The electrostatic interaction of the charge cluster of an amphipathic peptide antibiotic with microbial membranes is a salt-sensitive step that often determines organism specificity. We have examined the correlation between charge clusters and salt insensitivity and microbial specificity in linear, cyclic, and retro-isomeric cystine-stabilized β-strand (CSβ) tachyplesin (TP) in a panel of 10 test organisms. Cyclic tachyplesins consisting of 14 and 18 amino acids are constrained by an end-to-end peptide backbone and two or three disulfide bonds to cross-brace the anti-parallel β-strand that approximates a “β-tile” structure. Circular dichroism measurements of β-tile TPs showed that they displayed ordered structures. Control peptides containing the same number of basic amino acids as TP but lacking disulfides or disulfides were highly salt sensitive. Cyclic TP analogues with six cationic charges were more broadly active and salt-insensitive than those with fewer cationic charges. Reducing their proximity or number of cationic charges, particularly those with three or fewer basic amino acids, led to a significant decrease in potency and salt insensitivity, but an increased selectivity to certain Gram-positive bacteria. An end-group effect of the dibasic N-terminal Lys of TP in the open-chain TP and its retroisomer was observed in certain Gram-negative bacteria under high-salt conditions, an effect that was not found in the cyclic analogs. These results suggest that a stable folded structure together with three or more basic amino acids closely packed in a charged region in CSβ peptides is important for salt insensitivity and organism specificity.

A common feature shared among the 500 antimicrobial peptides found in nature is their tendency to form amphipathic structures that cluster basic and hydrophobic amino acids into distinctive regions (1). This secondary structure is important for productive interactions of antimicrobial peptides with the microbial surfaces in models proposed independently by Shai (2), Matsuzaki (3), and Huang and co-workers (4) to explain the microbial killing mechanisms. In these models, the charged region provides the initial interaction with the negatively charged headgroups of the microbial surfaces whereas the hydrophobic region facilitates the displacement of lipids and entry of the peptide into the cell interior.

Cystine-stabilized β-strand (CSβ) peptides belong to a major structural family of antimicrobial peptides that are characterized by one or more cystine bonds in their β-strand scaffolds. This family is richly represented by defensins, protegrins, and tachyplesins that generally possess a cluster of three or more basic amino acids in their charged regions. These CSβ peptides exhibit various degrees of salt sensitivity. The protegrins and tachyplesins are salt insensitive whereas the α- and β-defensins are rapidly inactivated at physiological salt concentration of about 100 mM (high salt condition). Furthermore, the salt-dependent inactivation of β-defensin-1, an antimicrobial peptide found in lung epithelia, may play an important role in innate immunity in cystic fibrosis (5). The charged region may be useful in designing membrane-active transportants for intracellular delivery of functionally active cargoes that include peptides and proteins to inhibit protein-protein interactions (6). Recent findings have shown that synthetic peptides containing six or more basic amino acids in continuous sequences are cell permeable and can act as transportants to eukaryotic cells (7). However, the importance of basic amino acids present in a charge cluster of an amphipathic antimicrobial peptides under physiological conditions is poorly understood. To this end, we have used tachyplesin-1 as a model because it is one of the most potent antimicrobial peptides known today (8, 9). TP-1 exhibits a broad-spectrum activity against bacteria and fungi with minimal inhibition concentration (MIC) values in the submicromolar range under high salt conditions that may render some antimicrobial peptides inactive. Furthermore, TP-1 has also been shown to play a role in the proinflammatory response because it forms complexes with bacterial lipopolysaccharides that neutralize the factor C-activating activity of LPS in a manner similar to that of anti-LPS factor (8).

Tachyplesins isolated from horseshoe crabs consisting of 17–19 amino acids contain two cross-bracing disulfides stabilizing an antiparallel β-strand connected by a reverse turn as determined by two-dimensional NMR (10). They are similar in structure and activity profile to protegrins (11), a family of peptides found in pig intestines. Because of their potency and

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1 The abbreviations used are: CSβ, cystine-stabilized β-strand; Acm, acetylamidomethyl; DCC, N,N-dicyclohexylcarbodiimide; DCM, dichloromethane; DIC, N,N-diisopropylcarbodiimide; DIAE, N,N-diisopropylmethylation; DMF, dimethylformamide; MeSO, dimethylsulfoxide; HOBT, N-hydroxybenzotriazole; MALDI/MS, matrix-assisted laser desorption/ionization mass spectrometry; MBHA resin, methylbenzhydrylamine resin; MeBzl, methylbenzyl; MIC, minimal inhibition concentration; PG-1, protegrin-1; RP-HPLC, reverse phase-high performance liquid chromatography; Rₜ, retention time; RTD-1, rhesus theta defensin; TCEP, tris(carboxyethyl)phosphine; TFE, trifluoroethanol; TP-1, tachyplesin-1; TSB, trypticase soy broth; Boe, t-butyloxycarbonyl; LPS, lipopolysaccharide.

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James P. Tam‡; Yi-An Lu, and Jin-Long Yang

From the Department of Microbiology and Immunology, Vanderbilt University, A519 MCN, Nashville, Tennessee 37232-2363

Correlations of Cationic Charges with Salt Sensitivity and Microbial Specificity of Cystine-stabilized β-Strand Antimicrobial Peptides

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relatively small sizes, these peptides are attractive targets for structure-activity studies that may lead to therapeutics to treat infections.

Cyclic CSβ antimicrobial peptides known as cyclotides have also been discovered in plants and animals (12–16). These end-to-end constrained peptides include the Rhesus theta defensin (RTD-1) that has been identified from Rhesus monkey leukocytes (17). RTD-1, an 18-amino acid and salt-insensitive cyclic peptide, contains a β-tile-like structure with two β-strand framework constrained by three evenly spaced cross-linking disulfide bonds that partition RTD-1 into a four ring-like structure (Fig. 1). Because the structures and TP-1 are relatively similar, the stable β-tile template of RTD-1 can be exploited for structure-activity study (18, 19). Previously, we have replaced the hydrophobic amino acids with glycine in β-tile tachyplesin analogues and found that they retain most of their antimicrobial activity and salt insensitivity (20), suggesting that the constraint elements of the β-tile design that provide the conformational stability are sufficient to exert activity in high salt conditions.

The topology of charge cluster in tachyplesins and the CSβ antimicrobial peptides is due to their folded structure. Their electrostatic interactions with the microbial surfaces can be weakened under high salt conditions of 100 mM NaCl similar to physiological ionic conditions. In tachyplesins, most of the charged residues are clustered on one side of the β-tile structure. By maintaining a β-tile structure that provides a stable structure, we reasoned that it may be possible to correlate the number of basic amino acids in tachyplesin analogues to salt sensitivity. In cyclic peptides, there appears to be a correlation between the number of charges and salt sensitivity. Cyclic peptides having about 30 residues such as plant cyclotides that contains similar amino acid compositions as TP-1 but contains an invariable basic amino acid, Lys or Arg, at their N terminus and a carboxamide at their C terminus (12–15) with a cystine-knot motif (21–23) are salt sensitive and are far less basic than the salt-insensitive tachyplesins, protegrins, and RTD-1 that contain six cationic charges.

The charge cluster in an amphipathic peptide may also be a result of the side chain and the N-terminal amines of a dibasic amino acid. The open-chain CSβ structures in tachyplesins, protegrins, and bactenecin-1 contain an invariable basic amino acid, Lys or Arg, at their N terminus and a carboxamide at their C terminus. The end group effect in providing localized cationic charges to confer salt insensitivity can be conveniently examined by comparing TP-1 with its retroisomer that maintains similar amino acid compositions as TP-1 but contains transposed N-terminal Lys and C-terminal Gln in its sequence. Furthermore, the end-group effect of the open-chain TP-1 can be compared with cyclic TP-1, which does not have end groups.

In this report, we describe the correlation of cationic charges with activity spectra and salt sensitivity using linear, cyclic, and retroisomeric tachyplesin-1 analogs. In particular, we have exploited the β-tile design to constrain 14- and 18-residue tachyplesin analogs with an up-and-down arrangement of side chains (Fig. 1). Our results show that the number, proximity, and topology of charged amino acids are important contributions to organism specificity and salt sensitivity.

### MATERIALS AND METHODS

#### Peptide Synthesis and Purification

All peptides were synthesized by a stepwise solid phase peptide synthesis (24) performed manually or on an ABI 431A synthesizer using t-butyloxycarbonyl chemistry on a thioester resin (25, 26). The peptides cleaved from the resin by HF and purified by preparative RP-HPLC have been reported previously (18, 20). The purity of linear and cyclic peptides was verified by analytic RP-HPLC and described below, and correct peptide masses were verified by mass spectrometry on PerSeptive Biosystems Voyager instrument.

**Analytic RP-HPLC**—Analytical HPLC was performed on Shimazu SCL-6A using a Vydac C18 column (5μm, 4.6 × 250 mm) at a flow rate of 1 ml/min with a linear gradient of 5–65% B/90 min. Buffer A was 0.045% trifluoroacetic acid in water and buffer B was 90% acetonitrile in H2O, 0.04% trifluoroacetic acid with UV detection at 225 nm.

### Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Basic amino acid</th>
<th>HPLC</th>
<th>MH +</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP-18</td>
<td></td>
<td>17.71</td>
<td>2321.82 (2320.58)</td>
</tr>
<tr>
<td>cTP-18</td>
<td></td>
<td>19.71</td>
<td>2321.82 (2322.09)</td>
</tr>
<tr>
<td>cTP-14a</td>
<td></td>
<td>18.72</td>
<td>2303.80 (2303.44)</td>
</tr>
<tr>
<td>cTP-14b</td>
<td></td>
<td>21.28</td>
<td>2303.80 (2303.18)</td>
</tr>
<tr>
<td>cTP-14c</td>
<td></td>
<td>21.25</td>
<td>2185.72 (2184.91)</td>
</tr>
<tr>
<td>ccTP-14a</td>
<td></td>
<td>15.90</td>
<td>1823.21 (1823.10)</td>
</tr>
<tr>
<td>ccTP-14b</td>
<td></td>
<td>16.44</td>
<td>1823.21 (1823.61)</td>
</tr>
<tr>
<td>ccTP-14c</td>
<td></td>
<td>17.32</td>
<td>1684.81 (1684.35)</td>
</tr>
<tr>
<td>ccTP-14d</td>
<td></td>
<td>19.29</td>
<td>1577.92 (1578.10)</td>
</tr>
<tr>
<td>CP-1</td>
<td></td>
<td>17.65</td>
<td>1729.10 (1729.52)</td>
</tr>
<tr>
<td>CP-2</td>
<td></td>
<td>19.40</td>
<td>1688.08 (1687.55)</td>
</tr>
<tr>
<td>CP-15</td>
<td></td>
<td>20.50</td>
<td>1715.11 (1715.41)</td>
</tr>
<tr>
<td>CP-16</td>
<td></td>
<td>14.12</td>
<td>1948.33 (1948.12)</td>
</tr>
<tr>
<td>CP-2</td>
<td></td>
<td>12.80</td>
<td>2220.23 (2218.52)</td>
</tr>
</tbody>
</table>

#### Thioester Resin—Thioester resins were prepared according to Zhang and Tam (25), based on a thioester resin described by Hojo and Aimoto (26). Briefly, four equivalents (eq) of each of 3-mercaptopyrrolpropionic acid, HOBT, and DIC were sequentially added to a suspension of MBHA resin in DMP (10 ml/g resin). The mixture was shaken at room temperature until on-resin ninhydrin testing indicated that free amino sites were absent. The resin was washed in order with DMF, DCM, CH3OH, DCM, and DMF, and then treated with a mixture of 1 eq each of cysteine methyl ester hydrochloride, triphenylphosphine, and DIEA in DMP/DCM (3:1, v/v) for 2 h. After thorough washing with DMF, DCM, CH3OH, DCM, and drying in vacuum, the mercaptopyrrolpropionyl MBHA resin was obtained in quantitative yield.

#### Boc amino acid (4 eq) preactivated with BOP (4 eq) and DIEA (6 eq) for 5 min was added to the suspension of mercaptopyrrolpropionyl MBHA resin (1 eq) in DMP (10 ml/g resin). The mixture was shaken at room temperature for 2 h, and free thiol groups were monitored by Ellman’s reagent (27). The resins were washed in order, with DMF, DCM, CH3OH, and DCM and used for stepwise peptide synthesis.

#### End-to-end Cyclization of N-terminal Cysteinyl Peptide Thioester—All cyclic peptide precursors were assembled by Boc-chemistry solid-phase peptide synthesis on thioester resin. The assembled peptides were cleaved from the resin by hydrofluoric acid. The crude peptide was extracted into an 8 ∼ urea solution (pH 7.8) containing TCEP. Aliquots of crude linear peptide thiosteres were purified to confirm the purity before cyclization and to determine their rates of cyclization. The 8 ∼ urea solution was dialyzed by sequentially lowering the urea concentration to 4 ∼ and then 2 ∼ to permit concurrent cyclization, which was monitored by analytical C18 reversed-phase HPLC and MALDI-MS. In general, all cyclization reactions were complete during the 12–18 h dialysis sequence (28–30).

#### Two Step Disulfide Bond Formation—A two step method for disulfide bond formation was used to prepare the cyclic peptides with two or more disulfide bonds. The detailed procedure was reported previously (29, 31). The peptide was purified on preparative HPLC and characterized by MS (Table 1).

#### Antimicrobial Activity—A sensitive two stage radial diffusion assay (32) was employed for testing the antimicrobial activity of these peptides. Ten organisms obtained from the American Type Culture Collection (ATCC) were used for these assays. The Gram-negative bacteria were Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Klebsiella oxytoca ATCC 49311, and Proteus vulgaris ATCC 43492. The three Gram-positive bacteria were Staphylococcus aureus 29213, Micrococcus luteus ATCC 49732, and Enterococcus faecalis ATCC 29212. The three fungi were Candida albicans ATCC 37092, Candida krav ATCC 37095, and Candida tropicalis ATCC 37097. The strains were incubated in tryptocase soy broth (TSB), which was prepared in double distilled water and autoclaved for sterilization. The test organism was mixed with molten underlay gel solution poured into 10 × 10-cm Petri dishes. The gel solution contained 10 mM sodium phosphate buffer, 0.03% TSB, and 0.02% Tween 20 (with high salt) or without (low salt) 100 mM NaCl. Aliquots from seven concentrations of a serial half-log dilution of test peptides were added to each well. The
RESULTS

Peptide Design—The odd-numbered 17-residue TP-1 was transformed to an even-numbered tachyplesin TP-18 by adding a glycine at its C terminus not only because of the need for a tile-like design, but also for the end-to-end circularization of 18-residue peptides favored by the $4n+2$ rule ($n =$ number of amino acids) (33). Circularization of TP-18 formed cTP-18 with three constraints, a lactam plus two cystine. Adding a third cystine to cTP-18 formed ccTP-18 with four constraints that approximates a perfect $\beta$-tile structure consisting of four consecutively fused rings that are denoted as rings A, B, C, and D partitioned by three evenly spaced cross-strand disulfides. The ccTP-18 template is similar to RTD-1 (10–12) and simplifies our design for structure-activity study because it contains an invariable three-cystine face and a variable face consisting of cationic and hydrophobic amino acids. To determine the end-group effect, we prepared TP-18 and cTP-18 and their corresponding retroisomers, rTP-18 and rcTP-18 (Fig. 1).

The basic amino acids in TP-18 and their cyclic analogs are clustered on one side of the amphipathic structure. Mutating basic to nonbasic amino acids will lead to increased distance between cationic charges. To provide a close-packed cationic cluster, we prepared the truncated peptide synthesis. Cyclic peptides prepared from linear peptide precursors were cyclized to form end-to-end peptides in aqueous solutions (Fig. 4). In this scheme, an N-terminal cysteine and a C-terminal thioester undergo an intramolecular thiol-assisted intramolecular rearrangements (30). Ultimately, the ccTP-14 series of 14-residue cyclic dicystine peptides were designed to correspond with the monocystine cTP-14 series. The two cross-bracing disulfide bonds partitioned this series of peptides to a three-ring structure with a perfect tile-like topology that is structurally similar to ccTP-18. Adding a cystine to this group of ccTP-14a to ccTP-14d (Fig. 3) resulted in a series of peptides that contained two to four basic amino acids and were less cationic than the TP-18, cTP-18 and cTP-14 series. The sequence of the tile-like ccTP-14a also corresponded to the single-cystine cTP-14a so that the effect of the additional constraint on the 14-residue cyclic peptide could be compared.

Two control peptides with six basic amino acids similar to TP-1 but without disulfide constraints were also prepared. A linear peptide containing repeats and degenerated repeats of BHHB (B = Arg or Lys, H = hydrophobic amino acids) tethered on the amino arms of a lysine was used to mimic TP-1 with the topological repeats of basic and hydrophobic amino acids. This control peptide was used in a previous study for a different purpose but was included here for comparison. A cyclic peptide of TP-18 without disulfide constraint was also prepared for comparison.

Cys Thioester Cyclization—Open-chain peptides TP-18 and retro-TP-18 were prepared by the conventional solid-phase peptide synthesis. Cyclic peptides prepared from linear peptide thioester precursors were cyclized to form end-to-end peptides in aqueous solutions (Fig. 4). In this scheme, an N-terminal cysteine and a C-terminal thioester undergo an intramolecular transthioesterification through the N-terminal thiol with the C-terminal thioester to form a thiolactone. A subsequent ring contraction through an $S,N$-acyl migration forms the lactam.

The Cys-thioester cyclization was performed in a phosphate buffer at pH 7.6 without the use of any coupling reagent. The process was assisted by a thia-zip reaction involving a series of thiol-assisted intramolecular rearrangements (30). Ultimately, an N-terminal thiolactone is formed, leading to a spontaneous ring contraction through an $S,N$-acyl isomerization to form the end-to-end peptide bond. The cyclization rates of these 14, 18 residue macrocyclic peptides were found to be facile and completed in 12–18 h as monitored by analytic RP-HPLC.

To avoid misformed disulfide isomers, a two step oxidation method was used to form chemoselectively the disulfide bonds in cTP-18, retro-cTP-18 and other analogues containing two or three cystine pairs. In the two step oxidation method, one pair of cysteiny1 side chains was protected by the p-methylbenzyl (MeBzl) group, and the other pair by the acetamidomethyl
formed by Me₂SO oxidation. The Cys(Acm)-protected disulfide is re-
peptide from thia-zip-assisted cyclization. The disulfide bond is first
was obtained by stepwise solid phase peptide synthesis and cyclic
zation and disulfide formation of cTP-18.

acids in ccTP and ccTP-14 analogues.

disulfide connectivity of TP-18 was determined by
ues of 0.1
active peptides, displaying comparable potencies with MIC val-
analogs, TP-18, cTP-18, and their retroisomers, were broadly
killed and oxidized by Me₂SO to form one disulfide bond. Then
the Acm-protecting group was removed and the second disul-
cyclization and disulfide formation of cTP-18.

FIG. 3. Amino acid sequences and topology of charged amino
acids in ccTP and ccTP-14 analogues.

FIG. 4. Representative synthetic scheme for end-to-end cycli-
zation and disulfide formation of cTP-18. The thioester peptide
was obtained by stepwise solid phase peptide synthesis and cyclic
peptide from thienyl-assisted cyclization. The disulfide bond is first
formed by Me₂SO oxidation. The Cys(Acm)-protected disulfide is re-

(ACm) group. After cleavage from resin, the peptide was cy-
clized and oxidized by Me₂SO to form one disulfide bond. Then
the Acm-protecting group was removed and the second disul-
fide bond was formed by treatment with I₂/MeOH (Fig. 4). The
disulfide connectivity of TP-18 was determined by “on-target”
trypsin digestion and MALDI mass spectral analysis (34).

Antimicrobial Assays—A two stage radial diffusion assay in
agarose gels (32) was employed for testing the tachyplesin
analogues against ten organisms under both low and high salt
conditions with or without 100 mM NaCl. Each peptide was
tested over a range of 5,000-fold concentrations, and activities
were expressed as MICs of 0.1–500 µM (inactive).

Table II compares the antimicrobial profiles of six full-length
TP-18 analogs containing six basic amino acids and appropri-
ate numbers of hydrophobic amino acids, four with disulfide
constraints and two control peptides. Four CSβ-tachyplesin
analogs, TP-18, cTP-18, and their retroisomers, were broadly
active peptides, displaying comparable potencies with MIC va-
values of 0.1–1.3 µM against ten test organisms under both low
and high salt conditions. End-to-end cyclization of TP-18 to
cTP-18 resulted in only minor reduction of antimicrobial po-
tency. Overall, cTP-18 lost potency under high salt conditions
against K. oxytoca, M. luteus, and C. kefyr, but regained its
activity in the retroisomer. Similarly, there was no significant
alteration of the retroisomers rTP-18 and rcTP-18 in the anti-
microbial profile or potency against 6 of 10 test organisms, but
rcTP-18 displayed 2–4-fold increase in potency against, M.
luteus and C. kefyr. In contrast, the control peptides whether
linear or cyclic were significantly less potent than TP-18 under
low salt conditions and lost most of their activity under high
salt conditions.

Table III compares the antimicrobial profiles of three trunc-
cated cTP-18 analogs with different cationic charges. Two of
these analogs, cTP-14a and cTP-14b, contained six basic amino
acids whereas cTP-14c contained only four. In general, all three
analogues were broadly active and were fairly salt insensitive,
displaying MIC values of 0.5–2.1 µM under low salt conditions
and 0.7–4.2 µM under high salt conditions. Compared with
cTP-18, cTP-14a was 2–9-fold less active against Gram-nega-
tive bacteria. The retroisomers of cTP-14a, rcTP-14b, showed
a similar profile as cTP-14a. Removing two positive charges from
TP-14 but maintaining a close-pack cluster of three cationic
charges in cTP-14c (Fig. 2) resulted in only minor decreases in
the antimicrobial activity against certain Gram-negative or-
ganisms such as P. vulgaris and E. coli under high salt
conditions.

Previously, we reported that the antimicrobial activity of
cTP-18 with four basic amino acids was significantly lower
than that of cTP-18 (18, 19). Table IV compares the truncated
analoguecs of cTP-18-TP with a range of cationic charges. The
antimicrobial activity of the cTP-18 analogues with four cationic
charges, cTP-14a and cTP-14b, is comparable to cTP-14a and cTP-14c with MIC values of 0.8–8.8 µM. Increasing the
proximity of the basic amino acid residues in a charge cluster
resulted in enhanced potency of antimicrobial activities of
cTP-14a and cTP-14b than cTP-18. For example, there was
a significant increase in potency against two Gram-negative
bacteria, K. oxytoca and P. aeruginosa and one Gram-positive
bacteria E. faecalis. When the positive charges were decreased
to three in ccTP-14a or two in ccTP-14d, their activities were
significantly decreased, particularly against the Gram-nega-
tive bacteria and fungi. The two or three cationic charged
analogs ccTP-14c and ccTP-14d exhibiting MIC values of 1–3.8
µM against Gram-positive bacteria but 4–112 µM against
Gram-negative bacteria and fungi under high salt conditions
essentially became Gram-positive selective analogs.

DISCUSSION

We have exploited the β-tile template based on the cyclic
cystine-stabilized β-strand structure of RTD-1 to study the
importance of charge clusters in the full-length and truncated
tachyplesin to salt sensitivity and organism specificity. The
β-tile has an advantage to maintain a stable structure due to
its multiple intramolecularly constraints. Our previous study
has shown that the 18-residue β-tile peptides display stable
β-strand structures (18–20). Conformational stability in a
folded structure appears to be an important factor contributing
to salt insensitivity. These β-tile peptides with four or more
cationic charges are generally broadly active and salt insensi-
tive whereas the linear or cyclic control peptides with six cat-
ionic charges as TP-18 but without disulfide constraint are
found to be considerably less active and more salt sensitive.
These results are in agreement with previous structure-activ-
ity studies that focus on the role of the disulfide bonds of
tachyplesins. Deletion of two disulfide bridges has caused a
significant decrease in all activities (35, 36). Although Rao (37)
has found that antimicrobial activity can be retained when all
four cysteines are simultaneously substituted with other amino
acids suggesting that disulfide-bonded β-strand structure may
not be absolutely essential for antimicrobial activity, it is likely
that the study by Rao is performed under low salt conditions
in which linear tachyplesins retain significant antimicrobial ac-
tivity as observed in our control peptides.

The topological clusters of cationic charges in CSβ-stabilized
antimicrobial peptides can be roughly classified into two cate-
gories, mono-cluster and bi-cluster. Tachyplesins belong to the
mono-cluster with four or more of the cationic charges clustered
on one side of the amphipathic surface. The mono-cluster ar-
rangement is also found in the one-disulfide bactenecin-1 with
four cationic charges clustered at one end of a CSβ structure.
RTD-1 is also mono-clustered with all its cationic charges distributed fairly uniformly on the top face of the β-strands. In contrast, protegrins are bi-clustered with three cationic charges distributed on each end of the anti-parallel strands separated a hydrophobic region. Similar observation can be made for α-helical peptides that are known to be salt-insensitive. Both cercopins from silk moth and magainins from frogs contain monoclusters of cationic residues contributed by four or more basic amino acids. Taken together, they suggest the cationic charge region in a monocluster amphipathic structure of an antimicrobial peptide may require four or more basic amino acids to be broadly active and salt insensitive.

### TABLE II

Antimicrobial activity of TP-18, cTP-18 and their retropeptides

Experiments were performed in the radial diffusion assay with underlay gel containing 1% agarose, 10 mM phosphate buffer with (high salt, H) or without (low salt, L) 100 mM NaCl. Activities against multiple strains are expressed as MIC μM.

| Organism      | CP-1 | CP-2 | TP-18 | rTP-18 | cTP-18 | rcTP-18
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>L</td>
<td>H</td>
<td>L</td>
<td>H</td>
<td>L</td>
<td>H</td>
</tr>
<tr>
<td>Gram-negative</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.7</td>
<td>29.8</td>
<td>0.8</td>
<td>&gt;500</td>
<td>0.4</td>
<td>0.1</td>
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<tr>
<td><em>P. aeruginosa</em></td>
<td>6.3</td>
<td>40.4</td>
<td>32.3</td>
<td>&gt;500</td>
<td>0.5</td>
<td>0.2</td>
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<tr>
<td><em>P. vulgaris</em></td>
<td>18.4</td>
<td>117</td>
<td>40.5</td>
<td>&gt;500</td>
<td>0.4</td>
<td>0.7</td>
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<td><em>K. oxytoca</em></td>
<td>1.4</td>
<td>29.6</td>
<td>45.5</td>
<td>&gt;500</td>
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<td>0.2</td>
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<tr>
<td>Gram-positive</td>
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<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>2.1</td>
<td>111</td>
<td>35.2</td>
<td>&gt;500</td>
<td>0.4</td>
<td>0.5</td>
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<tr>
<td><em>M. luteus</em></td>
<td>0.8</td>
<td>1.8</td>
<td>18.7</td>
<td>&gt;500</td>
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<td>0.1</td>
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<tr>
<td><em>E. faecalis</em></td>
<td>5.0</td>
<td>17.2</td>
<td>na</td>
<td>na</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Fungi</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>1.9</td>
<td>14.4</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>3.1</td>
<td>38.2</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>5.0</td>
<td>28.8</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>0.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

### TABLE III

Antimicrobial activity of cTP-14 and their retropeptide

Experiments were performed in the radial diffusion assay as described in Table II.

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC</th>
<th>cTP-18</th>
<th>cTP-14a</th>
<th>rcTP-14b</th>
<th>cTP-14c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L-salt</td>
<td>H-salt</td>
<td>L-salt</td>
<td>H-salt</td>
</tr>
<tr>
<td>Gram-negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.1</td>
<td>0.5</td>
<td>0.6</td>
<td>1.9</td>
<td>0.7</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>0.7</td>
<td>0.2</td>
<td>0.9</td>
<td>1.8</td>
<td>1.0</td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>0.5</td>
<td>0.8</td>
<td>0.9</td>
<td>2.1</td>
<td>1.2</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>0.4</td>
<td>0.7</td>
<td>0.6</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Gram-positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0.4</td>
<td>0.5</td>
<td>0.7</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td><em>M. luteus</em></td>
<td>1.0</td>
<td>1.1</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>0.3</td>
<td>0.4</td>
<td>0.8</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>0.7</td>
<td>0.9</td>
<td>1.2</td>
<td>1.8</td>
<td>1.0</td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>0.9</td>
<td>1.3</td>
<td>0.5</td>
<td>1.1</td>
<td>1.9</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>0.5</td>
<td>1.0</td>
<td>0.7</td>
<td>0.7</td>
<td>0.8</td>
</tr>
</tbody>
</table>

### TABLE IV

Antimicrobial activity of ccTP-14 and their analogues

Experiments were performed in the radial diffusion assay as described in Table II.

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC</th>
<th>ccTP-18</th>
<th>ccTP-14a</th>
<th>ccTP-14b</th>
<th>ccTP-14c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L-salt</td>
<td>H-salt</td>
<td>L-salt</td>
<td>H-salt</td>
</tr>
<tr>
<td>Gram-negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>2.2</td>
<td>1.7</td>
<td>0.9</td>
<td>0.9</td>
<td>2.1</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>1.2</td>
<td>7.8</td>
<td>1.0</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>1.9</td>
<td>23.8</td>
<td>1.1</td>
<td>2.2</td>
<td>1.5</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>8.2</td>
<td>18.9</td>
<td>0.8</td>
<td>8.8</td>
<td>0.7</td>
</tr>
<tr>
<td>Gram-positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>7.5</td>
<td>3.0</td>
<td>1.3</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td><em>M. luteus</em></td>
<td>0.7</td>
<td>0.6</td>
<td>1.2</td>
<td>2.8</td>
<td>0.8</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>3.9</td>
<td>19.8</td>
<td>1.2</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>4.2</td>
<td>12.4</td>
<td>0.8</td>
<td>2.8</td>
<td>2.0</td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>0.9</td>
<td>5.0</td>
<td>1.1</td>
<td>3.2</td>
<td>0.7</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>1.0</td>
<td>5.8</td>
<td>1.3</td>
<td>3.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>
The minimal number of basic amino acids in a charge cluster of the $\beta$-tile tachyplesin analogs appears to be four to afford broad organism specificity and salt insensitivity. TP-18, cTP-14, and their retroisomers containing a cluster of six basic amino acids are broadly active against four Gram-negative and three Gram-positive bacteria as well as three fungi, exhibiting MIC values in a narrow range, and generally, under 1 $\mu$m in low and high salt conditions. Truncating the 18- to 14-member ring and retaining a cluster of six basic amino acids in cTP-14a and cTP-14c does not appear to be critical in affecting their broad spectrum activity spectra, but generally lead to an increase in salt sensitivity. A possible explanation is that the ring truncation deletes hydrophobic amino acids and alters the hydrophobicity of these analogs that also contribute to salt sensitivity and organism specificity. For example, both cTP-14a and its retroisomer rcTP-14b are broadly active. Under low salt conditions, cTP-14a missing the four hydrophobic residues in the D ring of cTP-18 (Cys7, 12, Tyr8, and His13) but retaining six cationic amino acids shows comparable antimicrobial activity (within 2-fold difference) of cTP-18 in eight of ten test organisms with E. coli and E. faecalis being exceptions. Under high salt conditions and again comparing to cTP-18, cTP-14a shows a selective decrease of 3–9-fold in potency against three of the four test Gram-negative bacteria but only one of the three Gram-positive bacteria (Table III). The activity profile of rcTP-14b, the retro analog of cTP-14a, also shows increased salt sensitivity similar to cTP-14a. Interestingly, the activity profiles of cTP-14a and cTP-14b are fairly similar to RTD-1, which is generally more active in low than in high salt conditions.

The separation of basic amino acids in a cluster also contributes to organism specificity and salt sensitivity. An advantage of the $\beta$-tile design is that their proximity can be readily adjusted by shortening the ring size. The $\beta$-tile cCT-18 differs from cTP-18 and contains a cluster of four basic amino acids and an additional constraint, a cystine replacing two Arg in cTP-18. Consequently, it is less cationic but more hydrophobic than cTP-18, and also significantly less potent than cTP-18 and TP-18. Thus, the cCT-14 peptides designed to increase the proximity of the charge cluster and to restore the charge/hydrophobic balance as TP-18 are generally more active than cCT-18. The antimicrobial activity cCT-14a lacking the D ring of cCT-18 increases 3-fold in low salt and almost 5 fold in high salt conditions. The importance of increasing the proximity of charge clusters is evident in cCT-14c containing only three charged amino acids that are 2-to-3-fold more active than cCT-18 in both low and high conditions. Furthermore, the decrease of cationic charges also leads organism specificity. They are generally more active against Gram-positive bacteria and fungi. The cCT-14d containing two cationic charges increases its selectivity to Gram-positive bacteria with MICs 1–3.8 $\mu$m and is considerably less active against Gram-negative bacteria and fungi with MICs of 7–112 $\mu$m. It loses significant activity against P. aeruginosa and is nearly inactive against C. kefyr.

Another effect of increasing proximity of charge clustering is placing a dibasic amino acid as part of a cationic cluster at the N terminus of an open-chain peptide. Dibasic amino acids such as Arg or Lys are commonly found at the N termini of many antimicrobial peptides. TP-18 has a dibasic Lys at the amino terminus and the Gly at the C terminus. Thus, TP-18 contains both $\alpha$- and $\epsilon$-amino at amino position not found in the retroisomer rTP-18. We show that the presence of an N-terminal Arg or N-terminal Lys in a cationic cluster may also contribute to salt insensitivity. When TP-18 and rTP-18 are compared under high salt conditions, TP-18 is found to be 6-, 4-, and 2-fold more potent than rTP-18 in three of the four tested Gram-negative bacteria, E. coli, P. aeruginosa, and K. oxytoca, respectively. The end-group effect also has an adverse effect on M. luteus, one of three Gram-positive bacteria being tested. Under high-salt conditions and comparing with TP-18, the MIC values of rTP-18 decrease 10-fold from 0.1 to 1 $\mu$m. Because of the close-chain arrangement, cyclic peptides do not have the end-group problem seen in the open-chain peptides. Consequently, the end-group effect and the Gram-negative bacteria is not observed in the corresponding set of cyclic peptides cTP-18 and rTP-18. Our results are consistent with the antimicrobial effects of known retroisomers that contain open-chain peptides with helical or $\beta$-strand structures. Merrifield et al. (38, 39) have reported that retroisomers of the $\alpha$-helical ceporin-melitin hybrids are as active as the parent peptide against five test bacterial strains, only one bacterial strain is resistant to the retroisomer.

Although the retroisomers of TP-18, cTP-18, or cTP-14 and their parents have the same calculated hydrophobicity values, their experimental values as determined by RP-HPLC show small variations (Table I). In each pair, the retention time of a retroisomer is found to elute 0.5–2.5 min longer than its parent peptide. These results suggest that the direction of amide backbone may also play a subtle role in their interactions with microbial surfaces of interactions.

Based on the models on the mechanism of actions of antimicrobial peptides (2–4), their initial interactions with microbial membranes heavily populated by negatively charged phospholipids are believed to be electrostatic. Thus, increasing ionic strength under the high salt conditions will likely weaken the electrostatic charge interactions and the activity of the antimicrobial peptides. Our results suggest that the topology and quantity of a charge cluster may provide low affinity molecular recognition to microbial surfaces and confer organism specificity and salt insensitivity. The principle of clustering cationic and hydrophobic regions of antimicrobial peptides has inspired the design of novel antimicrobials with $\beta$- and $\gamma$-peptide backbones (40) as well as dendrimeric peptides with $\beta$- and $\epsilon$-peptide backbones (41). Studies in the requirements of cationic clusters in the amphipathic design may further the understanding and lead to the development of antimicrobial peptides with high specificity and potency that are useful as therapeutic agents.

REFERENCES

17. Tang, Y.-Q., Yuan, J., Osayap, G., Osayap, K., Tran, D., Miller, C. J., Ouellette,
Cyclic Tachyplesins