr>
<td>Fraction III Q load</td>
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</tr>
<tr>
<td>Fraction IV HIC load</td>
<td>2.6</td>
<td>4.3</td>
<td>43</td>
<td>6.1</td>
</tr>
<tr>
<td>Fraction V HIC pool</td>
<td>1.0</td>
<td>9.0</td>
<td>9.9</td>
<td>10</td>
</tr>
<tr>
<td>Fraction V Superose load&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.97</td>
<td>1.3</td>
<td>9.9</td>
<td>9.8</td>
</tr>
<tr>
<td>Fraction VI Superose pool&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.26</td>
<td>3.6</td>
<td>0.43</td>
<td>61</td>
</tr>
</tbody>
</table>

<sup>a</sup> Specific activity is based on stimulation of DNA synthesis in a reconstituted holoenzyme assay in the presence of saturating levels of PA αα and β as described under “Experimental Procedures.”

<sup>b</sup> An ~2-fold loss in activity was observed between the initial assay of Fraction II and the initiation of the subsequent chromatography step. A similar loss was observed between the two subsequent purification steps, but not in the later stages of the purification. This may be attributable to denaturation or to the action of a protease that was eliminated during the latter stages of purification.

<sup>c</sup> Only 40% of Fractino V was actually loaded onto Superose-6. The Superose load and pool values for total protein, volume, and activity have been normalized assuming a 100% loading of Fraction V.

Previously (42), enzyme titrations were performed to determine the linear range of the assay, and specific activities were calculated using points in the linear range. Units are defined as pmol of nucleotide incorporated per min at 22 °C. Unless otherwise stated, assays were performed under low salt conditions, defined as 10 mM magnesium acetate, and no additional monovalent salt aside from a low level of salt carried over from enzyme storage buffers (usually less than 5 mM NaCl). Purification of other PA proteins was performed as described previously (42). E. coli proteins were purified as described: χψ (35), SSB (44), and DnaG (45).

Protein Analysis—SDS-PAGE and gel analysis were performed as described previously (42). Peptide mass fingerprinting for protein identification was performed by Amprox, Inc. (Carlsbad, CA). Protein concentrations were determined using the Coomassie Plus Protein Assay (Pierce) and a bovine serum albumin standard. We assumed that the stoichiometries of PA protein assemblies were the same as their E. coli counterparts: αα, ββ, ταβ, χψ, and SSB.<sub>v</sub>

Construction of Expression Vectors—The sequences of each gene were amplified by PCR from PA01 genomic DNA and inserted in expression vectors. When necessary, E. coli low usage codons in the N terminus of PA genes were changed to high usage codons, and non-AUG start codons were altered to AUG to facilitate efficient translation in E. coli. The PCR primers were as follows: holC forward, 5′-GGATCTAGAGGAGGAGATTTGAGATCTCATTGCTCGATTTTACG; holC reverse, 5′-GGATATGCTTCTCTCTGATCCATCGAGGATGCACCCAG; PA4679 forward, 5′-GGATCTAGAGGAGGAGATTTGAGATCTCATTGCTCGATTTTACG; PA4679 reverse, 5′-GGATATGCTTCTCTCTGATCCATCGAGGATGCACCCAG. The vector pET14b (Novagen) was cut with XbaI and BamHI; the holC PCR fragment was cut with HindIII and BamHI; the PA4679 PCR fragment was cut with HindIII and BamHI; the PA4679 Superose 6 PCR fragment was cut with XbaI and HindIII, and the resulting fragments were ligated together, creating the plasmid pET14b-PA-4679holC. The vector pET14b and the PA4679 PCR fragment were cut with XbaI and Xhol, and the resulting fragments were ligated together, creating the plasmid pET14b-PA-4679.

Vectors expressing 32 upstream amino acids at the N terminus of PA4679 were constructed as follows. A 448-nucleotide fragment, PCR PA 4679-32, was PCR-amplified from PA genomic DNA using
the following primers: 5'-GGATTCTAGAAGGAGGTACCAAGC-GATGCAGATTACCAGTTGGC and 5'-CAGCAGCAGGTAACCCGGA. pET14b-PA-4679 and PCR PA 4679-32 were digested with XbaI and BstEII, and the resulting fragments were ligated together, creating the plasmid pET14b-PA-N4679. pET14b-PA-4679holC and PCR PA 4679-32 were digested with XbaI and BstEII, and the resulting fragments were ligated together, creating the plasmid pET14b-PA-N4679holC.

Vectors expressing 45 upstream amino acids at the N terminus of PA4679 were constructed as follows. A 487-nucleotide fragment, PCR PA 4679-45, was PCR-amplified from PA genomic DNA using the following primers: 5'-GGATTCTAGAAGGAGGTACCAACTAATGATCGAAGAACAGCGTCGC and 5'-CAGCAGCAGGTAACCCGGA. pET14b-PA-4679 and PCR PA 4679-45 were digested with XbaI and BstEII, and the resulting fragments were ligated together, creating the plasmid pET14b-PA-L4679. pET14b-PA-4679holC and PCR PA 4679L were digested with XbaI and BstEII, and the resulting fragments were ligated together, creating the plasmid pET14b-PA-L4679holC.

Expression of N-terminally His-tagged PA—A 455-nucleotide fragment containing the PA holC gene, PCR PA holC, was PCR-amplified from PA genomic DNA using the following primers: 5'-GATCATGCATACCCGCGTCGATTTCTACGTGATCCCCA and 5'-GCATACTAGT-GAGCTCTCATCAGATACGCGGCAGGCGAT. The vector pA1-NB-KpnI contains a pBR322 origin, the gene for laqIQ repressor protein and the pA1 promoter that is repressed by laqIQ repressor. pA1-NB-KpnI was designed to express proteins with a 13-amino acid peptide tag that is biotinylated in vivo; it also contains a hexahistidine moiety and a thrombin cleavage site (46). pA1-NB-KpnI was digested with PstI and SpeI, and PCR PA holC was cut with NsiI and SpeI. The resulting fragments were ligated together, creating the plasmid pA1-NB-PAholC.

Affinity Purification of Tagged PA and Untagged PA4679 Variants—Cells transfected with pA1-NB-PAholC (expressing N-terminally hexa-His-tagged PA), pET14b-PA-4679 (expressing untagged PA4679 protein), pET14b-PA-N4679 (expressing untagged PA4679N32 protein), and pET14b-PA-L4679 (expressing untagged PA4679N45 protein) were induced and lysed, and crude lysates (Fraction I) were prepared as described above. All subsequent steps were performed at 4 °C. Each Fraction I was concentrated by precipitation with ammonium sulfate (80% saturation). Pellets (Fraction II) were resuspended in one-fourth their original volume and desalted in buffer F to remove residual ammonium sulfate in preparation for Ni2+-NTA affinity purification. Tagged PA χ Fraction II (0.5 ml) was added to 50 μl of Ni2+-NTA magnetic beads (Qiagen), and the mixture was rocked gently for 1 h. The beads were immobilized on a magnet, the supernatant was removed, and the beads were washed twice with 0.4 ml of buffer F. 1.0 ml of Fraction II from each PA4679 variant of interest was then added to the beads, and the mixtures were rocked for 1 h. The supernatant was removed, and the beads were washed twice with 0.4 ml of buffer F, followed by one wash with 0.2 ml of

FIGURE 2. Stimulation of DNA synthesis by endogenous replicase. DNA synthesis activity was measured by the Picogreen assay at 22 °C as described under “Experimental Procedures.” Reactions contained saturating levels of PA αε (14 μg/ml; 2 pmol) and β (13 μg/ml; 4 pmol) and thus were designed to detect activity of clamp loader complex. The following curves are depicted: titration of PA αε,βε in the presence of 300 mM potassium glutamate (●) or no added salt (■), titration of PA αε,βε in the presence of saturating amounts of E. coli χα and 300 mM potassium glutamate (●) or no added salt (■), titration of endogenous replicase (Fraction VI, Superose-6 pool) in the presence of 300 mM potassium glutamate (●) or no added salt (●).

FIGURE 3. Composition of endogenous pol III* complex. Fraction VI (Superose-6 pool) was analyzed by SDS-PAGE. The letters indicate bands that were excised and analyzed by peptide mass fingerprinting (TABLE TWO). The asterisks indicate bands that were not analyzed because they appeared to correspond to contaminants, the bulk of which eluted from gel filtration at a lower molecular weight than the active complex.
buffer G. Proteins bound to Ni\textsuperscript{2+}-NTA were then eluted from the beads with 75 ml of buffer H. The eluate would include tagged PA χ as well as any proteins bound to χ.

**Purification of ψ from an Overexpression Vector**—Rosetta DE3 cells (Novagen) were transfected with pET14b-PA-L4679holC and grown to 1 A\textsubscript{600} in a shake flask at 37 °C in F broth (yeast extract (14 g/liter), tryptone 8 (g/liter), KH\textsubscript{2}PO\textsubscript{4} (12 g/liter), KH\textsubscript{2}PO\textsubscript{4} (1.2 g/liter), 1% glucose) with 100 μg/ml ampicillin and 10 μg/ml chloramphenicol. Expression was induced by the addition of isopropyl β-D-thiogalactoside to 1 mM. Cells were harvested at 2 h postinduction. Cells were suspended in an equal volume of cold Tris-sucrose (50 mM Tris-HCl, 10% sucrose) and frozen by liquid nitrogen. 9.7 g of frozen cells were lyzed as described for PA above. All purification steps were performed at 4 °C.

**RESULTS**

**Purification of an Endogenous DNA Replication Complex from P. aeruginosa**—The stimulatory effect of E. coli ψ on DNA synthesis by the recombinant PA minimal replicase (42) pointed to the existence of PA χ and ψ analogs. PA χ was apparent based on sequence similarity to its E. coli counterpart, but PA ψ could not be deduced. In order to identify the ψ subunit from PA, our initial strategy was to purify endogenous clamp loader complex from PA. Based on the known characteristics of the E. coli clamp loader complex, we hoped that PA ψ would copurify with PA τ, δ, and δ′ as a tightly bound complex. Purification was monitored by a functional assay in which saturating levels of recombinant PA αε and β were added to each column fraction.

As a first step, crude lysate from PA was subjected to ammonium sulfate fractionation. We found that the activity was relatively insoluble in solutions containing ammonium sulfate, providing a simple initial purification step to form Fraction III. The activity was further purified by column chromatography using S Sepharose/Heparin-Sepharose HP column (Amersham Biosciences) equilibrated in buffer A\textsubscript{30}. The column was washed with 35 ml of buffer A\textsubscript{30} at a flow rate of 1 ml/min. The protein was eluted using a 75-ml linear gradient from 30 to 600 mM NaCl at a flow rate of 1 ml/min. Fractions showing peak PA ψ activity were pooled to form Fraction III.

**Accession Numbers**—Accession numbers for ψ sequences from different bacteria were as follows. P. aeruginosa (TIGR NT03PA5405 includes primary annotation and TIGR annotation; see also NP_253368.1); Pseudomonas syringae (NP_790830.1); Pseudomonas fluorescens (YP_088781.1); Vibrio vulnificus (NP_935480.1); Vibrio parahemolyticus (NP_798821.1); Vibrio cholerae (NP_230305.1); Photobacterium profundum (YP_128846.1); Erwinia carotovora (YP_048846.1); Yersinia pestis (NP_995024.1); E. coli (NP_290985.1); Salmonella typhimurium (NP_463413.1); Photobacterium luminescens (NP_931428.1); Candidatus Blochmannia floridanus (NP_878417.1).

**Protein Sequence Analysis**—The E. coli ψ subunit amino acid sequence was used to identify related proteins using BLASTP (47). Proteins that were more than 90% identical to existing members of the set were excluded from further analysis, leaving 16 ψ sequences, which were aligned using ClustalW (48). Similarly, the PA ψ subunit amino acid sequence was used to identify and align related proteins. The E. coli and Pseudomonad ψ family alignments were then aligned to each other using ClustalWPROF (48).

**TABLE TWO**

<table>
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<tr>
<th>Gel band</th>
<th>Apparent mass</th>
<th>Relative abundance</th>
<th>Assignment</th>
<th>Sequence annotation</th>
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<td>A</td>
<td>110–140</td>
<td>2.0</td>
<td>PA3640</td>
<td>DNA pol III α chain</td>
<td>130.9</td>
</tr>
<tr>
<td>B</td>
<td>70–90</td>
<td>2.5</td>
<td>PA1532</td>
<td>DNA pol III τ subunit</td>
<td>73.3</td>
</tr>
<tr>
<td>C</td>
<td>37</td>
<td>1.0</td>
<td>PA3989</td>
<td>DNA pol III δ subunit</td>
<td>37.4</td>
</tr>
<tr>
<td>D</td>
<td>35</td>
<td>0.9</td>
<td>PA2961</td>
<td>Hypothetical protein</td>
<td>35.7</td>
</tr>
<tr>
<td>E</td>
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<td>1.0</td>
<td>PA4679</td>
<td>DNA pol III ε subunit</td>
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</tr>
<tr>
<td>F</td>
<td>26</td>
<td>2.2</td>
<td>PA1816</td>
<td>DNA pol III ε subunit</td>
<td>26.8</td>
</tr>
<tr>
<td>G</td>
<td>18</td>
<td>0.8</td>
<td>PA4232</td>
<td>Single-stranded DNA-binding protein</td>
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<tr>
<td>H</td>
<td>16.5</td>
<td>1.1</td>
<td>PA3832</td>
<td>DNA pol III χ subunit</td>
<td>16.1</td>
</tr>
</tbody>
</table>

\* Excised bands from SDS-PAGE shown in Fig. 3.

\* Relative abundance compared with δ subunit based on densitometry, normalized for molecular weight.

\* Protein identification was based on peptide mass fingerprinting (Amprox, Inc.) using publicly available protein sequence databases; the numbers indicate the PA genes encoding the detected proteins.
expected components of the clamp loader complex in roughly the expected stoichiometries and also the α and ι subunits of PA pol III holoenzyme. Thus, although the assays were designed to monitor purification of just the clamp loader complex, the purified complex actually resembled *E. coli* pol III*, which contains all of the holoenzyme subunits except β (10).* The complex also contained a 32-kDa protein of similar abundance relative to χ, δ, and ι. This protein of unknown function was encoded by PA gene PA4679 and appeared to be a candidate for the ψ subunit.

### TABLE THREE

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity, units $\times 10^{-9}$</th>
<th>Total volume, ml</th>
<th>Total protein, mg</th>
<th>Specific activity, units/mg $\times 10^{-9}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 1</td>
<td>2.3</td>
<td>25</td>
<td>400</td>
<td>4.4</td>
</tr>
<tr>
<td>Fraction II (30% AS)</td>
<td>4.8</td>
<td>53</td>
<td>44.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Fraction III (Q pool)</td>
<td>2.3</td>
<td>20</td>
<td>48</td>
<td>4.7</td>
</tr>
</tbody>
</table>

5 ψ-dependent DNA synthesis activity was measured in the presence of 200 mM potassium glutamate as described under "Experimental Procedures." A subsaturating level of PA $\tau_{\delta\delta'}$ was used (0.7 μg/m; 0.15 pmol), and all other components were present at saturating levels. Background from $\tau_{\delta\delta'}$ alone was subtracted prior to calculation of ψ specific activity.

Identification of ψ—As annotated in the *Pseudomonas* genome project (available on the World Wide Web at www.pseudomonas.com), PA4679 encodes a protein of 233 amino acids with a predicted molecular mass of 24.9 kDa. The apparent molecular mass of the native polypeptide based on gel mobility, however, was ~32 kDa. Whereas this discrepancy could have been a SDS-PAGE artifact, we also considered mechanisms that would produce a larger protein product. Translational read-through of the stop codon was not likely, since several other in-frame termination codons would be encountered in rapid succession. A +1 translational frameshift near the predicted C terminus would add 49 amino acids, a change of approximately the right magnitude; a −1 frameshift would add 149 amino acids, giving a much larger protein product.

We also considered whether the translational start site was correctly annotated. There was an in-frame upstream AUG that would encode a protein with 32 additional amino acids at the N terminus, for a total molecular mass of 28.5 kDa. Further upstream, initiation at an in-frame UUG would add 45 amino acids to the N terminus, for a total molecular mass of 30.0 kDa. Sequence similarity between these upstream translated regions and conserved hypothetical proteins in *P. putida* and *P. syringae* supported the upstream translational start hypothesis. Furthermore, peptide mass fingerprinting of the native protein did not reveal any pepids consistent with either of the translational frameshift models but did detect several pepids consistent with the upstream start hypotheses (data not shown). The actual N-terminal peptide was not detected in the mass spectral analysis, however, leaving the exact translational start site ambiguous. We were also unable to obtain N-terminal amino acid sequence of the native protein by Edman degradation, despite several attempts. Therefore, we cloned and expressed three versions of the ψ candidate gene, denoted PA4679 for the original gene and PA4679N32 and PA4679N45, respectively, for the genes encoding the 32- and 45-amino acid N-terminal extensions (Fig. 4A).

Each of the three ψ candidate proteins was capable of binding to immobilized N-terminally His-tagged PA χ (Fig. 4B). The three proteins migrated on SDS-PAGE at approximately the expected sizes relative to molecular weight markers. The largest of the three proteins (encoded by PA4679N45) showed mobility comparable with the endogenous polypeptide (Fig. 4C) and was the only one that stimulated DNA replication (Fig. 4D). We concluded that PA4679N45 corresponds to the *E. coli* hold gene and encodes the ψ subunit of PA pol III holoenzyme. The observed stimulation of pol III holoenzyme activity by the affinity-purified χ and ψ was completely dependent on $\tau_{\delta\delta'}$ (data not shown), indicating that the stimulation resulted from formation of a fully functional clamp loader complex.

Expression, Purification, and Characterization of PA χψ—An inducible expression vector was constructed to co-express PA4679N45 and the holC gene from *P. aeruginosa*. Untagged χ and ψ could thereby be expressed in *E. coli* and purified as a complex. χψ complex was meas-

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*Fraction VI showed DNA synthesis activity when assayed in the presence of β without exogenously added αε (data not shown), consistent with the expected properties of a pol III*-like complex.*

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### FIGURE 4

Identification of the authentic form of PA χψ. A, N-terminal sequences of translated polypeptides corresponding to PA4679N45 (a), PA4679N32 (b), and PA4679 (c) are shown. B, crude lysates from cells expressing PA4679 (lane 1), PA4679N32 (lane 2), and PA4679N45 (lane 3) were mixed with His-tagged PA χ immobilized to Ni$^{2+}$-NTA beads and eluted as described under "Experimental Procedures." Band a (~32 kDa), b (~31 kDa), and c (~28 kDa) correspond to PA4679N45, PA4679N32, and PA4679 proteins, respectively. Identical results were obtained when C-terminally His-tagged χ was used (data not shown). Controls were also performed to confirm that lysates from cells expressing PA4679 variants did not show any apparent binding of the recombinant proteins to Ni$^{2+}$-NTA in the absence of tagged χ (data not shown). C, SDS-PAGE comparison of endogenous PA clamp loader complex Fraction VI (lane 1; ψ candidate denoted by an asterisk) with affinity-purified expressed PA4679N45 protein (lane 2; same as B, lane 3). D, ψ-dependent DNA synthesis activity was measured in the presence of 200 mM potassium glutamate by the Picogreen assay as described under "Experimental Procedures." A subsaturating level of PA $\tau_{\delta\delta'}$ was used (0.7 μg/m; 0.15 pmol), and all other components were present at saturating levels. Affinity-purified samples were prepared as described under "Experimental Procedures." Lane 1, basal $\tau_{\delta\delta'}$ activity with no added χ or ψ; lane 2, E. coli ψ (12 μg/ml); lane 3, E. coli χ (11 μg/ml); lane 4, affinity-purified tagged PA χ (1 μg/ml); lane 5, affinity-purified PA4679 protein and tagged PA χ (1 μg/ml); lane 6, affinity-purified PA4679N32 protein and tagged PA χ (1 μg/ml); lane 7, affinity-purified PA4679N45 protein and tagged PA χ (1 μg/ml; corresponds to B, lane 3).
ured based on stimulation of DNA synthesis in the holoenzyme reconstruction assay. \( \chi \psi \) was purified by ammonium sulfate fractionation and Q-Sepharose chromatography (TABLE THREE and Fig. 5A). Based on densitometry, the purified complex appeared to contain a 1:1 ratio of \( \chi \) and \( \psi \) (data not shown).

When \( \tau_5 \beta^\delta \) was titrated at low salt in the presence of \( \alpha \) and \( \beta \), both \( \chi \psi \) and SSB were required to achieve optimal holoenzyme activity (Fig. 5B). In the absence of either \( \chi \psi \) or SSB, little activity was observed. The addition of SSB alone substantially increased the maximal extent of the reaction; the addition of \( \chi \psi \) alone had little effect. The addition of both \( \chi \psi \) and SSB\(^5 \) had a synergistic effect, increasing the apparent specific activity of \( \tau_5 \beta^\delta \) 25-fold, and further elevating the maximal extent of the reaction. In contrast, addition of Pseudomonas \( \chi \psi \) to \( E. \ coli \) \( \tau_5 \beta^\delta \) did not result in stimulation of reconstituted \( E. \ coli \) pol III holoenzyme (data not shown). This is consistent with the lower level of stimulation observed in the \( E. \ coli \) system upon the addition of \( E. \ coli \) \( \chi \psi \) (35, 36, 41).

Under the low salt conditions shown in Fig. 5B, high concentrations of \( \tau_5 \beta^\delta \) could largely overcome the requirement for \( \chi \psi \) (i.e. stimulation by \( \chi \psi \) was only evident when \( \tau_5 \beta^\delta \) levels were subsaturating). At elevated salt, however, the synergistic contributions of both SSB and \( \chi \psi \) were essential for efficient synthesis (Fig. 5C).\(^7 \) Titration of \( PA \chi \psi \) (Fig. 5D) achieved maximal stimulation at a 1:1 ratio with \( \tau_5 \beta^\delta \). In contrast, >50-fold molar excess of \( E. \ coli \) \( \chi \psi \) was required to achieve maximal stimulation, suggesting that binding of the noncognate \( \chi \psi \) was much weaker than that of \( PA \chi \psi \).

Identification of a Pseudomonad \( \chi \psi \) Family—The amino acid sequence of \( PA \chi \psi \) was used to search for similar proteins, resulting in identification of a family of highly related proteins (Fig. 6). These were all conserved hypothetical proteins of unknown function, derived either from other Pseudomonads or \( A. \ vinelandii \), which is sometimes classified as a Pseudomonad (49). Note that although the annotated sequence for \( P. \ putida \chi \psi \) starts 31 amino acids upstream of the leucine shown in this alignment, sequence homology with this family of proteins suggests that the true start site corresponds to the start of this alignment. In addition, the annotated start site for \( P. \ fluorescens \chi \psi \) is 13 amino acids downstream from the site shown here. Again, alignment with this protein family suggests that the correct N terminus would include the italicized amino acids (Fig. 6).

Conserved Domains between \( E. \ coli \) and \( PA \chi \psi \)—When global alignments were attempted, the \( E. \ coli \) and \( PA \chi \psi \) subunits showed little amino acid sequence similarity. When the \( E. \ coli \chi \psi \) family was aligned with the Pseudomonad \( \chi \psi \) family; however, local regions of similarity were observed (Fig. 7). These regions appear to correspond to regions important for interactions with other components of the holoenzyme complex based on the \( E. \ coli \chi \psi \) crystal structure (40). For example, in \( E. \ coli \chi \psi \), two helical regions, \( \alpha_1 \) and \( \alpha_4 \), form the interface with \( \chi \alpha_1 \) from \( E. \ coli \chi \psi \) aligns with a region in \( PA \chi \psi \), showing 45% identity and 73%

---

**FIGURE 5.** Purification and characterization of expressed \( PA \chi \psi \). A, SDS-PAGE of pooled fractions from each purification step. 10 \( \mu \)g of each fraction were loaded. Lane M shows the molecular weight markers. Fraction I is the crude lysate. Fraction II is the 30% ammonium sulfate pellet, and Fraction III is the Q-Sepharose pool. DNA synthesis was measured in the primer extension assay as described under “Experimental Procedures.” B, titration of \( \tau_5 \beta^\delta \) in the presence of saturating concentrations of \( \alpha \) (32 \( \mu \)g/ml; 5 pmol) and \( \beta \) (9 \( \mu \)g/ml; 2.6 pmol) without added salt. Where indicated, saturating levels of \( \chi \psi \) (15 \( \mu \)g/ml; 2.6 pmol) and PA SSB (26 \( \mu \)g/ml; 9 pmol) were included. The following curves are depicted. 1, neither \( \chi \psi \) nor SSB; 2, with SSB; 3, with \( \chi \psi \); 4, with \( \chi \psi \) and SSB. C, titration of potassium glutamate in holoenzyme reactions containing \( \alpha \beta \chi \psi \) and \( \alpha \beta \) and/or SSB as described in B. \( \tau_5 \beta^\delta \) was present at a saturating level (22 \( \mu \)g/ml; 2.6 pmol). D, titration of \( PA \chi \psi \) and \( E. \ coli \chi \psi \) in the presence of 200 \( \mu \)M potassium glutamate and PA \( \alpha \beta \chi \psi \); PA \( \alpha \beta \), and SSB (enzyme amounts as in B) and a saturating concentration of \( \tau_5 \beta^\delta \) (0.2 \( \mu \)g/ml; 0.044 pmol). The dotted line indicates the pmol amount of \( \tau_5 \beta^\delta \) present in the reaction.

---

**FIGURE 6.** Alignment of the Pseudomonad \( \psi \) protein family. Protein sequences were aligned as described under “Experimental Procedures.” Completely conserved residues are shown in black, identical residues are shown in dark gray, and similar residues are shown in light gray. P1, \( P. \ aeruginosa \); P2, \( P. \ syringae \); P3, \( P. \ fluorescens \) (13 N-terminal italicized amino acids were not present in the annotated protein entry but were added based on genome sequence); P4, \( P. \ putida \); P5, \( A. \ vinelandii \).
In the PA system, \( \tau_{\delta \delta'} \) specific activity increased 25-fold in the presence of \( \psi \). In contrast, \( E. coli \) \( \tau_{\delta \delta'} \) specific activity was insensitive to its cognate \( \psi \) when assayed under similar conditions (42). In the \( E. coli \) system, the reported effects of \( \psi \) were modest (e.g. a 3-fold increase in overall synthesis levels at elevated salt (36)), making it difficult to quantify \( \psi \) specific activity. By modulating \( \tau_{\delta \delta'} \) levels in the PA reconstituted holoenzyme assay, particularly at elevated salt concentrations, we were able to observe a nearly complete dependence on \( \psi \). This facilitated the quantitative characterization of \( \psi \).

Characterization of the endogenous PA pol III* also provided valuable information regarding various subunits. The subunits were present in approximately the following ratio: \( \alpha/\delta/\delta' = \chi/\psi/SSB \), 2:2:2.5:1:1:1:1:1. Notably absent was any evidence of a \( \gamma \)-like truncation of \( \tau \). Thus, the frameshift that is observed during translation of \( dnaX \) in bacteria such as \( E. coli \) and \( S. typhimurium \) (20–22) does not occur to PA. This confirmed our initial hypothesis, based on sequence analysis, that PA \( dnaX \) would produce only a single full-length product.

Identification of \( \psi \) from PA led to the discovery of a \( Pseudomonad \) branch of the \( \psi \) protein family, based on sequence similarity. Our evidence strongly suggests that translation of PA4679 initiates at a non-canonical UUG located 135 nucleotides upstream of the originally annotated GUG start site. Several other members of the \( \psi \) family also use non-AUG translational start codons, doubtless complicating the prediction of open reading frames in genomic sequences. Similarity to other members of the \( Pseudomonad \psi \) family suggests that the correct translational start site for \( P. putida \) \( holD \) is probably the UUG found 90 nucleotides downstream from the AUG start site annotated in the referenced open reading frame. In addition, \( P. fluorescens \) \( \psi \) appears to initiate at a UUG 13 amino acids upstream of the annotated AUG start site. Although other members of the \( E. coli \psi \) family utilize more common initiation codons, at least one apparently erroneous annotation was encountered. \( A. peregrinum \psi \) probably initiates at an AUG 10 amino acids upstream from the annotated start site.

It is interesting to note that the genomic sequence context of \( holD \), the gene that encodes \( \psi \), is conserved between PA and \( E. coli \). The \( rimI \) gene, which encodes an acetyltransferase, lies immediately adjacent to \( E. coli \) \( holD \). The \( rimI \) immediately adjacent to \( E. coli \) \( holD \). PA \( rimI \) is immediately adjacent to PA4679, which we have shown to be \( holD \). The \( holD \) and \( rimI \) genes are also adjacent in the genomes of the other organisms represented in the \( Pseudomonad \psi \) family.

\( Pseudomonad \) family members each had a readily identifiable \( \chi \) subunit with high sequence similarity to \( E. coli \). Clearly, our inability to deduce \( \psi \) based on sequence similarity alone was attributable to poor sequence conservation rather than a genuine lack of the subunit. The same may be true of other Gram-negative bacteria in which \( \psi \) is apparent but \( \psi \) is not. Structural work on \( E. coli \) \( \chi \psi \) (40) raised questions regarding the existence of the \( \psi \) subunit in many organisms and raised the possibility of structurally divergent proteins fulfilling the functional role of \( \psi \) in these organisms. This report represents the first identification of a highly divergent branch of the \( \psi \) family.

No \( \chi \) or \( \psi \) analogs have been identified in any Gram-positive bacteria to date, despite the fact that many other holoenzyme constituents are shared across a broad spectrum of bacterial species. It is not clear whether this is also a simple consequence of evolutionary divergence or whether the requisite functions of \( \chi \psi \) are encompassed by other subunits of the Gram-negative replicase.

The proteins of the \( Pseudomonad \psi \) family are almost twice as large as their counterparts in the \( E. coli \psi \) family. Much of this extra sequence consists of regions that are highly conserved across the \( Pseudomonad \psi \) family and yet are completely absent in the \( E. coli \psi \) family.
tional significance of the additional sequence is unclear. Although the overall sequence conservation of ψ subunits is generally poor, a few notable regions show significant homology across all ψ family members. Based on structural information for E. coli χψ, these conserved regions probably represent the functional interfaces of ψ with χ and with τ. In support of this model, the N-terminally truncated forms of PA ψ (i.e., the proteins encoded by PA4679 and PA4679N32) were competent to bind PA χ but not to stimulate holoenzyme DNA synthesis activity (Fig. 4). This result is consistent with the notion that α1 and α4, which are present in both the full-length and truncated versions, mediate the interface between χ and ψ. It also supports the hypothesis that the N-terminal domain of ψ interacts with τ, the ATPase subunit that powers the clamp loader subassembly.

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Discovery and Characterization of the Cryptic Ψ Subunit of the Pseudomonad DNA Replicase
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