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The Ancestral N-Terminal Domain of Big Defensins Drives Bacterially Triggered Assembly into Antimicrobial Nanonets

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ABSTRACT Big defensins, ancestors of β-defensins, are composed of a β-defensin-like C-terminal domain and a globular hydrophobic ancestral N-terminal domain. This unique structure is found in a limited number of phylogenetically distant species, including mollusks, ancestral chelicerates, and early-branching cephalochordates, mostly living in marine environments. One puzzling evolutionary issue concerns the advantage for these species of having maintained a hydrophobic domain lost during evolution toward β-defensins. Using native ligation chemistry, we produced the oyster Crassostrea gigas BigDef1 (Cg-BigDef1) and its separate domains. Cg-BigDef1 showed salt-stable and broad-range bactericidal activity, including against multidrug-resistant human clinical isolates of Staphylococcus aureus. We found that the ancestral N-terminal domain confers salt-stable antimicrobial activity to the β-defensin-like domain, which is otherwise inactive. Moreover, upon contact with bacteria, the N-terminal domain drives Cg-BigDef1 assembly into nanonets that entrap and kill bacteria. We speculate that the hydrophobic N-terminal domain of big defensins has been retained in marine phyla to confer salt-stable interactions with bacterial membranes in environments where electrostatic interactions are impaired. Those remarkable properties open the way to future drug developments when physiological salt concentrations inhibit the antimicrobial activity of vertebrate β-defensins.

IMPORTANT β-Defensins are host defense peptides controlling infections in species ranging from humans to invertebrates. However, the antimicrobial activity of most human β-defensins is impaired at physiological salt concentrations. We explored the properties of big defensins, the β-defensin ancestors, which have been conserved in a number of marine organisms, mainly mollusks. By focusing on a big defensin from oyster (Cg-BigDef1), we showed that the N-terminal domain lost during evolution toward β-defensins confers bactericidal activity to Cg-BigDef1, even at high salt concentrations. Cg-BigDef1 killed multidrug-resistant human clinical isolates of Staphylococcus aureus. Moreover, the ancestral N-terminal domain drove the assembly of the big defensin into nanonets in which bacteria are entrapped and killed. This discovery may explain why the ancestral N-terminal domain has been maintained in diverse marine phyla and creates a new path of discovery to design β-defensin derivatives active at physiological and high salt concentrations.
Defensins are essential components of innate immunity broadly found in vertebrates and invertebrates (1). Through their multiple functions (e.g., as antimicrobial peptides [AMPs], proinflammatory mediators of the immune response), these cationic host defense peptides (HDPs) contribute to protection against infections at almost all human epithelial surfaces (2, 3). Some human β-defensins were shown to compromise bacterial membrane integrity (4). However, like many human (5–7) and avian (8) β-defensins, direct antimicrobial activity at physiological salt concentrations is significantly impaired. This suggests that electrostatic interactions initiating interactions between these cationic peptides and the negatively charged membranes of bacteria (9) are altered by salts.

Big defensins are a group of AMPs related to β-defensins, initially isolated from the hemocytes of a marine chelicerate, the horseshoe crab (Tachypleus tridentatus) (Tt-BigDef) (10). They contain a N-terminal hydrophobic domain and a C-terminal β-defensin-like domain (11). The two domains are encoded by separate exons (12, 13). On the basis of their gene structure and amino acid sequence and three-dimensional (3D) structure similarities, it was proposed that vertebrate β-defensins originated from an ancestral big defensin via intronization of exonic sequences or exon shuffling, thereby losing the ancestral N-terminal domain (1, 13). Big defensins have been predominantly described in marine organisms, mainly mollusks (12, 14), and, to a much lower extent, in ancestral chelicerates (horseshoe crabs) (10) and early-branching chordates (amphioxus) (15). The evolutionary advantage for those phylogenetically distant species of having conserved the N-terminal hydrophobic domain represents one puzzling unsolved issue.

Although they were isolated more than 20 years ago (10), technical limitations impeding the production of sufficient quantities of big defensins have precluded investigations on their structure and antimicrobial activities and of the roles of their respective domains. Sufficient amounts of native Tt-BigDef were obtained from horseshoe crab hemocytes to determine its 3D structure (Protein Data Bank [PDB] identifier: 2RNG), which remains the only one solved to date. Tt-BigDef is tightly packed in solution: the hydrophobic N-terminal domain adopts a β1-α1-α2-β2 fold, whereas the cationic C-terminal domain shows the cysteine pairing expected for β-defensins (11). To date, data on big defensin antimicrobial activities remain scarce (10, 14, 15).

Achievements that opened the way to structure and activity studies in big defensins included developments in peptide chemistry related to native chemical ligation (NCL) strategies (16, 17). Using this methodology, we synthetized Cg-BigDef1, one of the best-characterized big defensins in terms of gene structure, expression, and role in immunity (12, 18–20). We obtained several dozen milligrams, solved its solution structure, and investigated its interactions with bacteria. By using a combination of antimicrobial assays, immune detection, scanning electron microscopy, and mass spectrometry analyses, we determined that Cg-BigDef1 is a highly salt-stable AMP that entraps and kills bacteria in nanonet structures. Moreover, we found that Cg-BigDef1 is (i) active against clinical strains of Staphylococcus aureus multiresistant to antibiotics, which represent a major concern for human health, and (ii) devoid of cytotoxicity toward mammalian cells. Our findings pave the way for future drug developments inspired by the evolution-based molecular design of big defensins.

RESULTS

Big defensins are mainly found in marine organisms. We performed an exhaustive search for sequences containing a β-defensin domain in publicly available genomic and transcriptomic databases. Sequences of β-defensins were found in diverse groups of vertebrates (from fish to mammals) and invertebrates (mollusks and crustaceans), whereas big defensins were present in a limited number of species belonging to
Lophotrochozoa, Arthropoda, and Cephalochordata (Fig. 1). Remarkably, of the 78 obtained distinct big defensins, only one sequence (GenBank accession no. AEP26934) was found in a nonmarine species, the sequence corresponded to the freshwater mussel *Hyriopsis cumingii* (21). Mollusks (Lophotrochozoa) represented the superphylum containing the highest diversity of big defensins by far (see Fig. S1 in the supplemental material). Multiple-sequence alignments revealed a canonical conserved motif but differently spaced cysteines for big defensins [Cys-Xaa(4-14)-Cys-Xaa(3)-Cys-Xaa(13-14)-Cys-Xaa(4-7)-Cys-Cys] and β-defensins [Cys-Xaa(4-6)-Cys-Xaa(3–4)-Cys-Xaa(7–12)-Cys-Xaa(5–7)-Cys-Cys] (Fig. 1). The two defensin families also differed by the presence of a hydrophobic N-terminal domain (20 to 64 residues) in big defensins only (Fig. 1). This domain, which contains some highly conserved amino acids (Fig. 1), does not show any homology with sequences present in public databases outside big defensins.

Native chemical ligation-based chemical synthesis gives access to the exploration of multiple-domain Cg-BigDef1 structure and activity. To explore the role of the ancestral N-terminal domain in big defensin structure and activity, we focused on the mollusk big defensin Cg-BigDef1 (GenBank accession no. AEE92768). We first synthesized the entirety of Cg-BigDef1. Cg-BigDef1[1–93] corresponds to mature Cg-BigDef1 with a pyroglutamic acid (Pca) at the N terminus, an amidated C terminus, and six cysteines involved in three disulfide bridges (12) (Table 1). Total synthesis of Cg-BigDef1 (Fig. 2) was achieved through NCL (16) of Cg-BigDef1[57–93] with a Cg-BigDef1[1–56] cryptothioester (22) and subsequent oxidative folding (Fig. S2d). The resulting peptide was characterized by liquid chromatography-mass spectrometry (LC-MS) (Fig. S2e) and nuclear magnetic resonance (NMR) spectroscopy (see below).
Cg-BigDef1[1–42] and Cg-BigDef1[44–93], which correspond to the N-terminal and C-terminal domains of Cg-BigDef1, respectively (Table 1), were synthesized by solid-phase peptide synthesis according to the position of Cg-bigdef1 exons (12) (Fig. S2a to c).

**Cg-BigDef1 adopts a highly compact hydrophobic fold in solution.** The 3D structures of Cg-BigDef1[1–93] and Cg-BigDef1[44–93] were determined by NMR spectroscopy (Tables S1 and S2). Cg-BigDef1[1–93] is composed of two distinct globular domains connected by a flexible linker (Fig. 3). The N-terminal domain (Pca1 to Glu42) is hydrophobic and adopts a β1-α1-α2-β2 fold. A hydrophobic core composed of residues Tyr11, Val16, Val20, Leu24, Leu33, and Ile38 stabilizes this highly compact fold. The C-terminal domain adopts a β-defensin-like fold with a cysteine pairing identical to that of β-defensins (C1-C5, C2-C4, and C3-C6), although the cysteine spacings differ; it also displays a typical four-stranded antiparallel β-sheet and an α-helix (Fig. 3). The linker is composed of 10 residues (Asn43 to His52) and is located at the interface of the two globular domains (strong polar contacts are formed during the structure calculation as follows: Arg49/Thr15, Asp51/Ser54, His52/Asp53, Arg65/Asp51, and His71/Asn43). This allows Cg-BigDef1[1–93] to be highly compact in solution.

We found that the fold of Cg-BigDef1[44–93] is identical to that of the C-terminal domain of Cg-BigDef1[1–93] except for the first nine residues (44 to 52), viz., the linker, as the network of polar contacts cannot be formed in the absence of the N-terminal domain (Fig. S3 and S4). The surface of Cg-BigDef1[1–93] is mainly hydrophobic, with no amphipathic properties. Indeed, all the positively charged residues (Arg35, Arg39, Arg40, and Arg41) of the N-terminal domain are exposed at the surface. Arg39 is surrounded by four tyrosine residues (Tyr28, Tyr31, Tyr34, and Tyr36) forming a hydrophilic patch. Arg40 and Arg41 create an extended hydrophilic belt at the interface between the β-sheet of the hydrophobic domain, the linker, and the C-terminal domain (Ser10, Thr15, Arg40, Arg41, Glu42, Arg47, Arg49, Ser50, Asp51, His52, Asp53, Thr67, Ser70, His51, and Arg92). The C-terminal domain (net charge, +3) exhibits three positively charged residues (Arg61, Arg89, and Arg91) and two negatively charged acidic residues (Asp53 and Asp80) at the surface, the other charged residues being buried. Overall, the N-terminal domain does not alter the fold of the conserved β-defensin-like domain but confers hydrophobic properties to this molecule, which is otherwise mainly hydrophilic.

**Cg-BigDef1 has broad range bactericidal activity.** Cg-BigDef1 antimicrobial activities were tested at 150 mM NaCl and 400 mM NaCl, physiological salt concentrations for humans and marine bacteria, respectively (Table 2). Cg-BigDef1 was active against a range of reference, environmental, oyster, and human clinical strains, with various profiles of susceptibility to antibiotics (see Table S3 in the supplemental material). The lowest minimal inhibitory concentrations (MICs) were observed against Gram-positive strains (Table 2). All *S. aureus* strains tested, including methicillin-resistant *S. aureus* (MRSA) clinical isolates from cystic fibrosis (CF) and non-CF patients, were susceptible to Cg-BigDef1 (MICs in the 1.25 to 5 μM range). *Vibrio* isolates, including *V. tasmaniensis*...
and V. crassostreae strains pathogenic for oysters, were inhibited in the 1.25 to 10 μM range. Full inhibition of human clinical isolates of Pseudomonas and Burkholderia was not reached at the highest concentration tested. A bactericidal effect against most susceptible strains was determined with minimum bactericidal concentrations (MBCs) in the range of 0.6 to 10 μM (Table 2).

**FIG 3** Solution structure of Cg-BigDef1 (PDB entry 6QBL). The global fold and surface potentials of Cg-BigDef1[1–93] are displayed. (A) Cg-BigDef1[1–93] sequence. “Z” stands for pyroglutamic acid (Pca) and NH₂ for the C-terminal amidation. Cysteine residues are indicated in bold and their pairing by black lines. The secondary structure elements of the protein are indicated below the sequence in yellow, red, and green for β-strand, α-helix, and coil, respectively. The N-terminal domain (Pca1 to Glu42) is hydrophobic and adopts a β1-α1-β2-β2 fold (β1, Thr15-Val16; α1, Ala18-Val27; β2, Gly29-Arg35; β2, Arg39-Arg40). The C-terminal domain adopts a β-defensin-like fold (Cys57-Cys87, Cys64-Cys82, Cys68-Cys88), a four-stranded antiparallel β-sheet (β3, Ser56-Cys57; β4, Gly62-Arg65; β5, His71-Asp75; β6, Tyr86-Pro91), and an α-helix (α3, Trp76-Cys82). (B) Superimposition of the 10 models representative of Cg-BigDef1[1–93] solution structure with a root mean square deviation (RMSD) at 0.567 Å. (C) Electrostatic positive (blue) and negative (red) areas calculated at the Connolly surface by the use of the Adaptive Poisson-Boltzmann Solver (APBS) (54). (D) Hydrophobic (yellow) and hydrophilic (green) potential areas calculated at the Connolly surface by the use of the Platinum (55).
Covalent association of Cg-BigDef1 domains is essential for salt-stable antimicrobial activity. The separate domains were markedly less bactericidal than full-length Cg-BigDef1 (Table 2). Synergies between the two domains were therefore measured by exposing bacteria to Cg-BigDef1[1–42] and Cg-BigDef1[44–93] simultaneously or to Cg-BigDef1[1–93]. The separate domains acted synergistically against both Gram-positive and Gram-negative strains, with fractional inhibitory concentration (FIC) index values in the 0.625 to 0.740 range (i.e., <1) (Table 3). Strong synergy was observed when domains were linked covalently (FIC values in the 0.067 to 0.154 range; <0.5) (Table 3). This shows that the antimicrobial activity of the β-defensin domain

**TABLE 2** Antimicrobial activities of the full Cg-BigDef1 and its isolated domainsa

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<tr>
<td></td>
<td></td>
<td>MIC (μM)</td>
<td>MBC (μM)</td>
<td>MIC (μM)</td>
<td>MBC (μM)</td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>400</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
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<td>150</td>
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<td>&gt;10</td>
<td>&gt;10</td>
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<tr>
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<td>150</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC 9027</td>
<td>Ref</td>
<td>150</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (Pa25) 13/07/11-B-3003</td>
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<td>150</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
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<td>Clin/h</td>
<td>150</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
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<tr>
<td><em>Vibrio tasmaniensis</em> LGP32</td>
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<td>400</td>
<td>10</td>
<td>&gt;10</td>
<td>&gt;10</td>
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<tr>
<td><em>Vibrio tasmaniensis</em> 3T8_11</td>
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<td>400</td>
<td>10</td>
<td>&gt;10</td>
<td>&gt;10</td>
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<td><em>Vibrio tasmaniensis</em> 7G7_3</td>
<td>Clin/o</td>
<td>400</td>
<td>5</td>
<td>20</td>
<td>&gt;10</td>
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<tr>
<td><em>Vibrio crassostreae</em> 7T4_12</td>
<td>Clin/o</td>
<td>400</td>
<td>5</td>
<td>&gt;10</td>
<td>&gt;10</td>
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<tr>
<td><em>Vibrio crassostreae</em> 7F5_29</td>
<td>Clin/o</td>
<td>400</td>
<td>1.25</td>
<td>1.25</td>
<td>10</td>
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<tr>
<td><em>Vibrio oritaridis</em> 8F5_42</td>
<td>Env</td>
<td>400</td>
<td>10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td><em>Vibrio breogani</em> 7F1_16</td>
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<td>400</td>
<td>10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td><em>Vibrio harveyi</em> 7GS_1</td>
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<td>400</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Gram-positive bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Corynebacterium maritum</em> CIP 101282</td>
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<td>400</td>
<td>0.15</td>
<td>0.6</td>
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<tr>
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<td>&gt;10</td>
<td>&gt;10</td>
<td>10</td>
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<td>0.3</td>
<td>1.25</td>
<td>2.5</td>
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<td>150</td>
<td>2.5</td>
<td>5</td>
<td>&gt;10</td>
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<td>2.5</td>
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<td>1.25</td>
<td>&gt;10</td>
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<td>150</td>
<td>2.5</td>
<td>&gt;10</td>
<td>&gt;10</td>
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<tr>
<td><em>Staphylococcus aureus</em> (MSSA) 07/02/14-B-5264</td>
<td>Clin/h</td>
<td>150</td>
<td>5</td>
<td>10</td>
<td>&gt;10</td>
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<tr>
<td><em>Staphylococcus aureus</em> Newman</td>
<td>Ref</td>
<td>150</td>
<td>2.5</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> SG511</td>
<td>Ref</td>
<td>150</td>
<td>1.25</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
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</table>

*MIC values (reported in micromoles per liter [μM]) refer to the minimal concentration required to achieve 100% growth inhibition. MBC values (reported in micromoles per liter) refer to the minimal concentration required to kill 100% of bacteria. The NaCl concentrations at which assays were performed are indicated in millimoles per liter (mM). The origin of the clinical and environmental isolates is specified in Table S3. Env, environmental isolate; Clin, clinical isolate from either human (Clin/h) or oyster (Clin/o); Ref, reference strain; NT, not tested; CIP, Collection de l’Institut Pasteur; ATCC, American Type Culture Collection; MSSA, methicillin-susceptible *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*; GISA, glycopeptide-intermediate *Staphylococcus aureus*.

**TABLE 3** FIC index values of the N- and C-terminal domains of Cg-BigDef1a

<table>
<thead>
<tr>
<th>Strain</th>
<th>FIC index</th>
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<tr>
<td></td>
<td>Covalently linked domains</td>
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<tr>
<td><em>Staphylococcus aureus</em> SG511</td>
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<tr>
<td><em>Corynebacterium maritum</em> CIP 101282</td>
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<td><em>Micrococcus luteum</em> CIP 53.45</td>
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<tr>
<td><em>Vibrio crassostreae</em> 7F5_29</td>
<td>0.125</td>
</tr>
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</table>

aThe synergies of the N- and C-terminal domains were measured as described previously (51) by incubating either both domains or the full-length Cg-BigDef1 (i.e., covalently linked domains) with bacterial suspensions of 4 strains displaying the lowest MICs for Cg-BigDef1[1–93]. Results are expressed as FIC index values according to the following formula: FIC = (N-terminal)/MICN-ter + (C-terminal)/MICC-ter where MICN-ter and MICC-ter are the MICs of the N- and C-terminal domains tested alone and (N-ter) and (C-ter) are the MICs of the two peptides tested in combination. FIC index values are interpreted as follows: <0.5, strong synergy; 0.5 to 1, synergy; 1 to 2: additive effect; 2, no effect; >2, antagonism.
Cg-BigDef1 is dependent on a covalent association with the N-terminal Cg-BigDef1[1–42] domain.

Next, we focused our subsequent studies on S. aureus, which was highly sensitive to Cg-BigDef1 activity. We showed that Cg-BigDef1[1–93] was bactericidal up to 300 mM NaCl against both the laboratory strain S. aureus Newman and the multidrug-resistant S. aureus clinical isolates 7877 and 53863 (Fig. 4A). At 5 μM, Cg-BigDef1[1–42] at 20 μM, and Cg-BigDef1[44–93] at 20 μM against S. aureus Newman. Bacterial cells were incubated with the indicated peptides (gray bars) or the corresponding solvents (black bars) in killing buffer (KB) in the presence of 0, 20, 100, or 300 mM NaCl. After 2 h, bacterial suspensions were serially diluted with phosphate-buffered saline (PBS) and aliquots were streaked on Luria-Bertani (LB) agar plates and incubated for 24 h at 37°C. Bactericidal effects were monitored by counting the bacterial CFU on LB agar plates and expressed either as percent killing compared with that seen with treatment without the antimicrobial peptide (A) or as CFU counts per milliliter (B). ND, not detected (<100 CFU per milliliter).

Cg-BigDef1[44–93] is dependent on a covalent association with the N-terminal Cg-BigDef1[1–42] domain.

We further explored the mechanism of action of Cg-BigDef1 on S. aureus. We showed that Cg-BigDef1[1–93] was bactericidal up to 300 mM NaCl against both the laboratory strain S. aureus Newman and the multidrug-resistant S. aureus clinical isolates 7877 and 53863 (Fig. 4A). At 5 μM, Cg-BigDef1[1–93] was bactericidal against S. aureus Newman in the range of 0 to 300 mM NaCl whereas NaCl by itself had no effect on bacterial growth (Fig. 4B). A bactericidal effect was recorded for Cg-BigDef1[1–42] in the absence of NaCl as a consequence of raising the peptide concentration to 20 μM. This effect was lost at concentrations over 100 mM NaCl (Fig. 4B). No bactericidal activity was recorded for 20 μM Cg-BigDef1[44–93] in the NaCl concentration range of 0 to 300 mM (Fig. 4B). Therefore, unlike its separate domains, Cg-BigDef1[1–93] shows salt-stable bactericidal activity.

Cg-BigDef1 entraps bacteria in nanonets without inducing membrane permeabilization. We further explored the mechanism of action of Cg-BigDef1 on S. aureus. Cg-BigDef1[1–93] killed cells of the SGS11 reference strain at 1.25 μM, with a 2-log reduction in colony-forming unit (CFU) counts after 2 h. At 10 μM, no CFU could be counted after 60 min (Fig. 5A) and no membrane permeabilization was detected (Fig. 5B). Therefore, Cg-BigDef1 bactericidal activity is independent of membrane permeabilization in S. aureus. Remarkably, by observing Cg-BigDef1-treated S. aureus by scanning electron microscopy (SEM), we found that Cg-BigDef1[1–93] undergoes intense fibrilar aggregation upon contact with bacteria. Cellular cells were entrapped in highly branched nanonets and/or covered with large fibers that adhered to the bacterial surfaces when incubated with 5 μM Cg-BigDef1[1–93] (Fig. 6A). Such structures were not observed in the absence of bacteria (data not shown), which indicates that bacteria play an essential role in triggering their assembly. Similar structures were observed when bacteria were incubated with 5 μM Cg-BigDef1[1–42] but not when...
they were incubated with β-defensin-like Cg-BigDef1[44–93] (5 μM) (Fig. 6A). This supramolecular assembly is consistent with the loss of solubility of Cg-BigDef1[1–93] and Cg-BigDef1[1–42] after 30 min of contact with S. aureus (Fig. S5 in the supplemental material). By using polyclonal antibodies raised against Cg-BigDef1[1–93], we observed intense immune staining of large areas surrounding S. aureus under conditions of incubation with Cg-BigDef1[1–93] (5 μM) (Fig. 6B). This showed that the observed fibers represented Cg-BigDef1[1–93] nanonets. Confocal sections revealed the absence of immune staining at positions occupied by bacterial cells, showing that Cg-BigDef1[1–93] did not enter bacteria intracellular space, at least during 30 min of contact (Fig. 6B).

**Cg-BigDef1 is neither cytotoxic nor proinflammatory toward mammalian cells.** We finally examined Cg-BigDef1 and its separate domains for potential toxic and/or proinflammatory effects on eukaryotic cells. None of the three peptides were toxic toward bronchial epithelial cell line IB3 isolated from CF patients, as they did not induce any detectable release of lactate dehydrogenase compared to the 100% release seen in the Triton X-100 positive control (Fig. 7A). Moreover, they did not induce a proinflammatory response in mouse alveolar macrophage cell line J774. Indeed, none of the peptides triggered secretion of keratinocyte-derived protein chemokine (KC) (Fig. 7B), the mouse homologue of interleukin-8 (IL-8) known to induce neutrophil chemotactic activity (23), leading to the accumulation of these cells in the site of infection. Similar results were obtained for IL-1β (Fig. 7C), which promotes bacterial killing by alveolar macrophages (24). In contrast, infection of J774 cells by Pseudomonas aeruginosa PAO1 (positive control) led to high levels of secretion of both KC and IL-1β (Fig. 7B and C).
During evolution, the activity of β-defensins has broadened beyond direct antimicrobial action such that in mammals, some peptides have adopted a series of additional functions in immunity (e.g., immune modulation, chemotraction) and reproduction; they are currently considered to represent multifunctional HDPs rather than solely AMPs (3, 25). Here, by studying big defensins, the ancestors of Δ2-defensins, we

**FIG 6** The N-terminal domain drives bacterially triggered assembly of Cg-BigDef1 into nanonets. (A) SEM observation of Cg-BigDef1 nanonets. Large and branched fibers entrapping S. aureus SG511 were observed in S. aureus samples subjected to 24 h of exposure to 5 μM Cg-BigDef1[1–93] or Cg-BigDef1[1–42] but not 5 μM Cg-BigDef1[44–93], which is indistinguishable from the no-peptide control results (not shown). (B) Immune staining of Cg-BigDef1 nanonets in contact with S. aureus SG511. Confocal microscopy images were acquired after 30 min of contact between bacteria and peptides. DNA was stained with DAPI (blue), and Cg-BigDef1 was stained with polyclonal antibodies (anti-Cg-BigDef1) revealed with a secondary antibody coupled to Alexa Fluor 488 (green). Merged images show that space occupied by S. aureus cells is left empty by Cg-BigDef1 nanonets. Bars represent 3 μm.

**DISCUSSION**

During evolution, the activity of β-defensins has broadened beyond direct antimicrobial action such that in mammals, some peptides have adopted a series of additional functions in immunity (e.g., immune modulation, chemotraction) and reproduction; they are currently considered to represent multifunctional HDPs rather than solely AMPs (3, 25). Here, by studying big defensins, the ancestors of β-defensins, we

**FIG 7** Cg-BigDef1 is neither cytotoxic nor proinflammatory toward eukaryotic cells. (A) Cytotoxicity assay. NCI-H292 cells were incubated with 5 μM Cg-BigDef1[1–93], 20 μM Cg-BigDef1[1–42], or 20 μM Cg-BigDef1[44–93]. The release of lactate dehydrogenase (LDH) by the cells was measured after 24 h using the CytoTox 96 nonradioactive cytotoxicity assay (Promega). Triton X-100 (1%) was used as a positive control (100% LDH release). Untreated cells were used as a negative control (Ctrl). Data are expressed as a percentage of the total LDH release compared to cells treated with Triton X-100 (for more details, see reference 22). (B and C) Proinflammatory assays. Macrophage cells (cell line J774) were incubated with peptides at the same concentrations. Secretion of KC (B) and IL-1β (C) was measured after 24 h using DuoSet ELISA kits as previously described (24).

As a positive control for cytokine/chemokine secretion, J774 cells were infected with P. aeruginosa PA01 (multiplicity of infection [MOI] 1:1).
uncovered unique physicochemical properties essential for their ancestral antimicrobial activity. These properties have been lost during evolution toward β-defensins but have been preserved in a limited number of marine species.

We found that oyster Cg-BigDef1 has conserved salt-stable antimicrobial activity. Indeed, Cg-BigDef1 showed a broad spectrum of activity in the 1 to 10 μM range, even at high salt concentrations. It was active at up to 300 mM NaCl against human pathogens and 400 mM NaCl against oyster pathogens (marine microorganisms). The two domains of Cg-BigDef1 did not display complementary spectra of activities, as initially proposed for the horseshoe crab Tt-BigDef (10). Instead, they were highly synergistic, highlighting the importance of their covalent association for the whole peptide activity. NMR data revealed that oyster Cg-BigDef1, like horseshoe crab Tt-BigDef (26), possesses two structural domains. The hydrophobic N-terminal domain adopts a unique globular fold, and the C-terminal domain adopts a β-defensin-like conformation. The two domains are in close contact, giving rise to a very compact 3D structure in solution. While cationic and anionic patches are displayed at the surface of Cg-BigDef1, the overall structure is mainly hydrophobic and does not display the typical amphipathic structure of cationic AMPs (27). This has important consequences for Cg-BigDef1 interactions with bacteria, which were shown to require the hydrophobic N-terminal domain and are not impaired at high salt concentrations. This suggests that Cg-BigDef1 interacts with bacterial membranes through hydrophobic interactions rather than electrostatic interactions.

The 3D structure of Cg-BigDef1 is only the second from the big defensin family. It differs from Tt-BigDef by exhibiting a longer linker and a different orientation of the N- and C-terminal domains (Fig. 8). By modifying the orientation of the domains, we found that linkers drastically modified the surface properties of big defensins. Indeed, Tt-BigDef is amphipathic whereas Cg-BigDef1 is hydrophobic. Whether this orientation modifies peptide activity and/or stability with respect to salts remains to be established. Indeed, information is still missing on the activities of Tt-BigDef and other big defensins, precluding further interpretations. In a micellar (membrane-like) environment, the N-terminal domain of Tt-BigDef adopts a single α-helix structure, which penetrates into micelles; it was hypothesized that insertion of this helix into target membranes may be involved in the Tt-BigDef antimicrobial activity (11). According to our results, such a membrane activity is not responsible for Cg-BigDef1 activity, although it could confer antimicrobial activity to the Cg-BigDef1 N-terminal domain at a high concentration (20 μM).

The ancestral N-terminal domain of Cg-BigDef1 was shown to drive bacterially triggered assembly of Cg-BigDef1 into nanonets, while Cg-BigDef1 appeared highly soluble and monomeric in solution. These nanonets entrapped and killed S. aureus. The N-terminal domain alone also produced nanonets but did not kill S. aureus. This suggests that Cg-BigDef1 antimicrobial activity is carried by the β-defensin-like domain but requires the N-terminal domain to promote close contact with bacteria. The mechanism by which the N-terminal drives nanonet formation remains to be characterized. In human α-defensin 6 (HD6), which self-assembles into elongated fibrils and agglutinates bacteria (28), hydrophobic amino acids play a key role (29). For Cg-BigDef1 to achieve self-assembly, the amino acids of the hydrophobic core need to be exposed through a partial unfolding process or conformational change. Micelles of dodecyl-phosphocholine induced such a conformational change of Tt-BigDef N-terminal domain (26). However, no report has been published on the possibility that Tt-BigDef can form nanonets. Surprisingly, whereas the big defensin N-terminal domain was lost during evolution toward β-defensins, human BD1 (hBD1) was recently shown to form nanonets in its reduced form (30), which is the active form of hBD1 in the colonic environment (31). This suggests that nanonet formation may have emerged independently in diverse families of AMPs, highlighting a neglected function of AMPs, which can entrap bacteria and prevent subsequent host colonization (29, 30). Whether all AMPs that self-assemble into nanonets form highly ordered structures, as observed for HD6 in vitro (29), or protein aggregates remains unknown. According to the results of
the HD6 study, nanonets are highly distinct from β-amyloid fibrils, although assembly benefits from hydrophobic interactions in both cases. The mechanisms triggering nanonet assembly in structurally unrelated peptide families remain largely unknown. It has been proposed for HD6 that bacterial surface proteins provide a nucleation site for peptide self-assembly (28).

We showed here that oyster Cg-BigDef1 exerts antimicrobial activity, even at the high physiological salt concentrations of its marine host. This salt-stable antimicrobial activity is conferred by the hydrophobic properties of the ancestral N-terminal domain lost in β-defensins. The ability to osmoregulate, when present, is not as efficient in chelicerates and mollusks as in vertebrates, and oysters themselves are osmoconformers (32, 33). Consequently, the hemolymph concentration of Na\(^+\) and Cl\(^-\) ions is very close to their concentration in seawater. We believe that such strong selection pressures imposed by marine environments could have preserved ancestral big defensins from evolving toward (salt-sensitive) β-defensins in organisms with poor control of their blood osmolarity. In contrast, an evolutionary cost may have accelerated big defensin molecular evolution in species that osmoregulate their body fluids and have more efficient immune systems, such as vertebrates, or that live in freshwater. The only big defensin gene present in a freshwater organism (mussel) remains a puzzling exception (21); this gene may have evolved toward other functions after transition of mussel species from seawater to freshwater.

Interestingly, we found that Cg-BigDef1 is active against methicillin-resistant S. aureus at micromolar concentrations and under high salt conditions without being

![FIG 8](http://mbio.asm.org/) Cg-BigDef1[1–93] and Tt-BigDef 3D structure comparison. The hydrophobic domain, the linker, and the β-defensin-like domain are colored in deep olive, red, and blue, respectively. (A) Alignment of Cg-BigDef[1–93] (top) and Tt-BigDef (bottom) primary sequences. Conserved residues are indicated in bold. (B) 3D structure (cartoon representation) of Cg-BigDef[1–93] (PDB entry 6QBL). (C) 3D structure (cartoon representation) of Tt-BigDef (PDB entry 2RNG).
cytotoxic or proinflammatory toward mammalian cells. This is important, as small peptides that self-assemble can be toxic to mammals (34). MRSA is a major cause of mortality due to antibiotic-resistant infections (35). As antimicrobial resistance threatens human health and the core of modern medicine, the WHO is calling upon the design of new drugs active against multiresistant bacteria (36). On the basis of the complementary action of two domains, the mechanism of action of the Cg-BigDef1 complex would be an interesting trait to explore. We can indeed speculate that grafting the ancient N-terminal domain to vertebrate β-defensin would confer high stability with respect to salt and increase the spectrum of activity, as shown here for the two domains of Cg-BigDef1. This reconstruction of ancient big defensins not only may have synergistic effects, as reported for other AMP combinations (37) but may also confer a great advantage, as resistance rises at lower rates when combinations of AMPs are used (38). Considering the current interest in AMPs (39), we believe that analysis of ancestral big defensins can inspire the design of novel antimicrobials that will be efficient at physiological salt concentrations but that will also be applicable for treatment of diseases involving salt imbalance or for which salt treatment is used, such as cystic fibrosis (40).

**MATERIALS AND METHODS**

Database searches and sequence analysis. Sequences containing the β-defensin domain were collected from publicly accessible databases and were used for the search of homologous sequences in both annotated and nonannotated databases (tBLASTx at NCBI). All obtained sequences were manually inspected and translated using the ExPasy Translate Tool (http://web.expasy.org/translate/). Predictions of signal peptides and furin-like cleavage sites were performed with the ProP 1.0 Server (http://www.cbs.dtu.dk/services/ProP/). Multiple alignments of the deduced amino acid sequences were generated using the MAFFT program (https://mafft.cbrc.jp/alignment/server/).

Chemical synthesis. Peptides were synthesized by solid-phase peptide synthesis on a Prelude peptide synthesizer (Gyros-Protein Technologies) using standard Fmoc/tertBu chemistry at the 25-µmol scale starting from a Tentagel resin equipped with a Rink’s amide linker and including automated introduction of the N-2-hydroxy-5-nitrobenzyl (Hnb) group through on resin reductive amination (22). Cg-BigDef1 was obtained under standard conditions (19) through native chemical ligation (NCL) of an N-terminal cysteiny1 peptide segment (Cg-BigDef1[57–93]) and the crypto-thioester Cg-BigDef1[1–56]-(Hnb)Cys(S)Bu. After high-performance liquid chromatography (HPLC) purification, the reduced form was engaged into thermodynamically controlled oxidative folding to form the three disulfide bridges, using already-described protocols (41, 42). Purification by C4 RP-HPLC afforded the pure oxidized form of Cg-BigDef1 (see supplemental material).

Online liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). (i) Chemicals. MilliQ water (Merck Millipore, Billerica, MA) was used. LC-MS-grade formic acid (FA), trifluoroacetic acid (TFA), and acetonitrile (MeCN) were from Carlo-Erba (Val de Reuil, France). Reagent-grade chemicals for protein preparation and proline endopeptidase (E1411) were from Sigma-Aldrich (St. Louis, MO). Sequencing-grade modified trypsin and GluC were from Promega (Madison, WI). RapiGest surfactant (SF) was from Waters (Milford, MA).

(ii) Big defensin enzymatic digestion. Synthetic peptides were digested (overnight, 37°C) by adding RapiGest SF (0.1% to 1%) and protease (1:10 ratio). Following acidification, the digest was dried using a speed vacuum apparatus (FreeZone Plus 1.5-liter freeze-dry system; Labconco, Kansas City, MO, USA) and suspended in 2% MeCN–0.1% TFA (vol/vol).

(iii) Peptide analysis by LC-ESI-MS/MS. An Agilent HPLC HP-1290 system (Agilent Technologies, Santa Clara, CA) on-line coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) was used. Separation was performed on an Accucore C18 column (Thermo Scientific) (2.1 mm by 150 mm) at 35°C with a flow rate of 350 μL/min. Solvent A was 0.1% FA–water and solvent B was 0.1% FA–MeCN, and the gradient was from 2% solvent B to 15% in 26 min and then to 62% in 34 min. Typically, volumes of 0.5 to 2 μg of peptide were injected. The Q-Exactive Orbitrap mass spectrometer was operated as previously published (43, 44) except for the automatic lock mass function (enabled on the ion at m/z 593.15761).

NMR experiments and calculation of structures. Cg-BigDef1[44–93] and Cg-BigDef1[1–93] were dissolved in H2O:D2O (9:1 ratio) at concentrations of 1.5 mM and 1.0 mM, respectively. pH was adjusted to 4.6 for both samples. 2D 1H nuclear Overhauser effect spectroscopy (NOESY), 2D 1H total correlation spectroscopy (TOCSY), band-selective optimized flip angle short–heteronuclear multiple quantum coherence (sofam-HMQC) (45) (15N natural abundance), and 13C-HSQC (13C natural abundance) were performed at 298 K on an Avance III HD Bruker 700 MHz spectrometer equipped with a cryoprobe. 1H chemical shifts were referenced to the water signal (4.77 ppm at 298 K). NMR data were processed using Bruker’s Topspin 3.2 and analyzed with CCPNMR software (version 2.2.2) (46). Structures for both proteins were calculated using the Crystallography and NMR system (CNS) suite (47, 48) through the use of ARIA2 automatic assignment software (version 2.3) (49) with NOE derived distances and hydrogen bonds and three ambiguous disulfide bridges. For Cg-BigDef1[44–93], backbone dihedral angle restraints were
addicted (determined with the DANGLE program [50]). See detailed protocol in Text S1 in the supplemental material.

Strains and media. Strains and media are listed in Table S3 in the supplemental material. Marine strains from the genera *Alivibrio*, *Corynebacterium*, *Microbacterium*, and *Vibrio* were grown at 20°C in liquid Zobell 1/3 medium. Other strains were grown at 30°C in liquid Poor broth (PB) medium (Table S3). Zobell or Luria-Bertani (LB) agar plates were used as solid media.

Antimicrobial assays. (i) Determination of MICs and minimal bactericidal concentrations (MBCs). For antibacterial assays, MICs and MBCs were determined by the liquid growth inhibition assay previously described (51). For antifungal assays, inhibition of spore germination was monitored by a composite of MICs and MBCs. For antibacterial assays, MICs and MBCs were determined by the liquid growth inhibition assay previously described (51). For antifungal assays, inhibition of spore germination was monitored by a composite of MICs and MBCs.

(ii) Bacterial assays and kinetics of killing. Bacteria (10^6 to 10^9 CFU/ml) were incubated at 37°C with or without peptide in killing buffer (KB; 0.1 M Tris [pH 8.0], 1 mM CaCl_2) supplemented with 0.1% fatty acid-free bovine serum albumin and 0 to 300mM NaCl. CFU counts were performed by plating on LB agar plates at the indicated times. Data are expressed either as CFU per milliliter or as a percentage of killing compared to untreated samples. Tests were performed in triplicate, and mean values were calculated.

Membrane permeabilization assay. Stationary-phase cultures of *S. aureus* SG511 grown at 37°C in LB medium were washed and resuspended at 10^9 CFU/ml in 10 mM phosphate-buffered saline (PBS), supplemented with 1.38 mM NaCl and 2.7 mM KCl; pH was adjusted to 7.4 in 0.1 M NaOH. Bacteria were dispersed into 96-well microtiter plates containing 10 µl Cg-BigDef1 (10 μM or 1.25 μM final concentration), water (negative control), or Triton X-100 (0.1% final concentration; positive control). Fluorescence was measured every 5 min over 4 to 6 h (λ excitation [λex] = 480 nm; λ emission [λem] = 550 nm) at 20°C. The maximum permeabilization was given by the fluorescence of the positive control.

Cytotoxicity assays. Human airway epithelial NCI-H292 cells (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium supplemented with 200 mM L-glutamine, 10% (vol/vol) fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2.5 mg/liter glucose and buffered with 25 mM HEPES at 37°C in a humidified, 5% CO2 water-jacketed incubator. Then, the cells were cultured overnight under serum-free conditions and treated with the peptides (5 or 20 µM). After 24 h, the cell viability was assessed by measuring the release of lactate dehydrogenase (LDH) as previously reported (22).

Assays of cytokine production. Alveolar macrophage cell line J774 cells (ATCC TIB-67, ATCC, Manassas, VA, USA) were plated in complete RPMI medium supplemented with 1% sodium pyruvate, 200 mM l-glutamine, 20 U/ml penicillin, 200 µg/ml streptomycin, and 2.5 mg/liter glucose and buffered with 25 mM HEPES (pH 7.4). After 24 h, cells were incubated overnight with fresh medium and treated with the peptides (5 or 20 µM) for 24 h. As a positive control for cytokine secretion, cells were infected with *P. aeruginosa* PAO1. After 1 h, bacteria were removed, the cells were reincubated in fresh culture medium for 20 h, and cytokine levels were measured by DuoSet enzyme-linked immunosorbent assay (ELISA) kits as previously described (24).

Monitoring of peptide solubility. Stationary-phase cultures of *S. aureus* SG511 grown at 37°C in LB medium were washed twice and resuspended in KB supplemented with 150 mM NaCl. Bacteria (10^9 CFU/ml) were treated with 5 µM Cg-BigDef1[1–93], Cg-BigDef1[1–42], or Cg-BigDef1[44–93]. For negative controls, we used a same volume of water or 10% dimethyl sulfoxide (DMSO). After incubation for 30 min at 20°C, bacteria were removed by centrifugation and TFA-acidified supernatants were analyzed by HPLC on an Agilent HPLC HP-1290 system (as described above), with an Accucore C4 column (2.1 mm by 150 mm; flow rate, 300 µl/min); solvent A was 0.05% TFA–water, and solvent B was 0.04% TFA–MeCN. The gradient consisted of an increase from 2% solvent B to 60% in 30 min, and UV detection was performed at 225 nm.

Immunofluorescence. Overnight cultures of *S. aureus* SG511 were washed three times to remove all traces of culture media and adjusted to 10^7 CFU/ml in killing buffer (KB) before contact with Cg-BigDef1[1–93] (5 µM final concentration). After 30 min at 25°C, bacterial cells were washed three times in KB, centrifuged onto glass slides (10 min, 1,500 rpm), and fixed for 10 min in PBS containing 4% paraformaldehyde. After permeabilization (0.01% Triton X-100, 10 min), cells were immunostained with a polyclonal mouse anti-Cg-BigDef1 antibody (or preimmune serum), and a 1:1,000 dilution of secondary anti-mouse antibody coupled to Alexa Fluor 488 (Invitrogen). After three washes in PBS containing 0.05% Tween 20, cells were stained for 10 min with 0.25 µg/ml DAPI (4’6-diamidino-2-phenylindole). Coverslips were mounted with fluorescent mounting medium (Dako). Slides were observed with 63× objectives, and images were captured using a Leica TCS SPE confocal scanning laser microscope.

Scanning electron microscopy of nanonets. Overnight cultures of *S. aureus* SG511 or *P. aeruginosa* ATCC 9027 were washed three times in killing buffer supplemented with 150 or 300 mM NaCl. Bacterial suspensions adjusted to an A600 of 3 were deposited on a glass slide onto peptides (5 µM final concentration). After 24 h of incubation in a humid chamber, preparations were fixed with 2.5% glutaraldehyde. Fixed samples were dehydrated using a graded ethanol series (30% to 100%), followed by 10 min in graded ethanol-hexamethyldisilazane and then hexamethyldisilazane alone. Subsequently, the samples were sputter coated with an approximately 10-nm-thick gold film and then examined under a scanning electron microscope (Hitachi S4000; microscopy performed at CoMET, MRI-RIO Imaging, Biocampus, INM Montpellier, Montpellier, France) using a lens detector with an acceleration voltage of 10 kV at calibrated magnifications.
Data availability. Assignments were deposited as Biological Magnetic Resonance Bank (BMRB) entries 34345 and 34346 for Cg-BigDef1[1–93] and Cg-BigDef1[44–93], respectively. Coordinates were deposited as PDB entries 6Q8K and 6QLB for Cg-BigDef1[44–93] and Cg-BigDef1[1–93], respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01821-19.

TEXT S1, DOCX file, 0.03 MB.
FIG S1, DOCX file, 1.1 MB.
FIG S2, DOCX file, 0.5 MB.
FIG S3, DOCX file, 0.3 MB.
FIG S4, DOCX file, 0.1 MB.
FIG S5, DOCX file, 0.04 MB.

TABLE S1, DOCX file, 0.04 MB.
TABLE S2, DOCX file, 0.01 MB.
TABLE S3, DOCX file, 0.03 MB.

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