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Crystal structures and biochemical characterization of DNA sliding clamps from three Gram-negative bacterial pathogens

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Abstract
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Keywords
dna, sliding, clamps, three, gram-negative, bacterial, pathogens, structures, crystal, biochemical, characterization

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Crystal structures and biochemical characterization of DNA sliding clamps from three Gram-negative bacterial pathogens

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ABSTRACT

Bacterial sliding clamps bind to DNA and act as protein–protein interaction hubs for several proteins involved in DNA replication and repair. The partner proteins all bind to a common pocket on sliding clamps via conserved linear peptide sequence motifs, which suggest the pocket as an attractive target for development of new antibiotics. Herein we report the X-ray crystal structures and biochemical characterization of β sliding clamps from the Gram-negative pathogens Pseudomonas aeruginosa, Acinetobacter baumannii and Enterobacter cloacae. The structures reveal close similarity between the pathogen and Escherichia coli clamps and similar patterns of binding to linear clamp-binding motif peptides. The results suggest that linear motif–sliding clamp interactions are well conserved and an antibiotic targeting the sliding clamp should have broad-spectrum activity against Gram-negative pathogens.

Keywords: antimicrobials; ESKAPE pathogens; sliding clamp

Abbreviations: CBM, clamp-binding motif; THC, tetrahydrocarbazole; FP, fluorescence polarization
1. Introduction

Antibiotic-resistance presents a growing threat to public health (Rice, 2008, 2010; Devasahayam et al., 2010; Moellering, 2011) and drives the need for discovery of new antibacterials that act via novel mechanisms (Robinson et al., 2012; Kaguni, 2018). A prominent group of bacteria harboring multidrug resistance and causing nosocomial infections worldwide are known as the “ESKAPE” pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp.). As such, these pathogens are of greatest concern for the development of innovative antibiotics (Rice, 2008, 2010; Boucher et al., 2009).

A rich source of potential new targets is the bacterial DNA replication machinery, which carefully orchestrates a complex series of protein–protein and protein–DNA interactions to faithfully replicate DNA prior to cell division (Robinson et al., 2012; Kaguni, 2018). Central in this process is the DNA polymerase III β subunit, also known as the β sliding clamp (β), a ring-shaped homodimeric protein (Kong et al., 1992) that encircles double-stranded DNA (dsDNA) (Georgescu et al., 2008a), where it serves as an interaction hub that recruits protein-binding partners to DNA.

Bacterial β interacts with the replicative DNA polymerase (Pol) III α and ε subunits (responsible for synthesizing and proof-reading nascent DNA, respectively) and in doing so confers high processivity to bacterial DNA synthesis (McHenry and Crow, 1979; Stukenberg et al., 1991; Georgescu et al., 2008a; Jergic et al., 2013). The β clamps are loaded onto dsDNA by a clamp loader complex, comprised of Pol III subunits δ(γ/τ)3δ′-ψχ (Jeruzalmi et al., 2001). Other proteins requiring β for efficient function include Pols I, II, IV and V and DNA mismatch repair proteins MutL and MutS (Dalrymple et al., 2001; López de Saro and O’Donnell, 2001; Pillon et al., 2011), among others. All of these protein partners interact with β at a common binding site (Robinson et al., 2008, 2012) using linear clamp-binding motifs (CBM) with the consensus sequence QLx1Lx2F/L; S or D preferred at x1; x2 may be absent (Dalrymple et al., 2001; Dalrymple, 2007). The CBM-binding pocket on β is comprised of two distinct sites, termed subsites I and II. Subsite I binds linear motif residues Lx2F/L and subsite II binds the QL dipeptide, with residue x1 linking these groups between the two subsites. We have proposed an ‘anchor-based’ model of linear motif binding to the Escherichia coli β in which the C-terminal (Lx2F/L) motif first binds to subsite I (the “anchor site”), followed by binding of the QL motif into subsite II (Yin et al., 2013). Non-
peptidic inhibitors, including a thioxothiazoline derivative (Georgescu et al., 2008b) and a biphenyloxime ether (Wijffels et al., 2011), have been shown to occupy subsite I only, whereas the natural cyclic peptide antibiotic griselimycin is able to occupy both subsites in the *Mycobacterium smegmatis* β (Kling et al., 2015). Structure-based design efforts have yielded peptidic inhibitors incorporating unnatural amino acids (Wolff et al., 2011), and we have previously described tetrahydrocarbazole (THC)-based inhibitors identified from fragment-based screening that occupy subsite I (Yin et al., 2014) and mimic the ‘anchor-based’ binding mechanism of linear motifs (Yin et al., 2015).

The *E. coli* β sliding clamp (here termed βEco) is the best characterized in terms of structure and function. The need to characterize β from other important human pathogens motivated us to study homologs from Gram-negative bacteria. In the present work, we present the X-ray crystal structures of β from the ESKAPE pathogens *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterobacter cloacae*. Interactions of β with conserved linear motif peptides were also assessed using fluorescence polarization (FP)-based assays and molecular modeling/dynamics simulations. Single residue substitutions in the linear motifs identified the residues necessary for binding to these β subunits. We have tested the inhibition of the linear motif interaction with THC-based inhibitors reported previously. Overall, we demonstrate a high degree of conservation of β-binding pockets and peptide interactions among the highly-diverged species considered here.

2. Materials and methods

2.1. Chemo-informatics and organic synthesis

Preparation of compounds 1–5 were described previously (designated 1e, 5a, 5b, 5l and 5m, respectively in (Yin et al., 2014, 2015)). Preparation of compound 6 is described in the Supplementary Methods.

2.2. Expression and purification of β clamps

The *E. coli* β sliding clamp (βEco) (Oakley et al., 2003) and its N-terminally His6-tagged derivative βEco-His6 (Jergic et al., 2013) were isolated as previously described. The dnaN genes from *P. aeruginosa* PAO1 (GenBank accession no. NP_064722), *A. baumannii* ATCC 19606 (accession no. EEX01755) and *E. cloacae* EcWSU1 (accession no. AEW71442) were PCR amplified using
genomic or plasmid templates. An NdeI restriction site was incorporated at the start codon of the dnaN genes and an EcoRI site followed a TAA stop codon at the 3' end. Agarose gel-purified digested fragments were inserted into expression vectors between the same sites, and nucleotide sequences were verified. The phage λ promoter vector pND706 (Love et al., 1996) or the T7 promoter vector pETMCSIII (Neylon et al., 2000) were used for expression of native or N-terminal His6-tagged sliding clamps, respectively. Plasmids were transformed into E. coli BL21Star(λDE3) or BL21(λDE3)/pLysS strains for protein expression. For production of native β from P. aeruginosa (βPae), A. baumannii (βAbm) and E. cloacae (βEcl), cells were grown in LB broth at 30 °C to A600 = 0.6. Protein expression was induced by temperature shift to 42 °C for 2.5 h. Expression of βAbm-His6 was achieved by auto-induction using the Studier system (Studier and Moffatt, 1986; Studier, 2005) with cultures being incubated at 25 °C for 2 days. Cells were collected by centrifugation and pellets snap frozen in liquid nitrogen for storage at −80 °C.

The βPae, βAbm, βAbm-His6 and βEcl clamps were purified using modifications of methods described for βEco (Oakley et al., 2003) and βEco-His6 (Jergic et al., 2013), as detailed in the Supplementary Methods. Their purity and integrity were confirmed by SDS-PAGE and electrospray ionization mass spectrometry of solutions in 0.1% formic acid (Supplementary Material, Fig. S1).

2.3. Crystallization and data collection

Crystallization experiments were conducted using the vapor-diffusion technique. Initial crystallization conditions were found using the PEGs and JSCG+ suites (Qiagen). The sitting-drop configuration was used in 96-well crystallization trays (Corning) at 21 °C. Reservoirs contained 50 μL of screen solution, and drops consisted of 1 μL of protein mixed with 1 μL of reservoir solution. Optimization experiments were conducted using the hanging-drop vapor-diffusion technique at 21 °C. VDX plates with siliconized coverslips were used. Crystals of βPae grew in JSCG+ condition B6: 100 mM phosphate-citrate pH 6.0, 40% (v/v) ethanol, 5% (w/v) PEG 1,000, with a protein concentration of 6.8 mg/mL. Crystals of βAbm were found in the PEGs screen, condition C12: 100 mM MES pH 6.5, 15% (w/v) PEG 20,000. The structure was solved using crystals formed by microseeding in 100 mM Tris-HCl pH 7.6, 100 mM MgCl2, 7.5% (w/v) PEG 3,350 and 1% (v/v) DMSO with a protein concentration of 10 mg/mL. Crystals of βEcl were obtained using the PEGs screen, condition G8: 200 mM MgSO4, 20% (w/v) PEG 3,350, and were optimized by
microseeding in 100 mM citrate pH 6.0, 150 mM MgSO₄ and 10% (w/v) PEG 3,350 in a seed stock dilution of 1 x 10⁻⁴, with a protein concentration of 7 mg/mL.

Crystals were cryoprotected by increasing the concentration of PEG in each condition in 5% (v/v) increments to 25% with 2 min soaking intervals. Crystals were flash frozen in a stream of nitrogen gas and using MiTeGen loops. For synchrotron data collection, the SSRL automated mounting system was used and data were collected at 100 K using MX1 and MX2 beamlines at the Australian Synchrotron with Blu-Ice (McPhillips et al., 2002). For in-house data collection, crystals were flash frozen at 100 K with an Oxford Cryo-stream and diffraction data were collected with an MAR345 desktop beamline using Cu Kα X-rays from a Rigaku 007HF rotating anode generator with Varimax optics.

2.4. Data processing, structure determination, and refinement

Data sets were integrated, merged and scaled with HKL2000 (Otwinowski and Minor, 1997). Structures were solved by molecular replacement either with CCP4 PHASER (Winn et al., 2011) or PHENIX (Adams et al., 2010) using βEco (PDB ID: 4K3S) as the search model. Alternate rounds of rebuilding and refinement, including non-crystallographic symmetry restraints, were performed with PHENIX (Adams et al., 2010), COOT (Emsley and Cowtan, 2004) and REFMAC (Vagin et al., 2004). Structural illustrations were created using VMD (Humphrey et al., 1996) and PyMOL (PyMOL Molecular Graphics System, Version 1.7, Schrödinger, LLC).

2.5. Coordinates

The atomic coordinates and crystallographic structure factors (PDB ID: 6AMS for βPae, PDB ID: 6AP4 for βAbm and PDB ID: 6AMQ for βEcl) have been deposited in the Protein Data Bank (http://www.rcsb.org/).

2.6. Subunit exchange by nanoESI-mass spectrometry

Samples of β were dialyzed against four changes of 2 L of NS buffer (200 mM ammonium acetate, 1 mM β-mercaptoethanol, pH 7.6). ESI-mass spectra were acquired in positive ion mode using nano-electrospray ionization on a Waters Synapt HDMS spectrometer with a Z-spray source. Spectra were acquired over m/z range of 500–10,000; 100–150 acquisitions were combined and
spectra were baseline subtracted and smoothed using the Savitzky-Golay algorithm. Native state spectra of each β clamp were collected prior to subunit exchange to identify optimal conditions (Supplementary Material, Fig. S2). For subunit exchange between bacterial β clamps, equimolar amounts of proteins (5 μM, as dimers) were mixed and left at 30 °C for the duration of the experiment. Samples of 3 μL were taken hourly for the first 7 h then at 24 h and analyzed on the mass spectrometer. The time from sample extraction to measurement was <1 min. In the cases of β\textsubscript{Pae} and β\textsubscript{Ecl}, β\textsubscript{Eco-His}\textsubscript{6} was used for subunit exchange to increase the molecular weight difference for peak separation. The relative abundance of heterodimers was calculated from the intensities of their most abundant ions (19+ for β\textsubscript{Pae} – β\textsubscript{Eco} and β\textsubscript{Abm} – β\textsubscript{Eco}, and 19+ and 20+ for β\textsubscript{Ecl} – β\textsubscript{Eco} pairwise combinations) and expressed as a percentage of the sum of the intensities for those ions. Methods for the subunit exchange in the presence of 1 M NaCl are given in the Supplementary Methods.

2.7. Molecular dynamics simulations

The peptide AcQLDLF was modeled into the binding sites of β based on the crystal structure of the β\textsubscript{Eco} complex with this peptide (PDB ID: 3Q4J). All MD trajectories were calculated by NAMD (Phillips et al., 2005) using the CHARMM27 all-atom force field (Mackerell et al., 1998, 2004). Structures were embedded in cubic water boxes. Sodium and chloride ions were added (target concentration 100 mM) such that the systems had zero net charge. All simulations were run as NpT ensembles (temperature 310 K; pressure 101.325 kPa) with periodic boundary conditions. Temperature was controlled using Langevin dynamics (damping constant 5 ps\textsuperscript{−1}). Pressure control used the Nosé-Hoover Langevin piston (period 100 fs; decay rate 50 fs). A multiple time-step approach was used with 1, 2, and 4 fs for bonded, non-bonded and long-range electrostatic calculations, respectively. The particle-mesh Ewald with a grid resolution of ≤1 Å was used to calculate long-range electrostatic forces. All other non-bonded interactions were smoothly scaled to zero between 10 and 12 Å. All systems in this study were subjected to energy minimization (10,000 steps) prior to equilibration by MD.

2.8. Fluorescence polarization assays

FP experiments were carried out as described previously (Yin et al., 2013, 2014, 2015). The fluorescent peptides, at 10 nM concentration, and proteins were in 10 mM HEPES buffer, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.07% (v/v) nonidet-P40 and 5% (v/v) DMSO. To generate
data points for saturation curves, the β proteins were serially diluted from 10 to 0.0098 μM, and a 0 μM control was also used (all concentrations of β are given as monomers, each with a single binding site). Experiments were carried out in quadruplicates. Curves were fit using the one site, specific binding equation, Eq. (1) in GraphPad Prism v7.0d to determine values of the dissociation constants, $K_D$.

$$Y = \frac{B_{\text{max}} \cdot X}{K_D + X}$$

where $Y$ is the polarization in mP units, $B_{\text{max}}$ is the maximum response at saturation in mP and $X$ is the concentration of β.

For competition assays, the fluorescent tracer used was Ac-QL-(5FAM)K-LF (GL Biochem, China) at 10 nM. Blank control (buffer), negative control (buffer and the tracer), and positive control (buffer, tracer, and β) were used for data normalization to yield the extent of inhibition. Concentrations of β used were: $\beta^{Eco}$, 80 nM; $\beta^{Eue}$, 500 nM; $\beta^{Abm}$, 500 nM; and $\beta^{Ecl}$, 100 nM (as monomers). Inhibition of tracer binding to β was measured using a two-fold dilution series of the competitors; mP was converted to % inhibition using Eq. (2) and plotted against peptide/inhibitor concentrations to determine their IC$_{50}$ values using Eq. (3). IC$_{50}$ values were then transformed into inhibition constants ($K_I$ values) using the measured $K_D$ values in Table 1 with Eq. (4) which assumes competitive binding and uses the Kenakin correction for ligand depletion (Munson and Rodbard, 1988; Kenakin, 1993; Huang, 2003).

$$\text{Inhibition} \% = \frac{Y_{\text{max}} - X}{Y_{\text{max}} - Y_{\text{min}}} \times 100$$

where $Y_{\text{max}}$ is the mP value of the β–tracer complex, $X$ is the mP value following addition of the competing peptide, and $Y_{\text{min}}$ is the mP value for the tracer alone.

$$Y = Y_{\text{min}} + \frac{Y_{\text{max}} - Y_{\text{min}}}{1 + ([X]^{h} / \text{IC}_{50}^{h})}$$

where $Y$ is the inhibition %, $Y_{\text{max}}$ is the maximum inhibition (fixed at 100%), $Y_{\text{min}}$ is the minimum inhibition (fixed at 0%), IC$_{50}$ is the concentration of competitor that gives $Y = 50\%$, $[X]$ is the concentration of the competitor and $h$ is the Hill slope.
\[
K_1 = \frac{IC_{50}}{1/(1-f_o) + l_o(2-f_o)/2K_D} - K_D[f_o/(2-f_o)]
\]  
(4)

where \(f_o\) is the fraction of tracer bound, \(l_o\) is the tracer concentration and \(K_D\) is the equilibrium dissociation constant of the tracer–\(\beta\) complex.

3. Results and Discussion

3.1. X-ray crystal structures of bacterial sliding clamps

The \(\beta\) sliding clamp proteins from three Gram-negative pathogens were purified and their integrity confirmed by SDS-PAGE and nanoESI-MS (Supplementary Material, Figs S1 and S2). The X-ray crystal structures of sliding clamps from \textit{P. aeruginosa} (\(\beta^{Pae}\), Fig. 1A), \textit{A. baumannii} (\(\beta^{Abm}\), Fig. 1B) and \textit{E. cloacae} (\(\beta^{Ecl}\), Fig. 1C) were determined at 2.40, 2.95 and 2.69 Å resolution, respectively (Supplementary Material, Table S1). The crystal structure of \(\beta^{Pae}\) has been solved previously at 1.8 Å resolution (with a His-tag; PDB ID: 4TR8 (Wolff et al., 2014)). The dimeric structure aligns with an RMSD of 0.82 Å over 637 C\(\alpha\) atoms with the \(\beta^{Pae}\) structure solved here. The three new structures all reveal head-to-tail dimers that formed closed rings (Fig. 1). Each monomer is comprised of three DNA-clamp domains (I, II and III) linked by extended loop regions. \(\beta^{Abm}\) is 16 amino acids longer than \(\beta^{Eco}\) (excluding the His6-tag), with the extra amino acids located in the interconnecting loop between domains II and III (S133), in domain I (E67, G68 and E102), in an extended loop in domain II (N239–D249), and in another loop in domain II (T197) (Supplementary Material, Figs S3 and S4). \(\beta^{Pae}\) is one amino acid longer than \(\beta^{Eco}\) (P187) (Supplementary Material, Fig. S3) while \(\beta^{Ecl}\) has the same number of residues. Sequence identities of \(\beta^{Pae}\), \(\beta^{Abm}\) and \(\beta^{Ecl}\) to \(\beta^{Eco}\) are 58, 45 and 95%, respectively. Backbone superimposition of these three new structures, in order, onto \(\beta^{Eco}\) (PDB ID: 4K3S) revealed very high conservation of structure among the clamps, with RMSD values of 1.05, 1.12 and 0.98 Å over 358, 346 and 357 C\(\alpha\) atoms, respectively (Fig. 1D). Computed electrostatic potential surfaces of the \(\beta\) dimers were also similar to the \(\beta^{Eco}\) (Supplementary Material, Fig. S5).

3.2. Comparison of dimerization interfaces
We used nanoESI-MS under native conditions to assess whether sliding-clamp subunits from different species could form heterodimers with $\beta^{Eco}$, with the rationale being that the dimerization interfaces would have to be chemically and structurally similar for formation of heterodimers, which would validate this interface as a broad-spectrum drug target. It is known that $\beta^{Eco}$ mutant subunits can be made to form heterodimers with the wild-type subunit (Scouten Ponticelli et al., 2009; Sutton et al., 2010; Jergic et al., 2013). Peaks in mass spectra corresponding to the $m/z$ values of heterodimeric ions were observed for all pairwise combinations (Fig. 2). Under the experimental conditions, equilibration was reached between $\beta^{Abm}$ and $\beta^{Eco}$ after 7 h, with the summed abundance of all ions from each $\beta$ after 24 h being approximately 15 and 70% of the total, respectively, and 15% corresponding to the heterodimer (Fig. 2A). Note that although the two $\beta$ clamps were mixed in equal concentrations, the abundance of $\beta^{Abm}$ ions at time zero was lower than those of $\beta^{Eco}$, indicating differences in ionization efficiency exist under these conditions.

For $\beta^{Eco}$-His6 and $\beta^{Pae}$, the heterodimer accounted for 25% of the total ion abundance at equilibrium (Fig. 2B), while the heterodimer formed by subunit exchange between $\beta^{Eco}$-His6 and $\beta^{Ecl}$ dimers was 50% of the total ions (Fig. 2C). The monomers from each bacterial $\beta$ can clearly exchange with $\beta^{Eco}$, but the exchange is relatively slow even at 30 °C, taking place on a timescale of hours. This reflects the high stability of the $\beta$ dimers, and the timescales are consistent with previous measurements of subunit exchange in $\beta^{Eco}$ (Jergic et al., 2013).

With $\beta$ concentrations in the $\mu$M range, monomer association (predicted rate constants $>10^6$ M$^{-1}$ s$^{-1}$) is likely orders of magnitude faster than dimer dissociation, so subunit exchange is limited by the lifetime of the dimer (Northrup and Erickson, 1992; Schreiber, 2002; Binder et al., 2014; Purohit et al., 2017). The $\beta^{Eco}$ dimer dissociation rate constant has been calculated at $6.5 \times 10^{-6}$ s$^{-1}$ and the equilibrium dissociation constant at $< 65 \text{ pM}$ (Yao et al., 1996; Binder et al., 2014). The $\beta$ homodimers studied here would be predicted to form heterodimers only if the chemical and structural environments at the dimer interfaces are similar. Observation of formation of heterodimeric $\beta$ on similar time scales suggests that the association/dissociation rates and equilibrium constants for $\beta^{Ecl}$, $\beta^{Abm}$ and $\beta^{Pae}$ dimers might be similar to $\beta^{Eco}$.

The dimer interfaces of $\beta$ are stabilized by three classes of interactions: a hydrophobic core, hydrogen bonding between $\beta$-strands, and salt bridges (Kong et al., 1992; Purohit et al., 2017). One monomer contributes the domain I residues I78, F106 and L108 ($\beta^{Eco}$ numbering is used) to the hydrophobic core. The adjacent monomer contributes A302, I272 and L273 of domain III. All of these residues are conserved among the relevant bacterial $\beta$ (Supplementary Material, Table S2 and Fig. S4), underscoring their importance for establishment of the dimer interfaces. It has been
shown that the double mutant I272A/L273A of $\beta^{Eco}$ favors monomerization at low $\mu$M concentrations and lacks activity in DNA replication assays (Stewart et al., 2001; Jeruzalmi et al., 2001; Fang et al., 2011). An extended $\beta$-sheet is formed by five hydrogen bonds between two $\beta$-strands from each monomer. Nine charged residues are found at the interface, comprised of five acidic (Glu) residues in the N-terminal $\beta$-strand and four basic (Arg or Lys) residues in the C-terminal $\beta$-strands and $\alpha$-helix. Six salt bridges are present in $\beta^{Eco}$: K74–E298 (3 Å), K74–E300 (2.8 Å), R96–E300 (6.5 Å), R105–E301 (8.9 Å), R105–E303 (7 Å), R103–E304 (9 Å) (Kong et al., 1992). Of these six interactions, three are conserved in $\beta^{Abm}$, five in $\beta^{Pae}$ and all are conserved in $\beta^{Ecl}$ (Supplementary Material, Table S2 and Fig. S4). Conservation of these interactions highlights the importance of the salt bridges for dimer formation. It has been observed that high salt concentrations lead to higher subunit exchange rates in $\beta^{Eco}$ dimers (Jergic et al., 2013; Purohit et al., 2017). Subunit exchange ESI-MS experiments conducted in buffer containing 1 M NaCl (Supplementary Material, Fig. S6) showed increased rates of subunit exchange between $\beta^{Eco}$ and all three $\beta^{Pae}$, $\beta^{Abm}$ and $\beta^{Ecl}$ dimers, with heterodimerization reaching equilibrium within 3.5 h. This confirms the importance of electrostatic interactions for interface stability among all of these clamps and suggests that the rate-limiting step for opening of the dimer interface probably involves disruption of the salt bridges.

3.3. Linear clamp-binding motif-binding pockets

The linear motif-binding pockets of $\beta$, which are critical for interactions with multiple protein partners, are located between domains II and III of each monomer. Superimposition of the $\beta$ structures shows that the residues lining the pocket that are known to interact with linear motif consensus peptides in $\beta^{Eco}$ are well conserved among the relevant species (Fig. 3). The pockets of $\beta^{Eco}$ and $\beta^{Ecl}$ are virtually identical (not shown) while the $\beta^{Pae}$ and $\beta^{Abm}$ pockets contain a single amino acid substitution (V344 to Asn) in subsite II.

3.4. Consensus peptide interactions with linear motif-binding pockets

Linear motif interactions with $\beta^{Eco}$ have been extensively characterized (Wijffels et al., 2004; Georgescu et al., 2008b; Wolff et al., 2011; Yin et al., 2013), and interactions between linear motif peptides and $\beta$ from *Mycobacterium tuberculosis*, *Bacillus subtilis*, *Staphylococcus aureus*, and *P. aeruginosa*, while less studied, have been reported (Gui et al., 2011; Wolff et al., 2014). In
the present study, binding of a fluorescently labeled linear motif-consensus peptide (5FAM-Q_{1}L_{2}D_{3}L_{4}F_{5}-OH; “P1”) to the bacterial β clamps studied by fluorescence polarization (FP). Affinity of P1 for β^{Eco} was measured as $K_D = 29 \text{ nM}$, which is similar to the value reported previously ($K_D = 70 \text{ nM}$) (Yin et al., 2013). Affinity of P1 for the three other β clamps varied from low to mid-nM (Table 1). Curiously, the observed values of $B_{\text{max}}$, the signal at saturation in mP units (data not shown), varied from 100–250 mP among the β proteins (with β^{Eco} having $B_{\text{max}} \sim 250$ mP). As the same concentration ranges were used for all of the β clamps, it was expected that $B_{\text{max}}$ should have been similar, corresponding to one peptide binding per monomer. The differences in $B_{\text{max}}$ were attributed to non-specific binding of the P1 fluorophore. This hypothesis was explored further using a different fluorescently-labeled linear motif consensus peptide. From the previous co-crystal structures of β^{Eco} bound to the linear motif consensus peptide, it was evident that the side chain of residue D_{3} made few interactions with the protein and was directed out of the pocket. Labeled peptide AcQ_{1}L_{2}K_{3}(5FAM)-L_{4}F_{5}-OH (“P2”) was therefore designed based on the likelihood that the 5FAM-labeled lysine side chain would make no interactions with the linear motif-binding pocket. The dissociation constant for P2 binding to β^{Eco} was measured at $K_D = 80 \text{ nM}$, in agreement with the value reported previously (Yin et al., 2013). Affinity of P2 for the β^{Pae} and β^{Abm} pockets was 4–5-fold lower, while for β^{Ecl}, $K_D$ was 2-fold higher (Table 1). The $B_{\text{max}}$ value was similar among all four β proteins with the P2 tracer, within 50 mP units of that for β^{Eco}, indicating that the fluorophore-labeled lysine was not contributing to the interaction.

While the β partner proteins from other bacteria are less studied than that from E. coli, sequence alignments have shown that the Q_{1} and L_{4} residues of the linear motif are largely conserved among bacterial species (Supplementary Material, Fig. S7). In some P. aeruginosa and A. baumannii binding partners (e.g., DnaE and HolA), the Q_{1} residue may be either His, Ile, Asn or Ser. Variation appears at position F/L_{5} in A. baumannii proteins, with either Trp or Met present. The weaker binding of tracer peptide P2 to β^{Pae} and β^{Abm} pockets compared to β^{Eco} may reflect these differences in putative linear motifs. Thus, the linear motif used in this study may be sub-optimal for β^{Pae} and β^{Abm}.

**Table 1**

<table>
<thead>
<tr>
<th>tracer</th>
<th>β^{Eco}</th>
<th>β^{Pae}</th>
<th>β^{Abm}</th>
<th>β^{Ecl}</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>0.029 ± 0.002</td>
<td>0.070 ± 0.006</td>
<td>0.280 ± 0.041</td>
<td>0.063 ± 0.005</td>
</tr>
</tbody>
</table>
The contributions of individual linear motif residues to $\beta^{Eco}$ binding have been extensively characterized by alanine and glycine scanning (Yin et al., 2013). Alanine scanning was used here to assess the relative side chain contributions towards binding of linear motif peptide to the three new $\beta$ clamps. Inhibition constants ($K_i$) were measured by a FP competition assay. In general, the patterns of peptide inhibition were found to be similar among the proteins, and the N-terminally acetylated peptide $AcQLDLF-OH$ showed very similar affinities, $K_i = 0.8–2.1 \, \mu M$. The un-acetylated peptide (NH$_2$-QLDLF-OH) showed much weaker binding to all clamps (Table 2).

**Table 2**

$K_i$ values (μM) for inhibition of linear motif binding to $\beta$ by linear motif-consensus peptide alanine mutants. Values are means ± standard error of the mean ($N = 2$).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$\beta^{Eco}$</th>
<th>$\beta^{Pae}$</th>
<th>$\beta^{Abm}$</th>
<th>$\beta^{Ecl}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_2$-QLDLF-OH</td>
<td>56 ± 9</td>
<td>530 ± 210</td>
<td>54 ± 8</td>
<td>52 ± 11</td>
</tr>
<tr>
<td>$AcQ$QLDLF-OH</td>
<td>0.8 ± 0.1</td>
<td>2.1 ± 0.4</td>
<td>0.9 ± 0.2</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>$AcQ$ALDLF-OH</td>
<td>170 ± 30</td>
<td>520 ± 70</td>
<td>130 ± 30</td>
<td>71 ± 9</td>
</tr>
<tr>
<td>$AcQ$ADLF-OH</td>
<td>10 ± 0</td>
<td>16 ± 2</td>
<td>12 ± 2</td>
<td>7.8 ± 1.6</td>
</tr>
<tr>
<td>$AcQ$LALF-OH</td>
<td>0.6 ± 0.4</td>
<td>2.8 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>$AcQ$LDAF-OH</td>
<td>26 ± 5</td>
<td>127 ± 23</td>
<td>106 ± 14</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>$AcQ$LDLA-OH</td>
<td>133 ± 25</td>
<td>— a</td>
<td>186 ± 42</td>
<td>74 ± 16</td>
</tr>
<tr>
<td>$AcQ$L-OH</td>
<td>— a</td>
<td>— a</td>
<td>— a</td>
<td>— a</td>
</tr>
<tr>
<td>$AcQ$LF-OH</td>
<td>188 ± 30</td>
<td>194 ± 41</td>
<td>240 ± 80</td>
<td>160 ± 80</td>
</tr>
<tr>
<td>$AcQ$LDAA-OH</td>
<td>— a</td>
<td>— a</td>
<td>— a</td>
<td>— a</td>
</tr>
</tbody>
</table>

* $K_i > 1000 \, \mu M$

Affinity for all $\beta$ clamps decreased markedly with the $AcQ_1$ to A mutant, highlighting the importance of this residue (Yin et al., 2013). The L$_2$ to A mutant peptide had 5–10-fold reduced affinity across all of the clamps, whereas the D$_3$ to A mutant peptide retained consensus motif affinity in all cases. The greatest effect on binding affinity for the majority of the $\beta$ clamps was observed for peptides with alanine mutations in the L$_4$F$_5$ dipeptide. The L$_4$ to A mutant showed ~30- and ~20-fold lower affinity for $\beta^{Eco}$ and $\beta^{Ecl}$, respectively, ~50-fold lower affinity for $\beta^{Pae}$ and ~120-fold for $\beta^{Abm}$. The F$_5$ to A mutant showed even greater loss in affinity across all species. Mutating both L$_4$ and F$_5$ to alanine resulted in all cases in total loss of binding ($K_i > 1 \, mM$).
Consistent with previous observations with $\beta^{Eco}$ (Yin et al., 2013), the truncated AcL4F5-OH dipeptide showed some affinity (albeit weak) for $\beta$ pockets across the species, and no binding was observed between any of the $\beta$ pockets and the AcQ1L2-OH dipeptide.

The reported X-ray co-crystal structure of $\beta^{Eco}$ in complex with AcQLDLF-OH (PDB ID: 3Q4J) (Wolff et al., 2011) was used to generate starting structures for molecular dynamics (MD) simulations probing the dynamics and stability of the peptide binding to the linear motif-binding pockets of $\beta^{Pae}$, $\beta^{Abm}$ and $\beta^{Ecl}$. In all cases, the peptides remained stably bound to the $\beta$ proteins, and both had a consistent radius of gyration and global root-mean-square-fluctuation (RMSF) over the course of the 20 ns simulation (Supplementary Material, Fig. S8). The interactions of each linear motif residue within the various $\beta$ binding pockets is similar.

The L4F5 dipeptide fully occupies subsite I and forms several contacts with the hydrophobic residues lining the cavity. Residue F5 samples multiple conformations within the pocket. By reducing the side chains to a methyl group (L4 to A and F5 to A linear motif peptides), these multiple stabilizing interactions are diminished, significantly reducing the affinity of the peptide for the pocket. The side chains of L2 and D3 do not form significant interactions with any of the $\beta$ clamps, reflected in the L2 to A and D3 to A linear motif peptides not affecting binding affinity relative to the consensus linear motif (Table 2). Furthermore, the D3 side chain faced into solution and sampled various orientation over the 20 ns simulation. By substituting the Asp for Lys in the P2 tracer, it can be speculated that the Lys side chain and 5FAM fluorescent probe would not form an interaction with the $\beta$ clamps studied here.

A buried water molecule ($W_1$) was observed from the beginning of the simulations to stabilize the buried Q1 side chain in subsite II. In all cases, $W_1$ was restrained by hydrogen bonds to the corresponding residues of the $\beta^{Eco}$ M362-O, P363-O and N320-N (Fig. 4). Interestingly, a second water molecule ($W_2$) binds after 5 ns into subsite II of $\beta^{Ecl}$. Both water molecules form hydrogen bonds with each other, and $W_2$ forms an additional hydrogen bond with the H175 carbonyl oxygen. The Q1 side chain, along with forming the stabilizing Q1Oe1–$W_1$H hydrogen bond (and Q1Oe1–$W_2$H in $\beta^{Ecl}$), can interact with the peptide backbone of N320$^{Eco}$ (Fig. 4). The Q1 to A mutation would result in loss of this vital water interaction, reflected as a loss in affinity in the FP assays (Table 2).

3.5. Binding of small-molecule inhibitors to linear motif-binding pockets of $\beta$
We previously identified tetrahydrocarbazole (THC) derivatives that bind to subsite I of $\beta^{Eco}$ with low micromolar affinity by soaking compounds into E. coli $\beta$ crystals (Yin et al., 2015). Six of these derivatives were examined here for binding to $\beta^{Pae}$, $\beta^{Abm}$ and $\beta^{Ecl}$ using the FP competition assay described earlier and the labeled linear motif consensus peptide P2 (Table 3). Compound 1 (1e in (Yin et al., 2015)) showed similar affinity for all $\beta$ pockets, with inhibition constants ($K_I$) between 49 and 77 $\mu$M (Table 3). Previous work indicated that the glycine carboxylates of 2 (5a) and 3 (5b) occupy the same region of the $\beta^{Eco}$ linear motif-binding pocket as the D$_3$ side chain of AcQLDLF-OH (Yin et al., 2015). Significantly increased affinity that varied only slightly relative to 1 among the $\beta$ clamps was observed for both compounds. The bromo-derivative 3 was consistently ~2-fold more potent than the chloro-analog 2, as noted previously with $\beta^{Eco}$ (Yin et al., 2015). Compounds 4 (5l) and 5 (5m), which substitute the appended glycine for the R and S enantiomer of phenylalanine, respectively, showed only small variations in affinity relative to 3 across all of the clamps.

In the X-ray co-crystal structure of $\beta^{Eco}$ with bound 4 (PDB ID: 4PNU), it was observed that the phenyl group was oriented towards subsite II. In contrast, the co-crystal structure of $\beta^{Eco}$ with bound 5 (PDB ID: 4PNW) revealed a conformational change in the H148–Y154 loop region that opened a new subsite III (Yin et al., 2015). This region appears to be conserved among the $\beta$ clamps, and since the affinities of 4 and 5 were all similar as for $\beta^{Eco}$, the data suggest that subsite III is most likely also revealed upon binding of 5 to the three newly-studied $\beta$ clamps. The co-crystal structures additionally showed that the phenyl group of 5 only partially occupied subsite III, suggesting extension from the para-position into the third subsite might lead to enhanced affinity. To test this, 5 was modified to include a 3,5-dichloroisonicotinamide group at the para-position of the phenyl ring, producing 6 (see Supplementary Materials). However, affinity of this compound for all $\beta$ clamps was decreased several-fold relative to 5 (Table 3).
Table 3
Inhibition of the β by THC-derivatives 1–6 (measured by FP). Values of \( K_I \) are in units of \( \mu \text{M} \). Values are mean ± standard error of the mean (\( N = 2 \)).

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \beta^{Eco} )</th>
<th>( \beta^{Pae} )</th>
<th>( \beta^{Abm} )</th>
<th>( \beta^{Ecl} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1e)</td>
<td>77 ± 19</td>
<td>75 ± 20</td>
<td>75 ± 25</td>
<td>49 ± 21</td>
</tr>
<tr>
<td>2 (5a)</td>
<td>12 ± 2</td>
<td>14 ± 1</td>
<td>11 ± 2</td>
<td>7.8 ± 1.2</td>
</tr>
<tr>
<td>3 (5b)</td>
<td>6.9 ± 1.8</td>
<td>7.9 ± 2.9</td>
<td>5.4 ± 2.2</td>
<td>3.1 ± 1.5</td>
</tr>
<tr>
<td>4 (5l)</td>
<td>1.2 ± 0.1</td>
<td>9.3 ± 1.1</td>
<td>9.3 ± 2.9</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>5 (5m)</td>
<td>3.8 ± 1.0</td>
<td>7.0 ± 1.1</td>
<td>4.2 ± 0.6</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>6</td>
<td>9.4 ± 2.6</td>
<td>29 ± 5</td>
<td>27 ± 5</td>
<td>8.3 ± 2.3</td>
</tr>
</tbody>
</table>
4. Conclusions

With the emergence of antibiotic-resistant bacteria, the development of new drugs is imperative. Bacterial β sliding clamps are targets for developing such compounds. Until recently, most studies of β utilized the E. coli homolog. We have now analyzed the structures of a range of Gram-negative bacterial β clamps, their binding pockets and their dimer interfaces. We propose the mechanism of linear motif interaction with the binding pocket observed in βEco is conserved in other Gram-negative bacterial homologs. In its interaction with βEco, the Q1, L4 and F5 residues of linear-motif peptides are crucial for strong interaction. Based on crystal structures and MD simulations, it appears that the same H-bond network of Q1 with subsite II is regulated by a structurally conserved buried water molecule in the clamps considered here. The β residue M362, the “methionine gate” in βEco that regulates the linear motif binding to subite II, is conserved in Gram-negative bacteria, but replaced by a threonine or leucine residue in Gram-positive clamps. Consequently, it has been proposed that there may be a different mechanism of linear motif interaction in the Gram-positive β pockets (Wolff et al., 2014). Analysis of the interaction of the linear motif with the Gram-positive β will highlight the differences between the β binding pockets of Gram-negative and Gram-positive bacteria.

The β binding pocket has been the target of therapeutic design (Georgescu et al., 2008b; Wijffels et al., 2011; Kling et al., 2015; Wolff et al., 2011; Yin et al., 2014, 2015; Wolff et al., 2014) due to its central role in essential bacterial processes, the moderate number of β per cell (300–600 dimers) (Burgers et al., 1981; Leu et al., 2000), its high degree of conservation across bacterial species and its structural distinctiveness from the human protein homolog, the proliferating cell nuclear antigen (PCNA) (Gulbis et al., 1996; Wegener et al., 2018). Human PCNA binds to different linear motif with consensus sequence Qxx[M/I/L]xxF(Y/F) (Warbrick, 1998). Consequently, antibacterial compounds developed for inhibition of the bacterial β should not interfere with the function of human PCNA. Our THC-derived compounds bind the Gram-negative β pocket in the three proteins studied here with similar affinities to that of βEco. Additionally, the most potent inhibitor, 5, opens a hydrophobic pocket deemed subsite III that is conserved in these bacterial β clamps (Yin et al., 2015). These inhibitors are promising scaffolds for further generations of inhibitors potentially fully occupying subsites I, II and III.
Acknowledgements

We are thankful to Dr. Z. Yin for invaluable help with fluorescence polarization data analysis. Part of this research was undertaken on MX1 and MX2 beam lines at the Australian Synchrotron, Victoria, Australia. This research was supported in part by Australian Government Research Training Program Scholarships (awarded to A.E.M. and A.P.M.), Australian Research Council Discovery Projects DP110100660 and DP180100805, and NHMRC Project Grant GNT1021479.

Author contributions

A.J.O., N.E.D., J.L.B and M.J.K. supervised the project. A.E.M. and F.E.D. expressed and purified sliding clamps. A.P.M. and L.R.W. performed synthesis of compounds. A.E.M. performed and analyzed the fluorescence polarization-based assays and mass spectrometry experiments. A.E.M. and A.J.O. performed and analyzed X-ray crystallography and molecular dynamics simulation studies. A.E.M. and A.J.O. initially drafted the manuscript with input from all authors. The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://dx.doi.org/10.1016/j.jsb.xxxx.xx.xx

References


protein interactions with the β2 sliding clamp of *Escherichia coli* DNA polymerase III by peptides from β2-binding proteins. Biochemistry 43, 5661–5671.


Fig. 1. Structural alignment of X-ray crystal structures of sliding clamps. $\beta^{Eco}$, in gray, is shown aligned with (A) $\beta^{Pac}$, in green, (B) $\beta^{Abm}$, in magenta, and (C) $\beta^{Ecl}$, in purple. (D) Orthogonal views of all four structurally aligned $\beta$ clamps. Domains I, II and III are labeled and arrows indicate the dimer interfaces.
Fig. 2. Subunit exchange between heterologous sliding clamps. NanoESI-MS showing subunit exchange in 200 mM ammonium acetate, pH 7.6 at 30 °C between: (A) $\beta^{\text{Eco}}$ and $\beta^{\text{Abm}}$, (B) $\beta^{\text{Eco-His6}}$ and $\beta^{\text{Pae}}$, and (C) $\beta^{\text{Eco-His6}}$ and $\beta^{\text{Ecl}}$. The protein concentrations were 5 μM (as dimers). Colored circles above each charge state show the identity of the dimer molecular ion based on $m/z$, with yellow indicating the heterodimer in all mass spectra. The three most abundant charge states are shown.
Fig. 3. Superimposition of the X-ray crystal structures highlighting residues of the linear motif-binding pockets. $\beta^{Pae}$ is in green, $\beta^{Abm}$ in magenta and $\beta^{Ecl}$ in purple. Conserved residues are labeled in black (according to $\beta^{Eco}$ numbering) and non-conserved residues red. V344 present in $\beta^{Eco}$ and $\beta^{Ecl}$ is replaced by Asn in $\beta^{Pae}$ and $\beta^{Abm}$. 
Fig. 4. MD simulations of AcQLDLF-OH linear motif-consensus peptide complexed with: (A) $\beta^{Eco}$, (B) $\beta^{Pae}$, (C) $\beta^{Abm}$ and (D) $\beta^{Ecl}$. Structures show the protein (left) and peptide (center) conformations after 20 ns. The water molecule $W_1$ (and $W_2$ in $\beta^{Ecl}$) buried in subsite II is highlighted. Graphs in the right panels show the bond distances (Å) between the side chain oxygen atom of Q$_1$ and the hydrogen of $W_1$ (and $W_2$ in (D)) over the course of the simulation. The linear motif peptide inset in the right panel of (A) shows the average $B$-factor distribution. Red indicates minimal movement and blue the largest movement over the 20 ns simulation. Near-identical $B$-factor distributions were observed in the other three simulations.
Pseudomonas aeruginosa
Acinetobacter baumannii
Enterobacter cloacae