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Reconstitution of a Minimal DNA Replicase from *Pseudomonas aeruginosa* and Stimulation by Non-cognate Auxiliary Factors*

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DNA polymerase III holoenzyme is responsible for chromosomal replication in bacteria. The components and functions of *Escherichia coli* DNA polymerase III holoenzyme have been studied extensively. Here, we report the reconstitution of replicase activity by essential components of DNA polymerase holoenzyme from the pathogen *Pseudomonas aeruginosa*. We have expressed and purified the processivity factor (β), single-stranded DNA-binding protein, a complex containing the polymerase (α) and exonuclease (ϵ) subunits, and the essential components of the DnaX complex ($\tau_3\delta\delta'$). Efficient primer elongation requires the presence of $\alpha\epsilon$, β, and $\tau_3\delta\delta'$. *Pseudomonas aeruginosa* $\alpha\epsilon$ can substitute completely for *E. coli* polymerase III in *E. coli* holoenzyme reconstitution assays. *Pseudomonas* β and $\tau_3\delta\delta'$ exhibit a 10-fold lower activity relative to their *E. coli* counterparts in *E. coli* holoenzyme reconstitution assays. Although the *Pseudomonas* counterpart to the *E. coli* ψ subunit was not apparent in sequence similarity searches, addition of purified *E. coli* χ and ψ (components of the DnaX complex) increases the apparent specific activity of the *Pseudomonas* $\tau_3\delta\delta'$ complex ~10-fold and enables the reconstituted enzyme to function better under physiological salt conditions.

Pseudomonas aeruginosa (PA)[1] is the causative agent in a wide range of infections, including bacteremia, urinary tract infections, burn wound infections, and pulmonary infections in patients on respirators. In hospitals, PA is responsible for about one-seventh of all infections with multidrug-resistant strains becoming increasingly common (1, 2). However, the most serious medical problem caused by PA is lung infection associated with cystic fibrosis (3, 4). Antimicrobial resistance among clinical isolates of PA is significant and growing (5). Multidrug-resistant PA is a major problem in hospital patients (6). Compounds that specifically inhibit PA growth relative to non-pathogenic flora would be useful to benefit cystic fibrosis and immunocompromised patients.

Extensive genetic and biochemical studies in *E. coli* have elucidated the functions of the critical replication proteins comprising pol III holoenzyme. pol III holoenzyme exhibits unique characteristics required for efficient replication, including high processivity, a rapid elongation rate, tolerance of physiological salt concentrations, and ability to utilize a long single-stranded template coated with SSB (7). Non-replicative polymerases cannot substitute effectively either *in vitro* or *in vivo* (8). Conditionally lethal mutations have been isolated for *dna*E, *dna*Q, *dna*N, and *dna*X genes that encode the α, ϵ, β, and τ subunits, respectively (9–13). Strains carrying a deletion of *hol*D, the gene encoding the ψ subunit, show chronic induction of the SOS response resulting in a temperature-sensitive phenotype (14). Knock-out mutants of *hol*A and *hol*B have shown that both δ and δ' are essential for cell viability (15), validating them along with *dna*E, *dna*X, *dna*Q, *hol*D, and *dna*N as targets for antibacterial development.

In *Escherichia coli*, pol III holoenzyme is composed of ten different subunits that function in concert to perform highly rapid and processive DNA chain elongation from a primed template. The α subunit serves as the polymerization subunit; ϵ catalyzes a 3′-5′ exonuclease activity that is necessary for proofreading. θ binds to the N-terminal region of ϵ (16, 17). Together, α, ϵ, and θ associate tightly to form pol III (18). The β subunit confers high processivity (19). It consists of a bracelet-shaped molecule that clamps around DNA, contacting the polymerase and preventing it from falling off of the template, thus ensuring high processivity (20, 21). The asymmetric DnaX complex is responsible for transferring the sliding clamp onto a primer terminus in an ATP-dependent reaction (22–27). The native holoenzyme appears to employ a DnaX complex containing two copies of the τ subunit and one copy of the shorter γ variant along with ancillary subunits ($\tau_2\gamma_1\delta\delta'\chi\psi$) (as discussed in Ref. 7). The *dna*X gene expresses two related proteins; τ is the full-length protein, and γ is a truncated version formed by frameshifting during translation of *dna*X (28–30). τ binds the α subunit of DNA polymerase III and causes it to dimerize, forming the scaffold upon which other auxiliary proteins can assemble to form a dimeric replicative complex (31, 32). δ and δ' are required for processive elongation in addition to their role in initiation complex formation (15). χ forms a 1:1 heterodimeric complex with ψ (33). $\chi\psi$ binds tightly to domain III of γ (34), whereas χ alone does not bind to γ (35). The interaction of ψ and γ is probably mediated through the conserved N-terminal region of ψ (36). $\chi\psi$ confers resistance to high salt on DNA synthesis catalyzed by holoenzyme, and this salt resistance requires the presence of SSB (37). χ interacts with the C terminus of SSB and enhances the binding of SSB to DNA,

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[1] The abbreviations used are: PA, *Pseudomonas aeruginosa*; SSB, single-stranded DNA-binding protein; DnaX complex, $DnaX_3\delta\delta'\chi\psi$ where DnaX can be either translational product γ or τ (from *E. coli*); pol, polymerase; pol III, (α ϵ θ); pol III holoenzyme, DNA polymerase III holoenzyme; IPTG, isopropyl β-D-thiogalactoside; DTT, dithiothreitol; RFU, relative fluorescent unit(s).

TABLE I
Primers used in construction of expression vectors

Gene amplified	Primer direction	Sequence (5′ → 3′)	Restriction site(s)
DnaN	Sense	AGTCTTAATTAACATGCATTTCACCATTCAACGCGAA	PacI
DnaN	Antisense	AGTCACTAGTTTATTAGAGGCGCATCGGCATGACGA	SpeI
DnaE	Sense	AGTCTTAATTAAGCTTATGACCGTATCCTTCGTTCA	PacI, HindIII
DnaE	Antisense	AGTCACTAGTTATTAACGATAATTGAGGAAGA	SpeI
DnaQ	Sense	AGTCCATATGCGTAGCGTCGTACTGGATA	NdeI
DnaQ	Antisense	AGTCGCTAGCAAGCTTATTCCTCCTCTACTATTCGCCGACCGGCGCCTCCA	NheI, HindIII
DnaX	Sense	AGTCTTAATTAAGCTTATGAGCTATCAAGTTCTTGCGCGTAAAT	PacI
DnaX	Antisense	AGTCACTAGTCTATCAGGCCTTGGCTTCCAAA	SpeI
HolA	Sense	AGTCTTAATTAAGCTAGCATGAAACTGACCCCGGCGCAACTCGCCAAGCACCT	PacI
HolA	Antisense	AGTCACTAGTATTAAGCTTTATCCTCCTCTATCAGGCTGCCGGCAGGCCGA	SpeI
HolB	Sense	AGTCCCATGGCTGATATCTATCCCT	NcoI
HolB	Antisense	AGTCACTAGTTATGCTAGCTTTCCTCCTCTACTAGCCCGGCCCGGGCAGGCTT	SpeI
Ssb	Sense	GATCCTGCAGGACGCGGTGCTGGTCCA	PstI
Ssb	Antisense	GTCAACTAGTCGCCTGAAAACGAAGA	SpeI

thereby preventing premature dissociation of SSB from the lagging strand and increasing holoenzyme processivity (38, 39).

DNA replication is central to the propagation of all bacteria. DNA replication components are highly conserved across bacterial genera and are essential to survival but are largely distinct from the human DNA replication components. To date, published work relating to DNA replication in PA has focused on either characterization of the origin of replication (40–42) or on the biophysical properties of the single-stranded binding protein (SSB) (43). No biochemical characterization of pol III holoenzyme from PA has been reported. Here we describe the reconstitution of the DNA replication elongation apparatus from PA. This system will enable us to screen for inhibitors of PA DNA replication that may possess novel antibacterial properties.

EXPERIMENTAL PROCEDURES

Construction of Overexpression Vectors—The sequences of each gene were amplified by PCR from PA (PAO1) genomic DNA and inserted in expression vectors. The PCR primers are shown in Table I. With the exception of the SSB expression vector, all of the expression vectors utilized the semi-synthetic highly inducible and repressible pA1 promoter (44). When necessary, *E. coli* low usage codons in the N terminus of PA genes were altered to high usage codons, and non-AUG start codons were changed to AUG, to facilitate efficient translation in *E. coli*. A plasmid containing a pBR322 origin of replication, a gene expressing the *lac*I^q repressor protein, and the semi-synthetic promoter, pA1, which is repressed by *lac*I, was used. The polylinker region was modified to introduce appropriate restriction sites to facilitate insertion of the desired PA genes. Details of each construct are included with the purification descriptions below. All purification steps were performed at 4 °C unless otherwise indicated.

Cell Growth and Lysis—Expression vectors were transfected into fermentation strains, AP1.L1 (F$^-$, *omp*T *hsd*SB(rB$^-$) (srl-recA)306::Tn10, T1 phage-resistant isolate) or MGC-1030 (*mcr*A *mcr*B λ$^-$ IN(*rrn*-D-*rrn*E)1 *lex*A3Δ(*uvr*D)::T$_c^r$Δ(*omp*T)::K$_m^r$). The time course of expression of recombinant proteins at 37 °C following IPTG induction was compared for the strains (data not shown). The strain giving optimal protein expression was chosen for large scale growth. No deleterious effects on cell growth were observed with any of the clones following induction. Cells expressing PA recombinant proteins were grown in a 180-liter fermentor at 37 °C (or 30 °C for αε expression) in F broth (yeast extract, 14 g/liter; Tryptone, 8 g/liter; K_2HPO_4, 12 g/liter; KH_2PO_4, 1.2 g/liter; glucose 1%) plus 100 μg/ml ampicillin to an A_{600} of ~0.75. The pH was maintained at 7.2 by the addition of ammonium hydroxide. Additional ampicillin was added (200 μg/ml), and expression was induced (except SSB) by addition of IPTG to 1 mM. Cells were harvested at 3 h post induction 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. Cells constitutively expressing PA SSB were grown in the same conditions as described above, but without the IPTG induction step, and were harvested at stationary phase. Lysis was accomplished via creation of spheroplasts by treatment of cells with lysozyme in the presence of 10% sucrose (45). The presence of 18 mM spermidine kept the nucleoid condensed within partially disrupted cells and displaced DNA-binding proteins. Centrifugation (16,000 × *g*, 1 h, 4 °C) resulted in a DNA-free supernatant (Fraction I).

Activity Assays—Units are defined as picomoles of total nucleotide incorporated per minute at 30 °C. DNA synthesis activity was monitored during purification of αε, β, and $\tau_3\delta\delta'$ using a reconstituted *E. coli* DNA pol III holoenzyme assay (33). M13Gori single-stranded phage DNA was used as template DNA and was purified as described previously (46). As purified PA subunits became available, these were substituted for their *E. coli* counterparts as indicated. Enzymes were diluted in EDB (50 mM HEPES (pH 7.5), 20% glycerol, 0.02% Nonidet P40, 0.2 mg/ml bovine serum albumin). Primase mix containing single-stranded DNA template, nucleotides, DnaG, and SSB was prepared first by incubating the following components for 20 min at 30 °C: 50 mM HEPES (pH 7.5), 20% glycerol, 0.02% Nonidet P40, 0.2 mg/ml bovine serum albumin, 13 mM $Mg(OAc)_2$, 5 mM DTT, 0.27 mM each of rGTP, rUTP, rATP, and rCTP, 64 μM each of dATP, dCTP, dGTP, 24 μM dTTP, ~60 cpm/pmol [^{3}H]dTTP, 2.4 nM M13Gori single-stranded DNA circles, 42 μg/ml *E. coli* SSB, and 1.0 μg/ml *E. coli* DnaG primase. Primase mix was then aliquoted and frozen for use in subsequent assays. Holoenzyme reactions were reconstituted by incubating 20 μl of primase mix with *E. coli* pol III ($\alpha\varepsilon\theta$), $\tau_3\delta\delta'\chi\psi$, and β in a 25-μl reaction volume for 5 min at 30 °C. Buffer conditions were comparable to the priming reaction except that each of the components was 25% more dilute. Incorporation of [^{3}H]dTTP was measured by trichloroacetic acid precipitation on GF-C filters (18). For the component being assayed, the corresponding *E. coli* subunit was omitted, and other components were used at saturating concentrations. Enzyme titrations were performed to determine the linear range of the assay, and specific activities were calculated using points in the linear range. In some cases where it was not necessary to determine absolute incorporation rates, formation of double-stranded DNA was measured by fluorescent detection of double-stranded DNA using PicoGreen (Molecular Probes) (47). For these assays 100 RFU approximately equals 15 pmol of nucleotide incorporated. In this case, 25-μl reactions were performed in opaque 96-well plates and stopped with 25 μl of 100 mM EDTA. PicoGreen was diluted 1:150 in 10 mM Tris-HCl (pH 7.5); 150 μl was added to each well. Fluorescence emission at 538 nm was measured in a GeminiEM platereader (Molecular Dynamics) with excitation at 485 nm. DNA synthesis was measured as relative fluorescent units (RFUs).

Primer extension assays were performed on single-stranded DNA templates that had been primed with a synthetic DNA oligonucleotide, 5′-AGGCTGGCTGACCTTCATCAAGAGTAATCT-3′. The oligonucleotide primer was annealed to M13Gori single-stranded DNA circles by mixing a 1:1 molar ratio of primer to template in the presence of 0.1 M KCl and 50 mM HEPES (pH 7.5), heating to 95 °C for 4 min and slowly cooling to room temperature. Primer extension reactions were performed in a 25-μl reaction volume for 10 min in 50 mM HEPES (pH 7.5), 20% glycerol, 0.02% Nonidet P-40, 0.2 mg/ml bovine serum albumin, 10 mM $Mg(OAc)_2$, 5 mM DTT, 0.2 mM rATP, 48 μM each of dATP, dCTP, and dGTP, 24 μM dTTP, ~60 cpm/pmol [^{3}H]dTTP, 2 nM primed template DNA, and enzyme components as indicated. Acid-precipitable ^{3}H label was measured to determine the picomoles of DNA incorporated. Reaction temperatures are indicated in the figure legends.

Estimates of PA Protein Complex Concentrations—We assumed that the stoichiometry of PA protein assemblies was the same as their *E. coli* counterparts: αε, β_2, $\tau_3\delta\delta'$, and SSB_4. Protein concentrations were determined using the Coomassie Plus Protein Assay (Pierce) and a bovine serum albumin standard.

Gel Electrophoresis and Protein Analysis—SDS-PAGE analysis of $\alpha\epsilon$, $\tau_3\delta\delta'$, and β samples was performed using 4–12% acrylamide gradient pre-cast gels (Novex NuPAGE; Invitrogen) using MOPS running buffer (Invitrogen). Benchmark unstained protein molecular weight markers were used (Invitrogen). SDS-PAGE analysis of SSB samples was performed on 10–20% acrylamide Novex Tris-glycine pre-case gels (Invitrogen) using Tris-glycine running buffer (Invitrogen). Gels were stained with SimplyBlue SafeStain (Invitrogen) or with Coomassie Brilliant Blue. Comparable results were obtained with either staining method. Densitometry was performed using a Kodak Image Station 440CF. Peptide mass fingerprinting for protein identification was performed by Amprox, Inc. (Carlsbad, CA). N-terminal sequencing was performed at the Molecular Resource Center (National Jewish Medical Center) using Edman degradation.

Buffers Used in Purifications—Subscripts indicate millimolar concentration of NaCl in each buffer (*i.e.* A_{40} is A_0 and 40 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), 0.19 g/ml ammonium sulfate (*i.e.* 34% ammonium sulfate saturation). A_0 is 20 mM Tris-HCl (pH 8.0), 10% glycerol, 0.1 mM EDTA, 0.1 mM EGTA, and 0.5 mM DTT. B_0 is 50 mM Tris-HCl (pH 7.5), 10% glycerol, 0.5 mM EDTA, and 1 mM DTT. C is 50 mM imidazole (pH 6.8), 10% glycerol, 50 mM NaCl, and 0.5 mM DTT. D is 25 mM HEPES (pH 7.5), 50 mM KCl, 10% glycerol, 0.1 mM EDTA, and 0.5 mM DTT. E_0 is 25 mM Tris-HCl (pH 7.5), 10% glycerol, 0.1 mM EDTA, and 0.5 mM DTT. F is 50 mM Tris-HCl (pH 7.5), 10% glycerol, 5 mM DTT, and 0.5 mM EDTA. G_{100} is 25 mM Tris-HCl (pH 8.0), 10% glycerol, 0.2 mM EDTA, 1 mM DTT, and 100 mM NaCl.

Expression and Purification of $\alpha\epsilon$—The vector pA1-CB-NdeI and the *dna*Q PCR fragment were cut with NdeI and NheI, and the *dna*Q PCR fragment was inserted, resulting in the plasmid pA1-PA-dnaQ. The vector pA1-CB-NcoI and the *dna*E PCR fragment were cut with SpeI and PacI, and the *dna*E PCR fragment was inserted, resulting in the plasmid pA1-PA-dnaE. An operon expressing *dna*Q and *dna*E was constructed by digesting pA1-PA-dnaE1 with HindIII and SpeI. The fragment containing the *dna*E gene (~3.5 kb) was inserted into pA1-PA-dnaQ that had been digested with the same two restriction enzymes, resulting in the plasmid pA1-PA-core1, containing the *dna*Q gene upstream of the *dna*E gene. Cells (2045 g) were grown, harvested, and lysed as described above. Ammonium sulfate (0.21 g to each initial milliliter of Fraction I; 37% saturation) was added slowly to Fraction I. The precipitate was collected by centrifugation (16,000 × *g*, 30 min, 4 °C). The pellet was resuspended using a Dounce homogenizer in 0.125 × Fraction I volume of BW buffer. The remaining precipitate was collected by centrifugation (16,000 × *g*, 45 min, 4 °C). The pellet was resuspended in buffer A_0 and diluted to match the conductivity of buffer A_{40}, forming Fraction II. Fraction II was applied to a 5-cm × 7-cm MacroPrep High S (Bio-Rad) column connected in series with a 5- × 35-cm DEAE-Sepharose Fast Flow (Amersham Biosciences) column equilibrated in A_{20}. The columns were washed with 300 ml of buffer A_{20}. Then the S column was disconnected, and the DEAE-column was washed with 3200 ml of buffer A_{40} followed by a 6.4-liter linear gradient from 40 to 400 mM NaCl. The PA $\alpha\epsilon$ complex eluted at ~150 mM NaCl, and fractions with the highest specific activity were pooled to form Fraction III. Fraction III was precipitated with 50% ammonium sulfate. Fraction III pellet was resuspended in buffer A_0 and was diluted to match the conductivity of buffer A_{30} and applied to a 2.5- × 41-cm heparin-Sepharose Fast Flow (Amersham Biosciences) column equilibrated in buffer A_{30}. The column was washed with ~17 column volumes of buffer A_{30}, followed by a 2-liter linear gradient from 30 to 500 mM NaCl. $\alpha\epsilon$ eluted at ~100 mM NaCl, and fractions with the highest specific activity were pooled to form Fraction IV.

Expression and Purification of β—The vector pA1-CB-NdeI and the *dna*N PCR fragment were cut with SpeI and PacI, and the *dna*N PCR fragment was inserted, resulting in the plasmid pA1-PA-dnaN. Cells (40 g) were grown, harvested, and lysed as described above. Ammonium sulfate (0.197 g to each initial milliliter of Fraction I; 35% saturation) was added slowly to Fraction I, and the precipitate was removed by centrifugation (16,000 × *g*, 60 min, 4 °C). Ammonium sulfate was added to the resulting supernatant (0.153 g to each milliliter of 35% supernatant; 60% saturation), and the precipitate was collected by centrifugation (16,000 × *g*, 60 min, 4 °C). The pellet containing the 35–60% ammonium sulfate fraction was resuspended in buffer B_0 diluted to match the conductivity of buffer B_{50}, forming Fraction II. Fraction II was applied a 2.5- × 40-cm DEAE-Sepharose Fast Flow column (Amersham Biosciences) equilibrated in buffer B_{50}. The column was washed with 400 ml of buffer B_{50}, followed by a 2-liter linear gradient from 50 to 600 mM NaCl gradient. The β subunit eluted at ~75 mM NaCl, and fractions with the highest specific activity were pooled to form Fraction III. Fraction III was applied to a 5- × 20-cm hydroxyapatite column (Hypatite C, Clarkson Chemical Co.) equilibrated in buffer C. The column was washed with 800 ml of buffer C, followed by a 4-liter linear gradient from 0–200 mM potassium phosphate. The β subunit eluted at ~100 mM phosphate, and fractions with the highest specific activity were pooled to form Fraction IV. Fraction IV was dialyzed into buffer D for storage.

Expression and Purification of $\tau_3\delta\delta'$—The vector pA1-CB-NcoI and the *hol*B PCR fragment were cut with SpeI and NcoI, and the *hol*B PCR fragment was inserted, resulting in the plasmid pA1-PA-holB. The vector pA1-CB-NcoI and the *hol*A PCR fragment were cut with SpeI and PacI, and the *hol*A PCR fragment was inserted, resulting in the plasmid pA1-PA-holA. The vector pA1-CB-NcoI and the *dna*X PCR fragment were cut with SpeI and PacI, and the *dna*X PCR fragment was inserted, resulting in the plasmid pA1-PA-dnaX. An operon expressing *hol*B, and *hol*A was constructed by digesting both pA1-PA-holA and pA1-PA-holB with NheI and SpeI. The fragment from pA1-PA-holA containing holA (~1 kb) was then inserted into the digested pA1-PA-holB, resulting in the plasmid pA1-PA-holBA. A three-gene operon was then constructed by digesting both pA1-PA-dnaX and pA1-PA-holBA with HindIII and SpeI. The fragment of pA1-PA-dnaX containing the *dna*X gene (~2 kb) was then inserted into the digested pA1-PA-holBA plasmid, resulting in the plasmid pA1-PA-BAX, with the *hol*B gene upstream, followed by *hol*A and *dna*X, respectively. Cells (400 g) were grown, harvested, and lysed as described above. Ammonium sulfate (0.197 g to each initial ml of Fraction I; 35% saturation) was added slowly to Fraction I, and the precipitate was collected by centrifugation (16,000 × *g*, 30 min, 4 °C). The ammonium sulfate pellet was resuspended in buffer E_0 and diluted to match the conductivity of buffer E_{20}, forming Fraction II. Fraction II was divided into three parts, and one-third was applied to 20-ml MacroPrep High S (Bio-Rad; 4- × 5-ml S Econo-Pac cartridges) connected in series to a 5- × 10.2-cm heparin-Sepharose 6 Fast Flow column (Amersham Biosciences) equilibrated in buffer E_{20}. The columns were washed with 600 ml of buffer E_{20}. The High S cartridges were removed, and the heparin column was washed with another 200 ml of buffer E_{20}, followed by a 2-liter linear gradient from 20 to 1000 mM NaCl. $\tau_3\delta\delta'$ eluted at ~150 mM NaCl, and fractions with the highest specific activity were pooled to form Fraction IIIA. The columns were then regenerated as per the manufacturer's recommendations, and additional Fraction II was purified, creating Fractions IIIB and IIIC. Ammonium sulfate (0.366 g/ml Fraction III; 60% saturation) was added slowly to each batch of Fraction III, and the precipitate was collected by centrifugation (16,000 × *g*, 30 min, 4 °C). Fraction III pellets were resuspended in buffer F_0 and diluted to match the conductivity of buffer F_{20}, and Fractions IIIA, IIIB, and IIIC were pooled and applied to a 2.5- × 20-cm DEAE-Fast Flow column (Amersham Biosciences) equilibrated in buffer F_{20}. The column was washed with 300 ml of buffer F_{20}, followed by a 1,000-ml linear gradient from 20 to 500 mM NaCl. $\tau_3\delta\delta'$ complex eluted at ~250 mM NaCl, and fractions with the highest specific activity were pooled to form Fraction IV.

Expression and Purification of SSB—The vector pBluescript II KS (Stratagene) and the *ssb* PCR fragment were digested with SpeI and PstI, and the *ssb* PCR fragment was inserted, resulting in the plasmid pBlue-lac-Pa-ssb. Unlike the vectors used for expression of the genes described above, this vector gave constitutive expression of the recombinant gene. Cells (1000 g) were grown, harvested, and lysed as described above. Ammonium sulfate (0.136 g to each initial milliliter of Fraction I; 25% saturation) was added slowly to Fraction I, and the precipitate was collected by centrifugation (16,000 × *g*, 30 min, 4 °C). The ammonium sulfate pellets were resuspended in buffer G_{100} and diluted in buffer G_0 to match the conductivity of buffer G_{100}, forming Fraction II. Insoluble material was removed by centrifugation and Fraction II was applied to a 5- × 10-cm column packed with Q-Sepharose Fast Flow (Amersham Biosciences) and equilibrated in buffer G_{100}. The column was washed with 1000 ml of buffer G_{100}, followed by a 2-liter linear gradient from 100 to 1000 mM NaCl. PA SSB eluted at ~320 mM NaCl, and fractions were pooled based on gel analysis of purity to form Fraction III.

Other Proteins—E. coli holoenzyme components were purified as described: pol III (44), $\tau_3\delta\delta'\chi\psi$ (48), τ (26), δ (49), δ' (50), β (46), and $\chi\psi$ (33). $\tau_3\delta\delta'$ was reconstituted by mixing individual components at the specified molar ratio.

RESULTS

Identification and Cloning of the Minimal Components of PA pol III Holoenzyme—Examination of the PA genomic sequence (51) revealed the complete sequences of the genes encoding a

TABLE II
DNA replication proteins from P. aeruginosa

PA gene name	PA gene number	Protein encoded	No. of amino acids	Identity/similarity to *E. coli* protein
				%
DnaE	PA3640	DnaE (α)	1173	58/71
DnaQ	PA1816	DnaQ (ϵ)	246	51/62
DnaN	PA0002	DnaN (β)	367	56/71
DnaX	PA1532	DnaX (τ)	681	42/53
HolA	PA3989	HolA (δ)	345	31/44
HolB	PA2961	HolB (δ')	328	29/45
HolC	PA3832	HolC (χ)	142	30/47
Ssb	PA4232	SSB	165	56/62

majority of the subunits of the pol III holoenzyme from PA as judged by amino acid sequence similarity to the known subunits in *E. coli* (Table II). No equivalent to the *E. coli* pol III θ subunit was apparent in the PA genome. Because θ is not essential (52), we assumed that the PA θ subunit would not be essential either. We found no apparent frameshifting site in the PA *dna*X gene that would lead to formation of a γ subunit. This did not appear to be a concern, because the full-length *E. coli* τ protein can substitute for γ *in vitro* (26) and *in vivo* (53). In *E. coli*, ψ is a binding partner to χ, and both associate with γ, τ, δ, and δ' to form the DnaX complex. In PA, the *hol*C gene, encoding χ, was apparent based on homology, but the *hol*D gene, encoding ψ, was not. The sequence of ψ is not highly conserved across proteobacteria (36). The apparent lack of identifiable ψ subunits in these bacteria and in PA may reflect sequence divergence rather than actual absence of the subunit. In *E. coli*, the reconstituted holoenzyme reaction is only moderately stimulated by the presence of $\chi\psi$ (37). Thus, we expected that a functional holoenzyme could be reconstituted from PA in the absence of $\chi\psi$.

Each identified replicase gene was amplified from PA genomic DNA by PCR and inserted in vectors for expression in *E. coli*. All of the proteins were expressed as native polypeptides without inclusion of any tag sequences. Proteins that function in a complex in *E. coli* were expressed in operons. Thus, *dna*X, *hol*A, and *hol*B were co-expressed, and *dna*E was co-expressed with *dna*Q. Expression vectors were transfected into fermentation strains, and induction conditions were optimized to give maximum expression and solubility of the recombinant proteins (data not shown). Cells were grown in large scale in a fermentor, and processed as described under "Experimental Procedures."

Purification of PA $\alpha\epsilon$—The α (polymerase) and ϵ (proofreading exonuclease) subunits were co-expressed and purified as a complex. PA $\alpha\epsilon$ was purified from cells transfected with pA1-PA-core1 and induced with IPTG. In initial trial purifications, a gap-filling assay was used to monitor non-processive polymerase activity (18). In subsequent purifications, activity was monitored in a holoenzyme reconstitution assay (see "Experimental Procedures"). Both α and ϵ expressed well with this system, comprising greater than 25% of the total cell protein. Unfortunately, when cells were grown and induced at 37 °C, α was found largely in inclusion bodies. When growth and induction were performed at 30 °C, the yield of soluble α increased ~3-fold.[2] Preliminary studies indicated that ϵ had exonuclease activity as expected (data not shown). The ϵ subunit from *E. coli* is insoluble when overproduced alone; upon co-expression with α it forms a 1:1 complex that is soluble, but excess ϵ is in inclusion bodies (54). By contrast, co-expression of the ϵ- and α-subunits from PA resulted in a large excess of soluble ϵ. Excess ϵ separated from the $\alpha\epsilon$ complex during purification (see below).

Initial purification attempts were complicated by the fact that $\alpha\epsilon$ exhibits a narrow pH tolerance, with optimal stability retained only at pH 7.5 or above. Exposure to pH 7.0 or below resulted in rapid loss of activity. In addition, we found that stability was markedly enhanced when EDTA or EGTA was included in the storage buffer (data not shown). Therefore the purification protocol described here was performed entirely at pH 8.0 in the presence of EDTA and EGTA.

As has been seen previously for *E. coli* pol III (55), PA $\alpha\epsilon$ was relatively insoluble in solutions containing ammonium sulfate. Fraction II was formed by precipitation with ammonium sulfate at 37% saturation, followed by backwashing with a 34% saturated ammonium sulfate solution. Fraction II was applied to an S-Sepharose column that was connected in series to a DEAE-Sepharose column. The S column served to remove the endogenous *E. coli* pol III and RNA polymerase as well as a number of other major contaminating proteins, while allowing the majority of the PA $\alpha\epsilon$ to pass through.[3] The S column was then disconnected, and the DEAE-Sepharose column was developed with a linear salt gradient. The fractions showing the highest specific activity (Fig. 1, *A* and *B*, and Table III) were pooled to form Fraction III. Excess ϵ subunit eluted early in the gradient, whereas the $\alpha\epsilon$ complex eluted later. Fraction III was applied to a heparin-Sepharose column. The column was washed extensively and then developed with a linear salt gradient. The fractions showing the highest specific activity (Fig. 1, *C* and *D*, and Table III) were pooled to form Fraction IV. SDS-PAGE analysis of the purification steps showed that Fraction IV was at least 95% pure (Fig. 1*E*). The purified polymerase complex exhibited an α:ϵ subunit stoichiometry of ~1:1 based on densitometric scans of gels of Fraction IV. Purified $\alpha\epsilon$ eluted as a single peak when subjected to high resolution gel filtration with Superdex-200 resin (data not shown).

Purification of β—The β subunit was purified from *E. coli* cells expressing the *dna*N gene from PA. The crude lysate was adjusted to 35% ammonium sulfate to precipitate contaminating proteins including the *E. coli* pol III holoenzyme. The supernatant was then adjusted to 60% ammonium sulfate to precipitate β, forming Fraction II. Fraction II was applied to a DEAE-Sepharose column; the column was washed and then

[2] A substantial percentage of α was still insoluble even under optimized fermentation growth conditions at 30 °C. Lowering temperature growth further resulted in lower overall induction and was not beneficial. Attempts to improve the yield of soluble α by co-expressing with molecular chaperones (DnaJ, DnaK, GrpE, and/or GroEL-GroES) were unsuccessful.

[3] Protein that bound to the S column was step-eluted with 1 M NaCl and analyzed for activity and composition. 95% of the DNA synthesis activity passed through the S column and bound to the DEAE-Sepharose column. SDS-PAGE analysis of the S eluate showed some high molecular mass proteins (~125–150 kDa) that were excised and subjected to peptide mass fingerprinting (Amprox, Inc.). The results indicated that both *E. coli* DNA polymerase α subunit and RNA polymerase β subunit were present in the S eluate (data not shown).

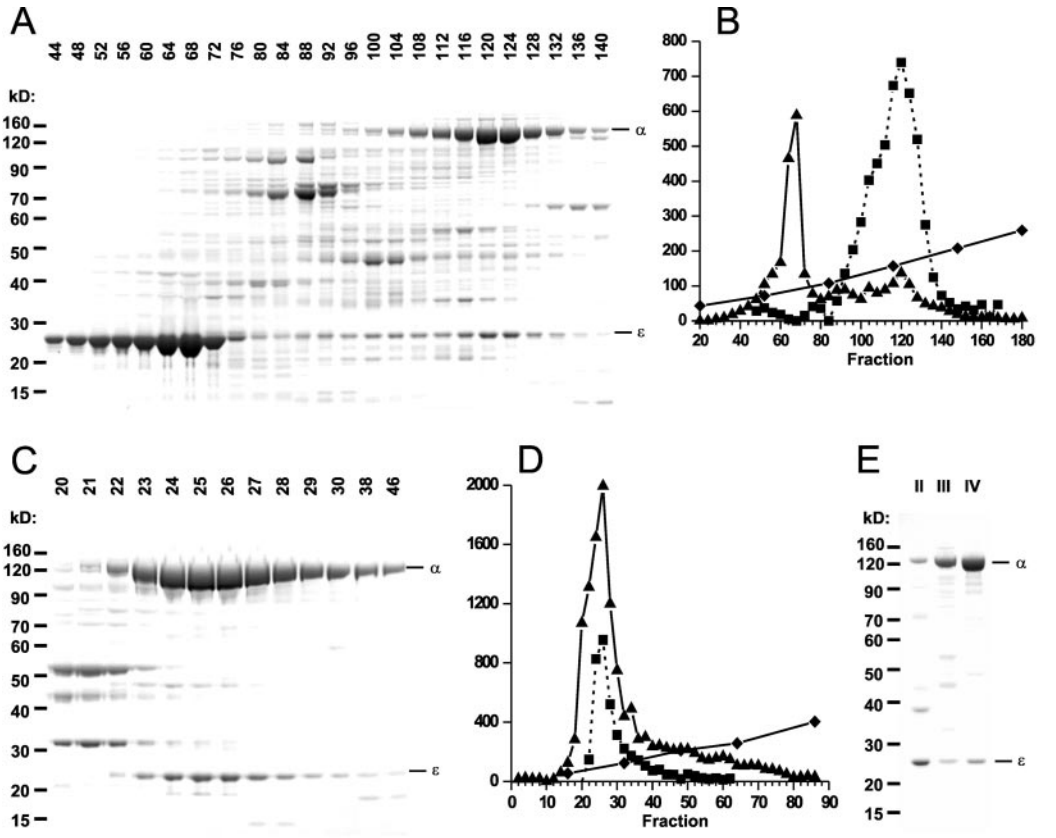

FIG. 1. **Purification of PA *αϵ*.** *A*, SDS-PAGE of fractions from DEAE-Sepharose chromatography. Fraction numbers are shown *above each lane*. *B*, profile of fractions resulting from DEAE-Sepharose chromatography. Indicated is the salt gradient (♦) in millimolar NaCl, the protein concentration (▲) in μg/ml ($\times 10^{-1}$), and the polymerase activity (■) in RFU (100 RFU approximately equals 15 pmol of nucleotide incorporated). Polymerase activity was measured in the holoenzyme reconstitution assay in the presence of 3.8 μg/ml *E. coli* DnaX complex ($\tau_3\delta\delta'\chi\psi$) and 8.6 μg/ml PA β. Fractions 104–133 were pooled to form Fraction III. *C*, SDS-PAGE of fractions from heparin-Sepharose chromatography. Fraction numbers are shown *above each lane*. *D*, profile of fractions from heparin-Sepharose chromatography. The plot indicates the salt gradient (♦) in millimolar NaCl, the protein concentration (▲) in μg/ml, and the polymerase activity (■) in RFU. Fractions 25–29 were pooled to form Fraction IV. *E*, SDS-PAGE of pooled fractions from each purification step. 2 μg of each fraction was loaded. Fraction II is the 34% ammonium sulfate cut, Fraction III is the DEAE-Sepharose pool, and Fraction IV is the heparin-Sepharose pool.

TABLE III
Summary data for purification of DNA polymerase III holoenzyme components

Protein	Fraction	Total activity	Total volume	Total protein	Specific activity
		Units $\times 10^{-8}$	*ml*	*mg*	*Units/mg* $\times 10^{-5}$
$\alpha\epsilon$	Fr II (34% AS)	10	2400	6240	1.7
	Fr III-DEAE pool	3.3	820	656	5.0
	Fr IV heparin pool	5.6	105	135	41
β	Fr II (35–60% AS)	130	1000	4430	29
	Fr III-DEAE pool	170	228	2054	85
	Fr IV HAP pool (dialyzed)	100	253	1075	97
$\tau\delta\delta'$	Fr II (35% AS)	13	1395	3125	4.1
	Fr III heparin pool[a]	9.6	950	1074	9.0
	Fr IV-DEAE pool	8.4	205	920	9.2
SSB	Fr II (25% AS)		1835	2881	
	Fr III Q pool		385	2503	

[a] Fractions (Fr) IIIA, Fr IIIB, and Fr IIIC from the three heparin chromatography batches were pooled to form Fr III.

developed with a linear salt gradient. The fractions showing the highest specific activity (Fig. 2, *A* and *B*, and Table III) were pooled to form Fraction III. Fraction III was applied to a hydroxyapatite column; the column was washed and then developed with a linear phosphate gradient. The fractions showing the highest specific activity (Fig. 2, *C* and *D*, and Table III) were pooled to form Fraction IV. SDS-PAGE analysis of the purification steps showed that β was purified to near homogeneity (Fig. 2*E*).

Purification of the DnaX Complex—We purified a complex of the essential components of the DnaX complex comprising τ, δ, and δ'. Unlike DnaX complex from *E. coli* (56), this complex lacks the χ and ψ subunits. The $\tau_3\delta\delta'$ complex[4] was purified from cells transfected with pA1-PA-BAX and induced with IPTG. The crude lysate was adjusted to 35% ammonium sulfate to precipitate $\tau_3\delta\delta'$ and form Fraction II. Fraction II was applied to a MacroPrep S column connected in series to a heparin-Sepharose column. $\tau_3\delta\delta'$ passed through the S column and bound to the heparin column. The heparin column was washed

[4] We assumed that the ratios of the subunits would be the same as in *E. coli*. Although crude densitometry supported this assumption, a more rigorous verification of the subunit stoichiometries has not been performed.

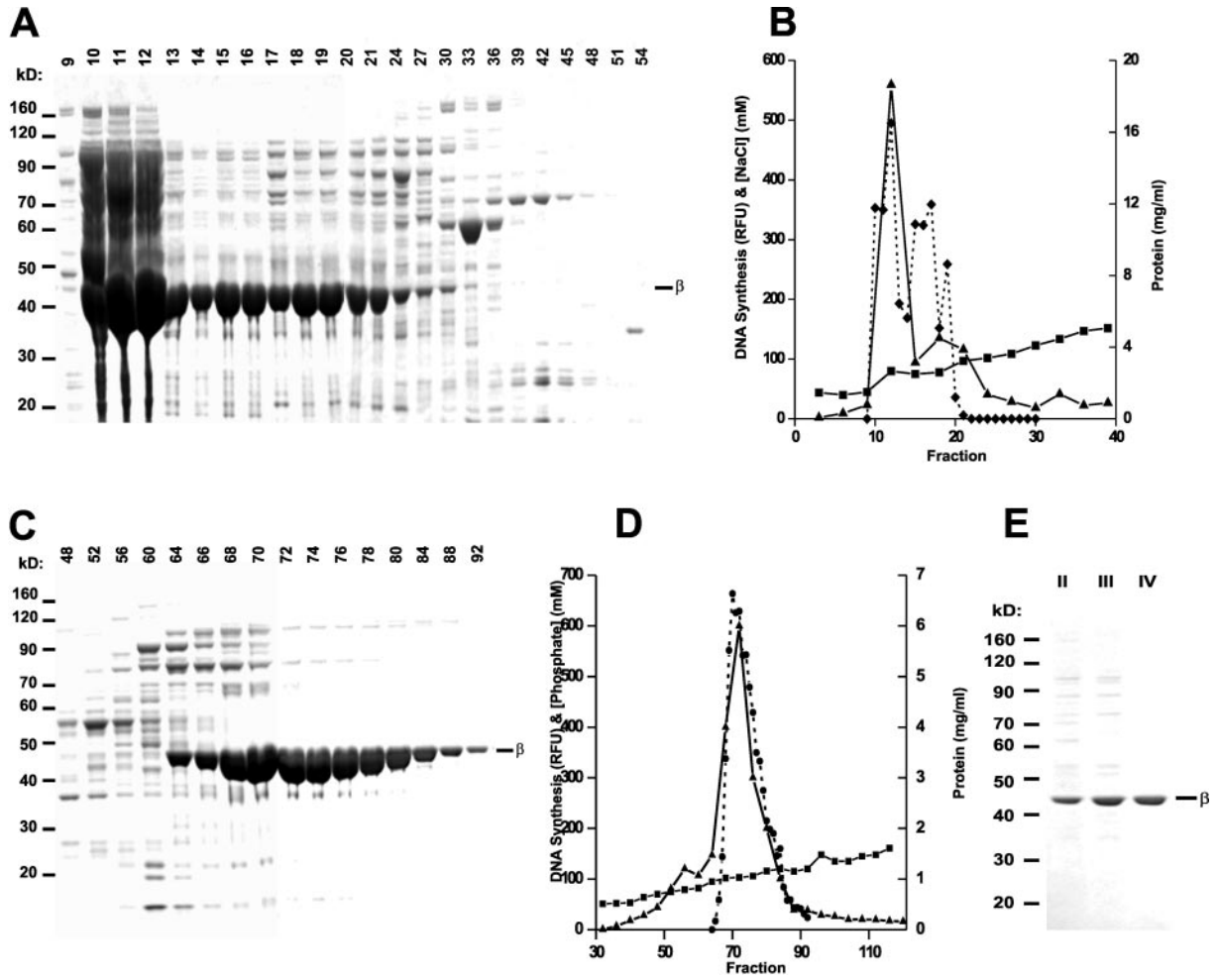

FIG. 2. **Purification of β sliding clamp.** *A*, SDS-PAGE of fractions from DEAE-Sepharose chromatography. Fraction numbers are shown *above each lane*. *B*, profile of fractions from DEAE-Sepharose chromatography. The plot indicates the salt gradient (■) in millimolar NaCl, the protein concentration (▲) in mg/ml, and β activity (♦) in RFU. The β activity was measured in the holoenzyme reconstitution assay in the presence of 3 μg/ml *E. coli* pol III and 6 μg/ml *E. coli* DnaX complex ($\tau_3\delta\delta'\chi\psi$). Fractions 10–19 were pooled to form Fraction III. *C*, SDS-PAGE of fractions from hydroxyapatite chromatography. Fraction numbers are shown *above each lane*. *D*, profile of fractions from hydroxyapatite chromatography. The plot indicates the salt gradient (■) in millimolar potassium phosphate, the protein concentration (♦) in mg/ml, and β activity (●) in RFU. Fractions 68–78 were pooled to form Fraction IV. *E*, SDS-PAGE of pooled fractions from each purification step. 1 μg of each fraction was loaded. Fraction II is the 35–60% ammonium sulfate cut, Fraction III is DEAE-Sepharose pool, and Fraction IV is the hydroxyapatite pool.

and then developed with a linear salt gradient. The fractions showing the highest specific activity (Fig. 3, *A* and *B*, and Table III) were pooled to form Fraction III. Fraction III was applied to a DEAE-Sepharose column; the column was washed and then developed with a linear salt gradient. The fractions showing the highest specific activity (see Fig. 3, *C* and *D*, and Table III) were pooled to form Fraction IV. SDS-PAGE analysis of the purification steps is shown in Fig. 3*E*.[5]

We were surprised to find that the τ subunit migrated with an apparent molecular mass that was almost 20 kDa higher than the expected 73 kDa. The identity of the protein was confirmed by N-terminal sequencing. Tryptic digest followed by mass spectrometry detected the expected C terminus of the protein and no additional peptides besides those encoded by the *dna*X gene. Therefore, the anomalous migration of the τ subunit does not appear to be the result of translational readthrough or frameshifting. The most likely explanation for the anomalous migration is that the protein has an unusually low isoelectric point (predicted pI ≈ 4.6) resulting in reduced SDS binding under the pH conditions used in standard electrophoresis. The band migrated with a progressively lower apparent molecular mass as the pH of the gel running buffer was lowered (data not shown).

A protein of about 45 kDa co-purified with $\tau_3\delta\delta'$ activity. N-terminal sequencing showed that this protein was an N-terminal fragment of τ. Relative to the amount of full-length DnaX, the amount of the N-terminal fragment was between 10–30% in different preparations. In *E. coli*, the γ subunit is formed by translational frameshifting, creating an N-terminal fragment of τ (29). The PA *dna*X gene lacks the consensus sequence thought to be responsible for the frameshift. The shorter protein is likely a proteolytic breakdown product of τ.[6]

Purification of SSB—Initial attempts to express PA SSB

[5] Like αε, the $\tau_3\delta\delta'$ complex was highly sensitive to changes in pH, and rapidly lost activity at pH below 7. Options for purification were therefore limited to higher pH conditions. Certain chromatography resins also caused separation of the τ, δ, and δ′ subunits. Thus, although a number of other chromatography resins were tested, no other useful purification steps were identified.

[6] Inclusion of protease inhibitors during purification did not alter the amount of the N-terminal τ fragment, but this doesn't rule out a proteolytic event occurring during cell growth or cleavage by an uninhibited protease. Samples of the fragment were subjected to digestion with three different proteases, and each digest was analyzed by mass spectrometry (data not shown). Based on this analysis of the C terminus appeared to be between amino acids 373 and 381. Unfortunately, the actual C-terminal peptide was not observed, so the identity of the C-terminal amino acid was not defined.

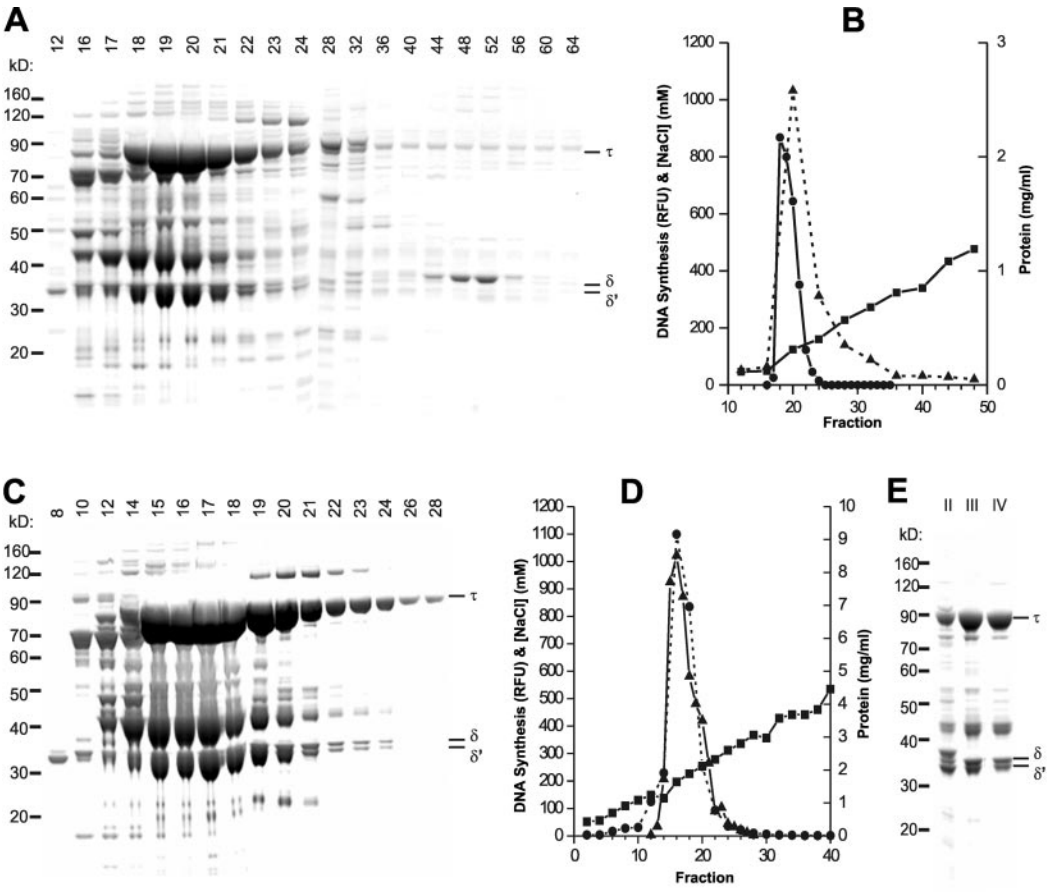

FIG. 3. **Purification of $\tau_3\delta\delta'$.** *A*, SDS-PAGE of fractions from heparin-Sepharose chromatography. Fraction numbers are shown above each lane. *B*, profile of fractions from heparin-Sepharose chromatography. The plot indicates the salt gradient (■) in millimolar NaCl, the protein concentration (▲) in mg/ml, and $\tau_3\delta\delta'$ activity (●) in RFU. $\tau_3\delta\delta'$ activity was measured in the holoenzyme reconstitution assay in the presence of 6 μg/ml *E. coli* pol III and 10 μg/ml PA β. Fractions 18–22 were pooled to form Fraction IIIA. *C*, SDS-PAGE analysis of fractions from DEAE-Sepharose chromatography. Fraction numbers are shown *above each lane*. *D*, profile of fractions from DEAE-Sepharose chromatography. The plot indicates the salt gradient (■) in millimolar potassium phosphate, the protein concentration (●) in mg/ml, and $\tau_3\delta\delta'$ activity (▲) in RFU. Fractions 15–23 were pooled to form Fraction IV. *E*, SDS-PAGE of pooled fractions from each purification step. 5 μg of each fraction were loaded. Fraction II is the 35% ammonium sulfate cut, Fraction III is the heparin-Sepharose pool and Fraction IV is the DEAE-Sepharose pool.

using an inducible pA1 promoter resulted in very low expression levels. An excellent overexpression of PA SSB using a constitutive expression vector that included substantial upstream and downstream genomic sequence flanking the *ssb* gene has been reported (43). Reasoning that the genomic sequence context may enhance transcription or translation and/or stabilize the mRNA transcript, we expressed PA SSB using a construct that included 178 nucleotides of upstream genomic sequence and 97 nucleotides of downstream genomic sequence. This system produced PA SSB constitutively at a level of about 10% of total cellular protein. Fraction II was prepared by precipitation of the crude lysate with 25% ammonium sulfate. The resulting Fraction II (>90% pure) was applied to a Q-Sepharose column. The column was developed with a linear salt gradient, and fractions containing SSB were pooled based on SDS-PAGE to form Fraction III (Fig. 4, *A* and *B*). The identity of the purified protein was verified by peptide mass fingerprinting.

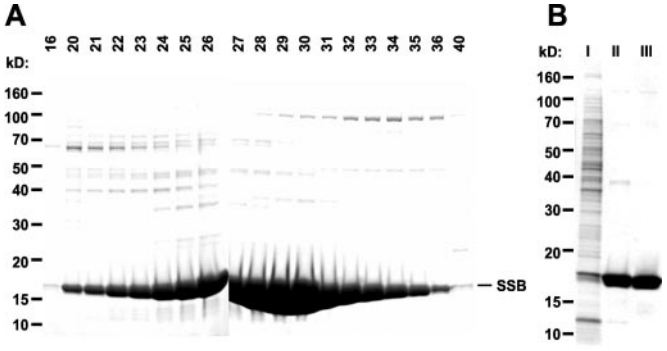

FIG. 4. **Purification of SSB.** *A*, SDS-PAGE analysis of fractions from DEAE-Sepharose chromatography. Fraction numbers are shown above each lane. Fractions 20–35 were pooled to form Fraction III. *B*, SDS-PAGE of pooled fractions from each purification step. 5 μg of each fraction was loaded. Fraction I is the crude lysate, Fraction II is the 25% ammonium sulfate cut, and Fraction III is the DEAE-Sepharose pool.

Reconstitution of DNA Polymerase Holoenzyme Activity—$\alpha\epsilon$, β, $\tau_3\delta\delta'$, and SSB were tested for their ability to reconstitute PA pol III holoenzyme activity (Fig. 5, *A–D*). The 8623-nucleotide single-stranded circular DNA template was primed by a single DNA oligonucleotide primer. As expected, nucleotide incorporation was completely dependent on $\alpha\epsilon$; under the conditions shown there was a 2- to 5-fold stimulation by each of the other components. We found that decreasing the $\alpha\epsilon$ concentration gave a concomitant increase in the dependence on both β and $\tau_3\delta\delta'$ (data not shown). Using a sub-saturating amount of $\alpha\epsilon$, the activity of each component was then measured alone and in combination with the other components (Fig. 5*E*). In the absence of SSB, only the combined presence of $\alpha\epsilon$, β, and $\tau_3\delta\delta$ was sufficient to produce a signal above background. In the presence of SSB, modest levels of synthesis by subassemblies were observed ($\alpha\epsilon$ plus β or $\alpha\epsilon$ plus $\tau_3\delta\delta'$), but a synergistic increase

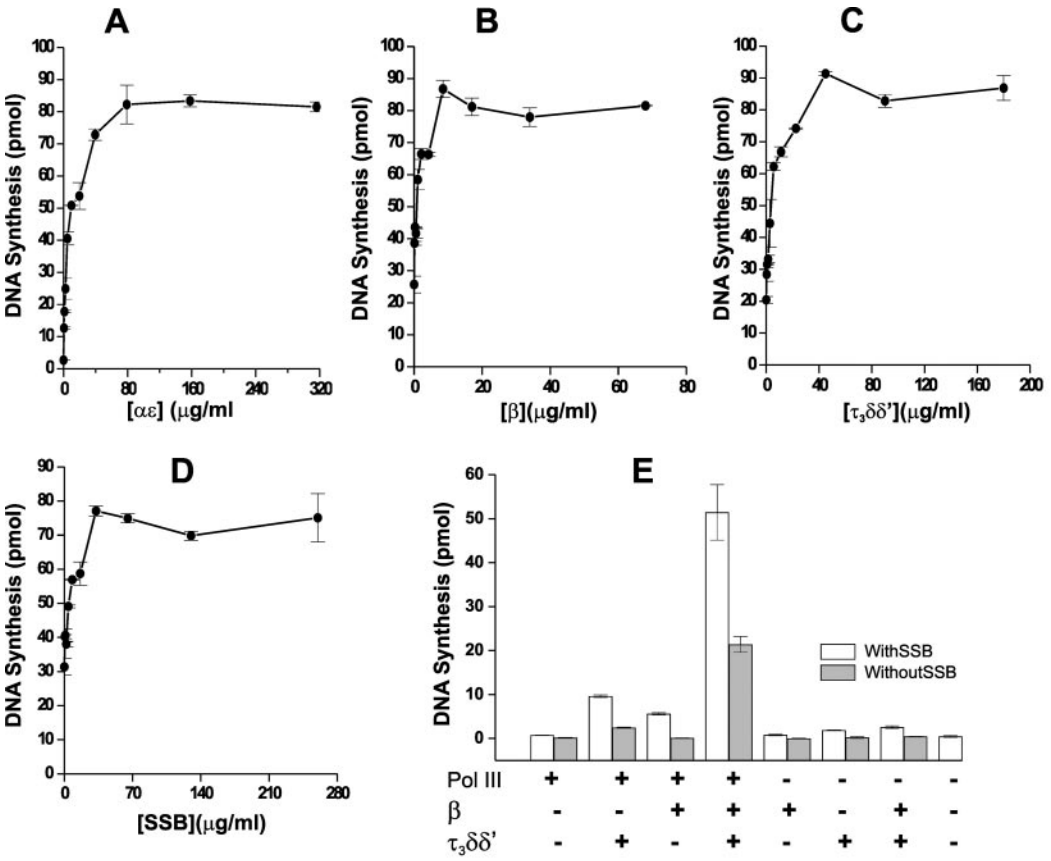

FIG. 5. **Reconstitution of PA pol III holoenzyme.** DNA synthesis was measured at 30 °C by the primer extension assay as described under "Experimental Procedures." *A*, titration of PA $\alpha\epsilon$ in the presence of saturating concentrations of PA β, $\tau_3\delta\delta'$, and SSB (17, 45, and 26 μg/ml, respectively). *B*, titration of PA β in the presence of saturating concentrations of PA $\alpha\epsilon$, $\tau_3\delta\delta'$, and SSB (79, 45, and 26 μg/ml, respectively). *C*, titration of PA $\tau_3\delta\delta'$ in the presence of saturating concentrations of PA $\alpha\epsilon$, β, and SSB (79, 17, and 26 μg/ml, respectively). *D*, titration of PA SSB in the presence of saturating concentrations of PA $\alpha\epsilon$, β, and $\tau_3\delta\delta'$ (79, 9, and 45 μg/ml, respectively). *E*, PA $\alpha\epsilon$ (labeled as *Pol III* in figure), β, $\tau_3\delta\delta'$, and SSB were present at 20, 4, 22, and 16 μg/ml, respectively, in combinations as indicated. Reactions were performed as described for the primer extension assay under "Experimental Procedures" with the addition of 100 mM potassium glutamate.

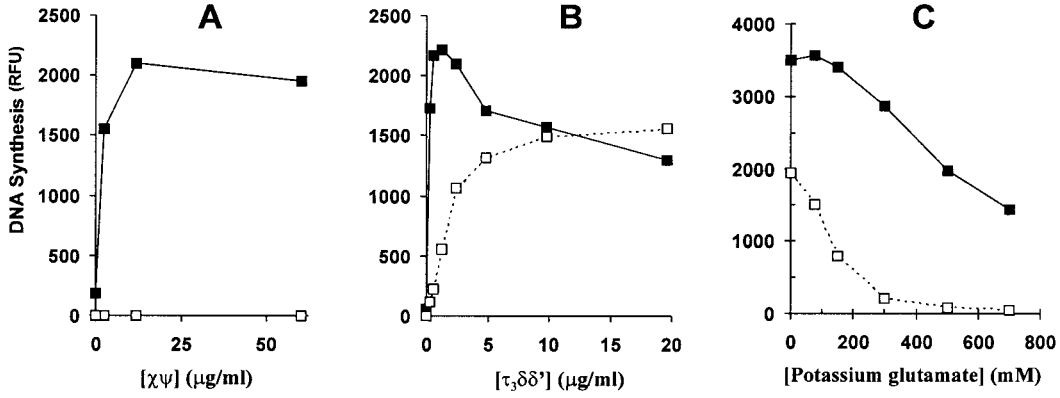

FIG. 6. **Stimulation of the PA-reconstituted holoenzyme reaction by *E. coli* $\chi\psi$.** DNA synthesis was measured by the holoenzyme reconstitution assay using PicoGreen detection of double-stranded DNA as described under "Experimental Procedures." Reactions were performed for 5 min at 22 °C using templates pre-primed with *E. coli* DnaG primase and SSB as described under "Experimental Procedures." Because these reactions were performed using different priming conditions and reaction temperatures than those used in the previous studies, the reaction components were re-titrated to determine the amounts needed to achieve saturation. Reaction buffers contained no added monovalent salt except as indicated in part *C*. In *A*: ■, titration of *E. coli* $\chi\psi$ in the presence of saturating amounts of PA pol $\alpha\epsilon$ and β (14 and 9 μg/ml, respectively) and a limiting concentration of PA $\tau_3\delta\delta'$ (1 μg/ml). □, titration of *E. coli* $\chi\psi$ alone. *B*, titration of PA $\tau_3\delta\delta'$ in the presence of saturating amounts of PA pol $\alpha\epsilon$ and β (14 and 9 μg/ml, respectively) with (■) or without (□) a saturating amount of *E. coli* $\chi\psi$ (12 μg/ml). *C*, titration of potassium glutamate in the presence of saturating amounts of PA pol $\alpha\epsilon$ and β (14 and 9 μg/ml, respectively) and a near-saturating level of $\tau_3\delta\delta'$ (3 μg/ml) with (■) or without (□) a saturating amount of *E. coli* $\chi\psi$ (12 μg/ml).

in synthesis was evident when all components were combined. Addition of both $\tau_3\delta\delta'$ and β to $\alpha\epsilon$ gave a 70-fold increase in activity over $\alpha\epsilon$ alone, consistent with the reconstitution of a highly processive holoenzyme. SSB only stimulated the reconstituted holoenzyme elongation reaction by about 2-fold. Changing the order of addition of SSB did not change the observed activity (data not shown).

α, ϵ, τ, δ, and δ' Form a Functional Complex That Co-purifies on Gel Filtration—We purified a subassembly of the PA pol III holoenzyme that contained α, ϵ, τ, δ, and δ' by co-lysing cells

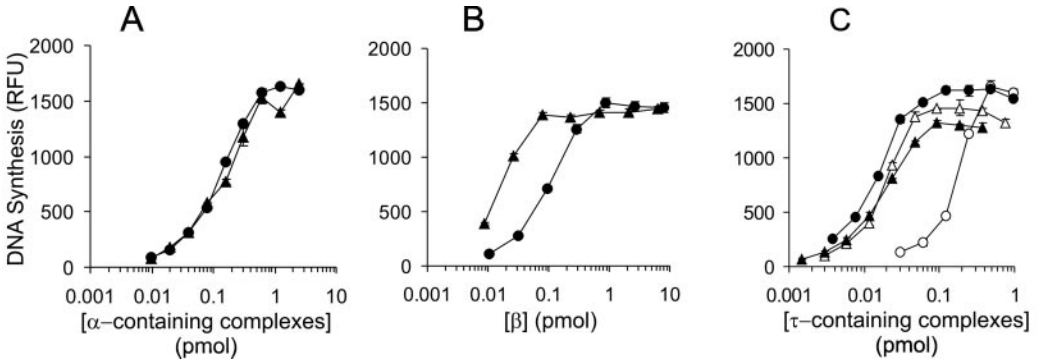

FIG. 7. **Substitution of PA components in *E. coli* holoenzyme reconstitution assays.** DNA synthesis by *E. coli* pol III holoenzyme was measured using PicoGreen detection of double-stranded DNA synthesized. 25-μl reactions were performed for 5 min at 22 °C using templates pre-annealed with DNA oligonucleotide primer as described under "Experimental Procedures." Reactions contained saturating levels of each *E. coli* component with the exception of the component being titrated. Saturating levels were: 0.9 pmol of pol III (6 μg/ml), 1.1 pmol of β_2 (3.6 μg/ml), 0.6 pmol of $\tau_3\delta\delta'\chi\psi$ (8 μg/ml), 11 pmol of SSB_4 (33 μg/ml), and where indicated 10 pmol of $\chi\psi$ (12 μg/ml). In *A*: ▲, titration of *E. coli* pol III. ●, titration of PA $\alpha\epsilon$. In *B*: ▲, titration of *E. coli* β; ●, titration of PA β. In *C*: △, titration of *E. coli* $\tau_3\delta\delta'$; ▲, titration of *E. coli* $\tau_3\delta\delta'\chi\psi$; ○, titration of PA $\tau_3\delta\delta'$; ●, titration of PA $\tau_3\delta\delta'$ in the presence of saturating amounts *E. coli* $\chi\psi$.

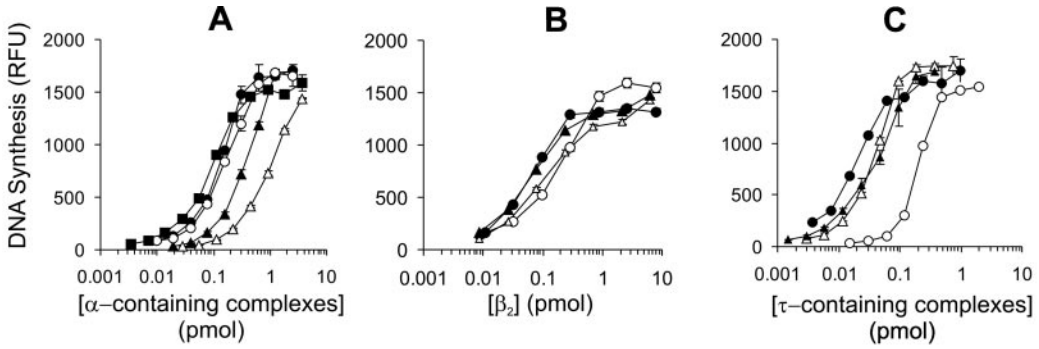

FIG. 8. **Substitution of *E. coli* components in PA holoenzyme reconstitution assays.** DNA synthesis by PA pol III holoenzyme was measured as described in the legend to Fig. 7. Reactions contained saturating levels of each PA component with the exception of the component being titrated; saturating levels were 5 pmol of $\alpha\epsilon$ (32 μg/ml), 3.6 pmol of β_2 (12 μg/ml), 1.1 pmol of $\tau_3\delta\delta'$ (13 μg/ml), 9 pmol of SSB (27 μg/ml), and where indicated 10 pmol of *E. coli* $\chi\psi$ (12 μg/ml). In *A*: △, titration of PA $\alpha\epsilon$; ▲, titration of PA $\alpha\epsilon$ in the presence of *E. coli* $\chi\psi$; ■, titration of PA $\alpha\epsilon$ in an assay where *E. coli* $\tau_3\delta\delta'\chi\psi$ substituted for PA $\tau_3\delta\delta'$; ●, titration of *E. coli* pol III; ○, titration of *E. coli* pol III in the presence of *E. coli* $\chi\psi$. In *B*: △, titration of *E. coli* β_2; ▲, titration of *E. coli* β_2 in the presence of *E. coli* $\chi\psi$; ○, titration of PA β_2; ●, titration of PA β_2 in the presence of *E. coli* $\chi\psi$. In *C*: △, titration of *E. coli* $\tau_3\delta\delta'$; ▲, titration of *E. coli* $\tau_3\delta\delta'\chi\psi$; ○, titration of PA $\tau_3\delta\delta'$; ●, titration of PA $\tau_3\delta\delta'$ in the presence of saturating amounts *E. coli* $\chi\psi$.

expressing $\alpha\epsilon$ with cells expressing $\tau_3\delta\delta'$. Activity was assessed in the reconstitution assay using purified PA β. The activity was purified by ammonium sulfate fractionation followed by Q-Sepharose chromatography and gel filtration (data not shown). The complex had an apparent molecular mass of 500–600 kDa based on gel filtration standards. The fact that these subunits formed a complex that was active in DNA synthesis and co-purified by gel filtration supports the functional relevance of the reconstituted system we have developed thus far. This complex was similar to the pol III* complex that has been reported from *E. coli* (57) except that it lacked χ and ψ.

E. coli $\chi\psi$ Stimulates DNA Synthesis by Minimal Reconstituted PA Replicase—In *E. coli*, the tightly associated χ and ψ subunits of the DnaX complex play a role in supporting DNA synthesis, especially at high salt and in increasing the affinity of the DnaX complex subunits for one another (33, 37). In PA, ψ was not apparent by sequence comparison, and therefore these studies were performed with a subassembly of only the essential τ, δ, and δ' subunits. In reconstituted PA pol III holoenzyme elongation reactions in the presence of limiting $\tau_3\delta\delta'$ (~10% of the amount required for saturation) *E. coli* $\chi\psi$ was found to stimulate the reaction 10-fold (Fig. 6*A*). This suggested that *E. coli* $\chi\psi$, although quite divergent, binds to PA $\tau_3\delta\delta'$ and stimulates the replicative reaction. A large molar excess of *E. coli* $\chi\psi$ was required to saturate the stimulatory response (*ca.* 100-fold), suggesting that the binding interaction was weak. Stimulation by *E. coli* $\chi\psi$ was most evident at subsaturating concentrations of $\tau_3\delta\delta'$. With addition of high concentrations of $\tau_3\delta\delta'$, the same levels of DNA synthesis could be obtained as in the $\chi\psi$-stimulated reactions (Fig. 6*B*). However, even when near saturating levels of $\tau_3\delta\delta'$ were used, the effect of *E. coli* $\chi\psi$ could be readily observed at elevated salt concentrations where $\chi\psi$ conferred an increase in salt tolerance (Fig. 6*C*).

Interchange of E. coli and PA pol III Holoenzyme Subunits—We had observed during purification that each of the PA subunits could substitute, at least to a degree, for its *E. coli* counterpart in an *E. coli* holoenzyme reconstitution assay. We wished to assess at a quantitative level the ability of each subunit to act as a surrogate in the non-cognate pol III holoenzyme. In the *E. coli* holoenzyme assay, PA $\alpha\epsilon$ substituted on an equivalent molar basis for *E. coli* pol III (Fig. 7*A*). In contrast, when β subunits were compared in the *E. coli* holoenzyme assay, 10-fold more PA β was required to achieve activity levels comparable to that exhibited by *E. coli* β, suggesting an impaired binding interaction between PA β and other *E. coli* holoenzyme components (Fig. 7*B*). Identical results were obtained for the PA $\alpha\epsilon$ and β titrations when $\chi\psi$ was omitted from the reaction (data not shown). In the *E. coli* holoenzyme assay, 10-fold more PA $\tau_3\delta\delta'$ was required to achieve the same levels of synthesis relative to *E. coli* $\tau_3\delta\delta'$. However, PA $\tau_3\delta\delta'$ could be rescued by addition of *E. coli* $\chi\psi$ such that it functioned as well as the *E. coli* counterparts (Fig. 7*C*).

In the PA holoenzyme assay, in the absence of additional components, we obtained the unexpected result that *E. coli* pol III functions better than PA $\alpha\epsilon$. Equivalent levels of synthesis are obtained at nearly 10-fold lower concentrations of *E. coli* pol III (Fig. 8*A*). This difference is largely suppressed by addition of *E. coli* $\chi\psi$ that results in stimulation of PA $\alpha\epsilon$ about 5-fold with negligible affect on *E. coli* pol III. Thus, as observed in the experiments reported in Figs. 6 and 7, the PA proteins are stimulated by *E. coli* $\chi\psi$ more than the cognate system! This observation surprised us at first, because it appeared to contradict the results reported in Fig. 7*A* where PA and *E. coli* polymerases were interchangeable in the *E. coli* system. We explored this observation further using various combinations of PA and *E. coli* components and found that the critical variable was DnaX complex. Replacement of PA $\tau_3\delta\delta'$ with *E. coli* DnaX complex in the PA holoenzyme reconstitution assay yielded a system where *E. coli* and PA polymerases gave equivalent synthesis (Fig. 8*A*). This and the results shown in Fig. 7*A* argue that the difference observed is not due to contamination of PA $\alpha\epsilon$ with inactive enzyme.

When β subunits were compared in the PA holoenzyme assay, *E. coli* β substituted on an equimolar basis with PA β (Fig. 8*B*), with $\chi\psi$ giving a slight stimulation of both *E. coli* or PA β activity. Thus, the 10-fold deficit observed for PA β is eliminated by using it in a cognate system. This result indicates that the deficiency observed in the *E. coli* holoenzyme reconstituted assay was not due to part of the PA β being inactive. As observed in the *E. coli* holoenzyme reconstitution assay (Fig. 7*C*), PA $\tau_3\delta\delta'$ exhibited 10-fold lower activity than its *E. coli* counterpart (Fig. 8*C*), but this deficit could be overcome by addition of *E. coli* $\chi\psi$.

DISCUSSION

We have expressed and purified key components of the pol III holoenzyme from PA. These proteins were sufficient to reconstitute an efficient DNA elongation reaction from a primed single-stranded DNA template. To our knowledge this is the first complete replication system that has been reconstituted from a Gram-negative bacterium aside from *E. coli*. PA is arguably the most evolutionarily distant organism from *E. coli* among the major Gram-negative pathogens. Further elucidation of the similarities and differences between the pol III holoenzymes from *E. coli* and PA may ultimately enrich our mechanistic understanding of these complex enzyme systems.

In the *E. coli* system, addition of β to pol III in the absence of the DnaX complex results in a modest increase in processivity. Thus, the stimulation of PA $\alpha\epsilon$ activity observed here with addition of PA β seemed consistent with the expected functions of these proteins (58, 59). We also observed a modest stimulation of $\alpha\epsilon$ activity with addition of $\tau_3\delta\delta'$ in the absence of β. Stimulation of pol III by τ, in the absence of other subunits, has also been observed in the *E. coli* system (32). However, in the PA system, the combined presence of $\alpha\epsilon$, $\tau_3\delta\delta'$, and β is necessary to achieve highly efficient DNA synthesis.

We found that when *E. coli* $\chi\psi$ was added to a reaction containing sub-saturating levels of $\tau_3\delta\delta'$, DNA synthesis could be stimulated dramatically to the same level as observed with saturating amounts of $\tau_3\delta\delta'$ in the absence of $\chi\psi$. In the absence of $\chi\psi$, the PA-reconstituted system was very sensitive to salt, with very little DNA synthesis seen at salt levels within a physiological range (>200 mM glutamate). Addition of *E. coli* $\chi\psi$ markedly increased the salt tolerance of the reaction to a level more typical of a replicase. Neither *E. coli* χ alone, nor tagged PA χ, was able to stimulate the synthesis reaction (data not shown). These results suggest the existence of ψ counterpart in PA and highlight its potentially important functional contributions.

It appears that our current system represents a minimal elongation system and that lack of the $\chi\psi$ component can be overcome by driving the binding equilibrium with high levels of $\tau_3\delta\delta'$. One contribution of $\chi\psi$ is to increase the affinity of the remaining DnaX complex components for one another (33). Identification of the PA ψ subunit would provide an important addition to the current system and would likely enhance the dependence of the system on each individual component, allowing one to work at lower $\tau_3\delta\delta'$ concentrations.

PA $\alpha\epsilon$ was able to substitute efficiently for *E. coli* pol III to reconstitute pol III holoenzyme in the presence of the required *E. coli* auxiliary proteins. PA $\tau_3\delta\delta'$ by itself was 10-fold less effective at limiting concentrations, but full activity could be attained by either addition of more $\tau_3\delta\delta'$ or by addition of saturating levels of *E. coli* $\chi\psi$. These observations would be consistent with the subunit interactions of PA $\tau_3\delta\delta'$ being weaker than their *E. coli* counterpart and their mutual affinities being increased by binding of *E. coli* $\chi\psi$. PA β was 10-fold less effective at limiting concentrations, but full synthesis was attained when higher levels are added. This would be consistent with a diminished affinity for *E. coli* holoenzyme subunits, either pol III or DnaX complex with which β interacts.

All *E. coli* pol III holoenzyme components, pol III, β, or $\tau_3\delta\delta'$, were able to substitute completely for the corresponding PA component in the presence of the required PA auxiliary proteins. The striking finding was that *E. coli* $\tau_3\delta\delta'$ and pol III worked better than the PA components. The relative defect in PA $\tau_3\delta\delta'$ could be restored by the addition of saturating *E. coli* $\chi\psi$, consistent with a diminished affinity of PA $\tau_3\delta\delta'$ subunits for one another, as discussed above. The defect in PA $\alpha\epsilon$ could be partially corrected by addition of high levels of *E. coli* $\chi\psi$. Because PA $\alpha\epsilon$ fully substituted for *E. coli* pol III in *E. coli* pol III holoenzyme reconstitution assays, we expected a component of the *E. coli* assay to fully restore PA $\alpha\epsilon$ activity. In hybrid assays, we found that *E. coli* DnaX complex ($\tau_3\delta\delta'\chi\psi$) restored full activity to PA $\alpha\epsilon$ in the PA holoenzyme reconstitution assay. Interestingly, *E. coli* $\tau_3\delta\delta'$ was also able to restore full activity to PA $\alpha\epsilon$ in the PA holoenzyme reconstitution assay (data not shown). These results suggest that PA $\alpha\epsilon$ lacks a function present in *E. coli* pol III ($\alpha\epsilon\theta$) and that *E. coli* DnaX complex contains a function not present in PA $\tau_3\delta\delta'$. The former deficiency might be explicable in terms of a missing PA θ subunit; the corresponding gene could not be found in sequence similarity searches. The latter function might be contained in authentic PA $\chi\psi$. If these hypotheses are true, use of these heterologous systems might be useful in detecting θ-DnaX complex functional interactions that have not been revealed by previous studies. A better understanding of these issues must await reconstitution of the complete PA replicase.

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Reconstitution of a Minimal DNA Replicase from *Pseudomonas aeruginosa* and Stimulation by Non-cognate Auxiliary Factors

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