Decoding Toxicity

DEDUCING THE SEQUENCE REQUIREMENTS OF IbsC, A TYPE I TOXIN IN ESCHERICHIA COLI

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Small peptides emerging from microbial genomes have been implicated in diverse catalytic and regulatory roles in cells. These functional peptides of only 16–60 amino acids are abundant in the genome of Escherichia coli and across other Gram-negative bacteria. The toxicity of this peptide was determined to be dependent on a stretch of highly hydrophobic residues near its center. Our results defined sequence-function relationship of IbsC and offered additional insights into properties common to membrane-targeting type I toxins in E. coli and related species.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Figs. S1–S6.

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8 This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC).

9 The abbreviations used are: TA, toxin-antitoxin; %N.G., normalized growth (%); Atc, anhydrotetracycline; IGR, intergenic region; PLtetO-1, tetracycline-inducible promoter; RBS, ribosome binding site; sRNA, small RNA; TFE, 2,2,2-trifluoroethanol.

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ing of the selected cells releases nutrients and other resources to sustain the surviving population. While the stress response model and the “altruistic killing” models seem contradictory, it is likely that the physiological role of chromosomally encoded TA pairs may not be defined by a single unifying model, but individual TA systems may serve different purposes in the cell. It is also possible that the observed toxicity of these peptides may be an artifact of their overexpression. They may have enzymatic or regulatory functions that are entirely independent of cell killing when expressed at endogenous levels (3). A recent global search for type I TA systems across bacterial species indicated that some TA families are well-conserved (18), implying that such TA systems may be important for bacterial fitness.

Among the six main families of type I TA pairs identified in E. coli K-12 is the Ibs/Sib family. Five Ibs/Sib homologs have been found in the E. coli K-12 genome, and they have been coined Ibs/SibA-E respectively (19). The ʻibs and ʻsib genes are arranged on opposite strands, with the antitoxin encoding sequences completely overlapping the toxin open reading frames (ORFs). These five TA repeats are distributed at three loci in the genome: ʻibs/sibA B and are found in tandem at the same intergenic region (IGR), while ʻibs/sibD E are arranged in the same manner at another IGR. Here, we are primarily interested in ʻibs/sibC, the sole pair detected in the IGR between ygfA and sibA. We initially came across ʻibs/sibC in a screen for antisense sequences that can knock down the expression of sRNAs and elicit growth defects in E. coli (20).

Through our screen, we observed growth suppression upon the induction of the reverse complement of the SibC sRNA. Our subsequent analyses demonstrated that the toxic phenotype was not attributed to sRNA interference. Rather, it was caused by the overproduction of a 19-amino acid peptide encoded in the antisense sequence. Our observations corroborate with the results reported in an earlier study published by the laboratory of Gisela Storz, who coined this peptide IbsC (for induction brings stasis) (19).

It has been postulated that IbsC and other members of the Ibs/Sib family localize to the inner membrane following overexpression, and their accumulation contributes to membrane depolarization (19). Like many type I TA pairs, the biological relevance and the exact mechanism of action of IbsC is currently unknown. It is also uncertain why this potentially deleterious element is maintained in the genome of various E. coli strains and in other proteobacterial species, including those from the Enterobacteriaceae, Pasteurellaceae, and Helicobacteraceae families (18, 21). Herein, we have established a comprehensive set of IbsC mutants to be used to address questions pertaining to the sequence conservation and functionality of this enigmatic peptide.

We carried out extensive sequence truncation studies on IbsC to deduce the minimal sequence for toxicity. A sequence randomization strategy was implemented to systematically introduce mutations in IbsC, allowing us to deduce the amino acid requirements for toxicity. IbsC was found to tolerate high frequencies of amino acid substitutions, considering that a large proportion of mutants retained their toxicity and membrane depolarization capabilities. Our mutagenesis data suggest that the sequence space for toxic peptides is quite large. In general, amino acids with hydrophobic side chains were strongly preferred at multiple positions, and mutations disrupting consecutive hydrophobic residues near the core of the peptide gave rise to inactive mutants. Using IbsC as a model, we defined additional sequence requirements for toxic peptides and refined current parameters used to guide searches for these elements across genomes in vivo and in silico.

EXPERIMENTAL PROCEDURES

Oligonucleotides—Sequences encoding IbsC and its derivatives were generated by PCR or by extending oligonucleotides with 15–20-nt complementary regions. The sequences of these oligonucleotides are presented in supplemental Table S1. Oligonucleotides were chemically synthesized by Integrated DNA Technologies (Coralville, IA). Oligonucleotides used for the sequence randomization studies were synthesized such that each nucleotide within the codon(s) of interest was prepared using a mixture of 25% adenine, cytosine, thymine, and guanine phosphorodiamidites. Oligonucleotides that were longer than 30 nt were purified by 10% (8 M urea) denaturing PAGE prior to usage.

Peptide Synthesis—Peptides, including wild-type IbsC and mutants IbsC (6–19), IbsC (L8G, L11L, S15A) and IbsC (L11R), were chemically synthesized by Genscript (Piscataway, NJ).

Strains and Plasmids—Information pertaining to the E. coli strain and plasmid used in this study are presented in supplemental Tables S2 and S3. The E. coli strain used in this study was DH5αZ1, which was previously described in Ref. 22. IbsC and its derivatives were cloned into pNYL-MCS11, a derivative of pZ21-MCS1 (courtesy of H. Bujard), downstream of the tetracycline-inducible promoter. Modifications were made to the parent vector, as previously described in Ref. 20. Briefly, we removed the ribosome binding site (RBS) present on pZ21-MCS1 and restored the multiple cloning sites that were lost during the RBS excision.

Molecular cloning procedures, including primer extension, restriction digestion, and ligation, were carried out following supplier-provided protocols. High Fidelity PCR Enzyme Mix, Klenow fragment (exo-), and T4 DNA ligase were purchased from Fermentas (Burlington, ON, Canada). EcoRI and BamHI restriction enzymes were purchased from New England BioLabs (Pickering, ON, Canada). Ligation products were transformed into DH5αZ1 cells by electroporation. With the exception of the mutants from the random sequence libraries, cloned constructs were confirmed by DNA sequencing at Mobix Lab (McMaster University).

Growth Media—Bacteria were cultured in Luria-Burtani (LB) medium at 37 °C in a shaking incubator. Growth media were routinely supplemented with 50 μg ml⁻¹ of kanamycin and spectinomycin (both purchased from Sigma-Aldrich). To induce the expression of IbsC and its derivatives, 200 ng ml⁻¹ of anhydrotetracycline (Atc; Sigma-Aldrich) was added to the medium.

Growth Curves and Lethality Screens—Growth curves were used to assess the toxicity of IbsC truncation and deletion mutants. Cells carrying pNYL-MCS11 (the negative plasmid...
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were grown at 37 °C with shaking until the A600 of the samples were ~0.3. Cells were subsequently induced with Atc. At 3 h post-induction, cells were harvested and treated with 10 μg ml−1 DiBAC4(3) (Sigma-Aldrich). Cells were incubated with the dye for 20 min before being pelleted and washed with phosphate-buffered saline (PBS). The final pellets were resuspended in different volumes of PBS to achieve uniform cell densities across samples. Fluorescence of each sample was measured using a Tecan Safire microplate reader (excitation at 490 ± 5 nm; emission at 516 ± 5 nm). Relative fluorescence of the mutants was calculated by dividing their fluorescence by the one corresponding to the plasmid control. The assay was repeated at least three times.

Circular Dichroism (CD) Spectroscopy—CD spectra were collected with an AVIV 410 CD instrument (AVIV, Lakewood, NJ). Scans were performed from 260 to 190 nm at a step resolution of 1 nm. A 1-mm pathlength quartz cuvette was used for the measurements. Sample temperature was maintained at 25 °C using a thermostatically controlled cell holder. Peptides were solubilized in 2,2,2-trifluoroethanol (TFE; Sigma-Aldrich) at a concentration of 50 μM. Background signal contributed by the solvent was subtracted for each sample. The CD spectra were converted to mean residual ellipticity. The secondary structure content of each sample was estimated from each spectrum using the CONTIN method available through the CDPro software (23).

RESULTS

Minimization of the IbsC Toxin—With 19 amino acids, IbsC is one of the smallest bacterial toxins identified to date (1, 19). We were interested in exploring whether this constitutes the lower size limit of type 1 toxins or whether shorter peptides can exhibit growth inhibitory potential in E. coli. We generated a series of terminal truncation mutants of IbsC by sequentially removing codons from the 5’- and 3’-ends of its ORF. We eliminated codons 2 through 7 from the 5’ truncation variants (Fig. 1A), while the 3’ truncation variants lacked codons 19 through 13 (Fig. 1B). The start codon was retained in the 5’ truncation mutants to enable translation initiation. Each sequence contained the RBS native to IbsC. The 13 IbsC truncation sequences were introduced into pNYL-MCS11, such that their expression was driven by a tetracycline-inducible promoter (P_LtetO-1) (20, 22). E. coli strain DH5αZ1, which constitutively expresses a tetracycline repressor to allow for tight regulation of genes governed by P_LtetO-1 (22), were transformed with these constructs. The production of these IbsC derivatives was induced with Atc.

Growth of cells carrying plasmids expressing the IbsC truncation mutants was monitored over 8 h following the induction of each IbsC truncation mutant (supplemental Figs. S1 and S2). Cell density was determined by measuring the A600. To facilitate comparison of the toxicity of each mutant, the A600 of each sample measured at t = 8 h was normalized with the A600 of cells carrying pNYL-MCS11, which served as our negative control. As observed in Fig. 1C, IbsC 5’ truncation mutants lacking amino acids 2–5 remained growth suppressive, indicating that these residues close to the N terminus of the peptide are not essential for toxicity. Removal of amino acids 6–13 and 14–18 led to a loss of toxicity.

Dye Uptake Assay—Cells carrying the plasmid control and the plasmid with ibsC or selected ibsC mutants were grown overnight and diluted 1:200 in fresh media. These cultures were grown at 37 °C with shaking until the A600 of the sample was ~0.3. Cells were subsequently induced with Atc. At 3 h post-induction, cells were harvested and treated with 10 μg ml−1 DiBAC4(3) (Sigma-Aldrich). Cells were incubated with the dye for 20 min before being pelleted and washed with phosphate-buffered saline (PBS). The final pellets were resuspended in different volumes of PBS to achieve uniform cell densities across samples. Fluorescence of each sample was measured using a Tecan Safire microplate reader (excitation at 490 ± 5 nm; emission at 516 ± 5 nm). Relative fluorescence of the mutants was calculated by dividing their fluorescence by the one corresponding to the plasmid control. The assay was repeated at least three times.

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Variability of Amino Acid Val-5—The significance of residue Val-5 in full-length IbsC was examined using a sequence randomization strategy. The sequence encoding the open reading frame of *ibsC* was designed with mutations at codon 5, such that each nucleotide in the codon was randomized using a mixture of 25% adenine, cytosine, thymine, and guanine phosphoramidites (Fig. 3A). This pool (or library) of *ibsC* mutants was subsequently cloned into pNYL-MCS11, downstream of the tetracycline-inducible promoter. *E. coli* DH5αZ1 cells carrying these recombinant vectors were grown in liquid media supplemented with Atc to screen for growth suppression associated with the overexpression of these IbsC derivatives lacking any of the last 10 amino acids led to a loss of toxicity (mutant Δ5). This observation was confirmed upon monitoring the effect of overexpressing this deletion mutant in cells grown in liquid medium over 8 h (supplemental Fig. S3). It is possible that the removal of residue Val-5 perturbed the hydrophobicity in its adjacent region or the active structure of the peptide.

Contribution of Individual Amino Acids to Toxicity—Data from our sequence truncation studies suggests that amino acids from positions 6–19 of IbsC are required for its toxicity. However, we were uncertain if all of the amino acids in this region are required for toxicity. To probe into amino acids that are potentially important for the function of IbsC, we designed a set of 15 IbsC deletion mutants, each lacking an individual amino acid (Fig. 2A). For amino acids that occur in tandem, such as the two isoleucine residues at positions 6 and 7, only the codon for the first amino acid was removed. Expression of the IbsC deletion mutants was induced in *E. coli* grown on LB-agar supplemented with Atc (Fig. 2B). Consistent with results from our sequence truncation analysis, IbsC derivatives lacking any of the last 10 amino acids failed to suppress growth following overexpression (mutants Δ10–Δ19), but amino acids Met-2, Arg-3, and Leu-4 can be deleted without interfering with the toxicity of IbsC mutants Δ2–Δ4. Interestingly, mutants lacking residues Ile-6 (mutant Δ6) remained growth inhibitory when overexpressed although 5′ truncation mutants lacking Ile-6 were non-toxic. While amino acids beyond the fifth residue led to inactive constructs, as observed with mutants IbsC (7–19) and IbsC (8–19), which lacked amino acids 2–6 and amino acids 2–7, respectively. On the other hand, elimination of amino acids near the C terminus was not tolerated, and cells expressing the 3′ truncation mutants grew to similar extents as the plasmid control (Fig. 1D). The data presented here suggests that the stretch of hydrophobic residues at the middle of the peptide and the few polar residues near the C terminus may have functional significance for IbsC. We also demonstrated that this toxin may be minimized to 15 amino acids without resulting in significant loss of toxicity.

FIGURE 1. Toxicity of IbsC 5′ and 3′ truncation mutants. A, amino acid sequence of wild-type IbsC and schematics of the 5′ truncation mutants. Codons 2–7 were sequentially removed from the ORF of *ibsC* in these constructs. B, schematics of the 3′ truncation mutants in which codons 19 through 13 were removed. C, toxicity of 5′ truncation mutants were assessed by comparing the relative A₆₀₀ of each mutant to that associated with the positive (IbsC) and negative (pNYL) controls at 8 h post-induction. Mutant IbsC (6–19), which lacked amino acids 2–5, was found to be the minimal toxic derivative of IbsC. D, toxicity of 3′ truncation mutants. Relative A₆₀₀ of each mutant was compared with that of the controls at 8 h post-induction. Removal of any of the amino acids near the C terminus of IbsC was found to be deleterious to its toxicity. n = 3.

FIGURE 2. Effect of single amino acid deletions on the toxicity of IbsC. A, design of IbsC deletion mutants. Starting from codon 2, codons were individually removed from *ibsC*. For amino acids that occur in tandem, such as Ile-6 and Ile-7, only the codon of the first amino acid was removed. B, phenotypic changes associated with the overexpression of each deletion mutant in *E. coli* DH5αZ1. Bacteria carrying pNYL-MCS11 with the deletion mutants were plated on LB-agar with Atc and incubated at 37 °C overnight. With the exception of amino acid Val-5, elimination of any amino acids from positions 2–9 did not seem to affect the toxicity of IbsC (left panel). Removal of any of the last 10 amino acids led to a loss of toxicity (right panel).
derivatives. Based on our design, we would theoretically need to sample 64 (4^3) sequences in order to cover all the possible mutations in this codon. If the randomization was unbiased, examining 64 mutants would encompass all 20 amino acids and the three stop codons. To account for unligated vectors and the isolation of identical mutants, we selected 100 clones to be screened using a growth assay.

In this assay, the A_600 of each sample was measured at 6 h post-induction. These values were normalized against the growth of bacteria expressing IbsC and the plasmid control (referred to as “normalized growth” or %N.G.). The data points and error bars represent average %N.G. and standard deviation calculated from triplicate. C, average toxicities of IbsC mutants with each of the 20 amino acid substitutions at position Val-5. The amino acids substituted at this position are noted in row 2 of the table. The number in each box in row 3 represents the average %N.G. associated with the overexpression of each mutant calculated from 3 experiments. Amino acid substitutions giving rise to active sequences (%N.G. <10%) are depicted by red boxes, those that led to peptides with intermediate toxicities (%N.G. between 10–20%) are indicated by orange boxes, and those that led to a loss of toxicity (%N.G. >40%) are shown in blue.

FIGURE 3. Randomization of the codon encoding amino acid Val-5 in IbsC. A, design of the IbsC-V5 library. Each nucleotide in codon 5 was synthesized with 25% adenine, guanine, cytosine, and thymine phosphoramidites (denoted by $N$). B, growth assay of IbsC-V5 mutants with the 20 amino acid substitutions. One mutant with each amino acid substitution was selected from the library and was subjected to a follow-up growth assay in which E. coli expressing each mutant was grown for 6 h. A_600 of each sample was measured thereafter, and these values were normalized against the growth of bacteria expressing IbsC and the plasmid control (referred to as “normalized growth” or %N.G.). The data points and error bars represent average %N.G. and standard deviation calculated from triplicate. C, average toxicities of IbsC mutants with each of the 20 amino acid substitutions at position Val-5. The amino acids substituted at this position are noted in row 2 of the table. The number in each box in row 3 represents the average %N.G. associated with the overexpression of each mutant calculated from 3 experiments. Amino acid substitutions giving rise to active sequences (%N.G. <10%) are depicted by red boxes, those that led to peptides with intermediate toxicities (%N.G. between 10–20%) are indicated by orange boxes, and those that led to a loss of toxicity (%N.G. >40%) are shown in blue.

Variability of C-terminal Amino Acids—In addition to amino acid Val-5, our single amino acid deletion experiments revealed that the loss of any of the last 10 amino acids in IbsC was deleterious to its toxicity. As such, we carried out a complete substitution analysis of each amino acid in this region using the aforementioned sequence randomization strategy to deduce the amino acid requirements in this region of IbsC (Fig. 4A). Following their synthesis, the 10 pools of mutant ibsC sequences were separately cloned into pNYL-MCS11. Each group of recombinant plasmids was then transformed into DH5αZ1 cells. Approximately 100 constructs were selected from each pool to establish our 10 libraries of ibsC de-
derivatives, and the toxicity of sequences from each library was independently assessed using growth assays. Active sequences were defined as those that can suppress growth by \( \frac{A_{600}}{\text{cells}} \) when overexpressed (%N.G. \( \leq 60\% \)).

From our initial screen, we observed that amino acid substitutions were tolerated at most positions in this segment of IbsC. An average of 40% of the sequences across the 10 libraries were found to be active (Fig. 4B). Amino acids near the C-terminus of the peptide, namely positions Phe-16, Ser-17, and Tyr-19, were found to be more mutatable than the rest of the sequence. Libraries corresponding to these amino acids contained nearly 70% of active sequences. On the other hand, the library corresponding to position Ser-15 displayed a much lower frequency of active sequences (12%). We sequenced all of the constructs that were examined. Upon analyzing the amino acid substitutions in each library, we noticed that our randomization approach generated near comprehensive sequence coverage. Amino acids that were absent from each library were often coded by non-degenerate codons, such as methionine and tryptophan (data not shown). We rationally designed and synthesized ibsC mutants that were not isolated from the initial screen in order to complete the set of substitutions. To verify the activity of each mutant, we selected 20 mutants from each library, each with a unique amino acid substitution, and repeated growth assays using these constructs (supplemental Fig. S4).

As presented in Fig. 4C, amino acids located near the center of IbsC, principally the ones at positions 10–14, exhibited a strong preference for hydrophobic residues (with Leu, Phe, Met \( \geq \) Cys \( \geq \) Val \( \geq \) Ile). Hydrophilic and charged residues are generally not favored at these positions. Substitutions by charged amino acids near the core of IbsC often led to a comed.
that hydrophobic residues (Cys, Val, Leu, and Ile) are favored. From these sequences, we note 3 sequences that displayed intermediate activity (with %N.G. associated with these mutants was comparable to IbsC were isolated (supplemental Fig. S5). From this library, only 5 sequences with toxicities comparable to the single mutation constructs from this library were selected and sequenced. We eliminated sequences that appeared to have base insertions, deletions, or contaminations, and the remaining sequences were subjected to additional growth assays to confirm our observations. From this library, only 5 sequences with toxicities comparable to IbsC were isolated (supplemental Fig. S5A). The %N.G. associated with these mutants was <10%. We also isolated 3 sequences that displayed intermediate activity (with %N.G. between 10 and 40%). From these sequences, we note that hydrophobic residues (Cys, Val, Leu, and Ile) are favored at both position 7 and 14. When the amino acids at these two loci were replaced by more hydrophilic residues, the toxicity of the resulting peptide diminished, although these mutants were still able to suppress growth by 60% or more. We also obtained the sequences of 10 inactive mutants. Most of these constructs were found to contain charged residues (Lys, Arg, Asp, or Glu) at one or both of these positions.

In addition to generating a library of double mutants, we screened triple mutants by randomizing the codons encoding amino acids Leu-8, Leu-11, and Ser-15 (Fig. 5B). These amino acids are hypothesized to be on the side of the putative α-helix opposite of Ile-7 and Ile-14. We expected to detect very few toxic triple mutants from our screen. Thus, we subjected nearly 350 constructs to growth assays. From this screen, we identified one active sequence with the expected length (supplemental Fig. S5B). However, further sequence analysis indicated that this mutant only contained two amino acid substitutions (L8G and S15A). The remaining mutants that we sequenced were inactive. Many of these mutants were found to contain proline at positions 8 and 15. Consistent with the inactive mutants we examined in the double mutant library, we observed that positively and negatively charged residues were generally disfavored in the triple mutants.

From our single amino acid substitution study, we observed that positions 11–14 of IbsC demonstrated a strong preference for hydrophobic residues (see Fig. 4C). We speculated that the presence of these consecutive hydrophobic amino acids is required for the toxicity of IbsC. They may be involved in mediating the interaction between IbsC and the inner membrane. Thus, we randomized the 12 nucleotides corresponding to codons 11–14 to examine the amino acid requirements in this region (Fig. 5C). Because of the scarcity of active sequences isolated from our double and triple mutant libraries, we examined the effect of the overexpression of around 450 sequences on the growth of E. coli. We did not isolate any active clones through this screen. Twenty constructs from this library were selected and sequenced. We eliminated sequences that appeared to have base insertions, deletions, or contaminations, and the remaining sequences were subjected to additional growth assays. We noticed that hydrophilic and charged residues were prevalent in these inactive mutants (supplemental Fig. S5C). One mutant, IbsC...
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(L11V, L12L, L13I, I14V), was found to be inactive even though the amino acids at positions 11–14 of this mutant remained highly hydrophobic, akin to the residues in the original sequence. This suggests that drastic changes to the sequence of IbsC are deleterious to its toxicity.

Structural Analysis of IbsC and Selected Derivatives—To further examine whether the activity of IbsC mutants is dependent on their structures, CD spectroscopy was carried out. Wild-type peptide and mutants IbsC (6–19) and IbsC (L8G, L11L, S15A) were chemically synthesized and subjected to CD analysis. The non-toxic mutant, IbsC (L11R), was also analyzed for comparison. With hydrophobicities of >75%, the peptides were insoluble in aqueous solvents. As such, 2,2,2-trifluoroethanol (TFE) was used as the solvent. The CD spectra collected for each peptide were analyzed using a secondary structure prediction program. As hypothesized, IbsC predominantly existed as an α-helix (supplemental Fig. S6, A–D). Interestingly, truncation mutant IbsC (6–19) and triple mutant IbsC (L8G, L11L, S15A), which displayed comparable toxicities as IbsC, were found to be comprised of β-aggregates along with α-helices. On the other hand, IbsC (L11R), the inactive point mutant, also seemed to favor the α-helical conformation under these conditions. Our structural analysis suggests that IbsC does preferentially adopt an α-helical conformation. However, the toxicities associated with these peptides may not be solely dependent on their secondary structures.

Overexpression of IbsC and Its Toxic Derivatives Causes Membrane Depolarization—IbsC has been proposed to interact with the inner membrane of E. coli. Its overexpression has been proposed to induce pore formation and elicit membrane depolarization (19). It is uncertain whether toxic IbsC derivatives disrupt the integrity of the inner membrane like their wild-type counterpart and if non-toxic variants have lost their ability to cause membrane defects. To examine the mechanism of toxicity of IbsC mutants, we subjected E. coli expressing a subset of these mutants to a dye uptake assay. We monitored the ability of cells to take up a fluorescent dye, DiBAC4(3) [Bis-(1,3-dibarbituric acid)-trimethine oxonol], following the overexpression of 14 active and inactive IbsC variants. Following membrane depolarization, DiBAC4(3) is able to enter cells, localize to highly hydrophobic environments, and interact with cytoplasmic proteins (24). This subsequently results in an increase in fluorescence, which is expected to be proportional to the change in membrane potential (24). In this assay, we induced the expression of IbsC and its derivatives in DH5αZ1 cells for 3 h before they were incubated with DiBAC4(3). We chose to assess dye uptake at 3 h post-induction, because maximal increase in fluorescence was detected at this time point following the induction of wild-type IbsC (data not shown).

We subjected 8 IbsC point mutants to the dye uptake assay. This set of mutants contained constructs that exhibited full activity [IbsC (V5R, V10T, S15C, and S17E)], intermediate activity [IbsC (V5D and V10N)], and no activity [IbsC (S15V and S17I)] in our growth assays. The overexpression of IbsC led to a 4-fold increase in fluorescence relative to the plasmid control (pNYL). Comparable to the wild-type toxin, active mutants led to a 3–4-fold fluorescence increase (Fig. 6A). The two inactive mutants demonstrated a slight increase in fluorescence (~1.4-fold relative to the negative control). It is possible that the accumulation of these small peptides resulted in stress on the inner membrane, yet the defect may not be severe enough to cause growth suppression. Overexpression of the IbsC (V10N) mutant did not result in apparent damage of the inner membrane. In previous growth assays, we saw that there is often variability in the toxicity of intermediately active constructs (supplemental Fig. S3). Here, our results suggest that this variability in toxicity may be attributed to impaired membrane interaction or membrane penetration by these mutants.

We further selected 6 double and triple mutants with varying toxicities and examined their effect on the integrity of the inner membrane (Fig. 6B). Change in fluorescence observed following the induction of the three inactive mutants approximated the change associated with the plasmid control. The expression of mutant IbsC (I7P, I14M), which displayed moderate toxicity, led to a 2-fold increase in fluorescence. Overexpression of mutant IbsC (I7C, I14V) resulted in fluorescence increase of ~2.5-fold. Consistent with this observation, this

FIGURE 6. Overexpression of toxic IbsC derivatives is disruptive to the integrity of the inner membrane. A, effect of IbsC point mutants on membrane potential. Eight IbsC point mutants with different levels of observed toxicity were overexpressed in E. coli DH5αZ1. At 3 h after inducing the expression of each mutant, E. coli was incubated with DiBAC4(3), a potential-sensitive fluorescent dye. DiBAC4(3) penetrates cells with compromised inner membranes, resulting in an increase in green fluorescence (λem = 516 nm). Similar to the overexpression of IbsC, cells expressing toxic mutants with V5D, V5R, V10T, S15C, and S17E substitutions were ~3–4-fold more fluorescent relative to the plasmid control. B, change in membrane polarization following the overexpression of IbsC combination mutants. We selected 3 IbsC double mutants and 3 IbsC triple mutants with varying toxicities and tested their effect on the inner membrane using the DiBAC4(3) uptake assay. Inducing the expression of active mutants IbsC (I7C, I14V) and IbsC (L8G, L11L, and S15A) dissipated the proton motive force, resulting in dye uptake and fluorescence enhancements of ~2.5–4-fold. The tables below each graph compare the toxicities (denoted by %N.G.) with the relative fluorescence (R.F.) observed following the overexpression of each construct. n = 3.
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Growth analyses conducted with the lbsC point mutants suggest that the hydrophobic residues spanning the middle of the peptide may be required for the proposed transmembrane localization of lbsC. From the combination mutants, we found that valine, leucine, and isoleucine are highly favored at multiple positions. From our mutagenesis studies, we noticed that polar and ionizable amino acids are well-tolerated at positions flanking the hydrophobic residue-rich region in the middle of the peptide. If lbsC is indeed a transmembrane peptide, it is possible that these hydrophilic and charged residues can facilitate initial contact between the peptide and polar head groups of the bilayer. These residues may further play a role in anchoring the peptide and stabilizing its interaction with the inner membrane. While we observed that an inactive point mutant with an arginine substitution at the core of the peptide was capable of adopting an α-helical structure in solvent, the presence of this charged residue may impede its interaction with the lipid bilayer. In the same experiments, we noticed that mutants lbsC (6–19) and lbsC (L8G, L11L, S15A) were not as helical as the wild-type toxin or as the lbsC (L11R) mutant in TFE. However, they retain their hydrophobic cores and may stably insert into the phospholipid bilayer when placed in the context of the membrane. In addition to causing structural changes and disrupting membrane insertion, mutations giving rise to non-toxic mutants may also perturb the peptides’ ability to self-aggregate or interact with other cellular targets.

Type I toxins have been implicated in regulatory processes that are important for the individual and communal survival and adaptability of bacteria. These elements are suggested to be abundant in microbial genomes. Currently, a number of biochemical approaches and computer algorithms are implemented in searches for peptides and sRNAs that comprise putative type I TA pairs (18, 27). As more TA pairs are identified and characterized, parameters guiding their searches are refined. Using lbsC as a model, we developed a better understanding of the sequence requirements for small peptide toxins found in prokaryotes. From our sequence truncation analysis, we learned that lbsC can be minimized to 15 amino acids and remain active, suggesting that the lower size limit of toxins can be set at 15 residues. Based on the minimal active truncation mutant, lbsC (6–19), we postulate that this toxin requires a minimum of 10 amino acids with highly hydrophobic side chains (e.g. Met, Cys, Phe, Leu, Val, or Ile) to retain its toxicity. Growth assays carried out with our lbsC point mutants indicated that only substitutions by other hydrophobic residues are tolerated at these positions near the core of the peptide. This is consistent with previous studies on transmembrane proteins, which suggest that 9 to 11 residues are sufficient to promote helix translocation across the membrane (28–30). At positions flanking the hydrophobic core, amino acids with different hydrophilic side chains were found to be able to substitute for native hydrophilic residues. This again demonstrates that many of lbsC residues can be substituted with similar functional groups. This knowledge of the general architecture of lbsC may aid in the design and search for novel toxin/antitoxin systems.
Decoding Sequence Requirements of a Toxic Peptide

Searches for novel TA systems are further fueled by the therapeutic potential of these elements. For example, expression of the HokC toxin from E. coli was induced in melanoma (31), breast (32), and lung (33) cancer cell lines. This toxin showed promise in hindering the growth and proliferation of these malignant cells. Type I toxins may also be considered as possible antimicrobial targets. Recent studies have shown that treating E. coli with certain antibiotics, such as ciprofloxacin, can induce the expression of toxins that have been linked to the SOS response, thereby promoting the formation of persistor cells and enhancing tolerance to these drugs (34). In another study, it was found that the overexpression of membrane-targeting peptides, including IbsC, can increase the sensitivity of bacteria toward tobramycin and other aminoglycosides (35). Given the possible applications of toxin-antitoxin systems, it would be of interest to further probe into the regulation and function of the Ibs family of toxins in the cell and to examine whether IbsC and the derivatives we generated through these studies would be suited for therapeutic purposes.

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REFERENCES

1. Hemm, M. R., Paul, B. J., Schneider, T. D., Storz, G., and Rudd, K. E. (2008) Mol. Microbiol. 70, 1487–1501
2. Alix, E., and Blanc-Potard, A. B. (2009) Mol. Microbiol. 72, 5–11
3. Fozo, E. M., Hemm, M. R., and Storz, G. (2008) Microbiol. Mol. Biol. Rev. 72, 579–589
4. Gerdes, K., Rasmussen, P. B., and Molin, S. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 3116–3120
5. Hayes, F. (2003) Science 301, 1496–1499
6. Magnuson, R. D. (2007) J. Bacteriol. 189, 6089–6092
7. Van Melderen, L., and Saavedra De Bast, M. (2009) PLoS Genet. 5, e1000437
8. Wozniak, R. A., and Waldor, M. K. (2009) Mol. Microbiol. Mol. Biol. 70, 1076–1093
9. Mok, W. W., Navani, N. K., Barker, C., Sawchyn, B. L., Gu, J., Pathania, R., Zhu, R. D., Brown, E. D., and Li, Y. (2009) Chembiochem 10, 238–241
10. Hershberg, R., Altvia, S., and Margalit, H. (2003) Nucleic Acids Res. 31, 1813–1820
11. Lutz, R., and Bujard, H. (1997) Nucleic Acids Res. 25, 1203–1210
12. Seerama, N., and Woody, R. W. (2000) Anal. Biochem. 283, 252–260
13. Epps, D. E., Wolfe, M. L., and Groppi, V. (1994) Chem. Phys. Lipids 73, 137–150
14. Papanfort, K., Pfeiffer, V., Lucchini, S., Sonawane, A., Hinton, J. C., and Vogel, J. (2008) Mol. Microbiol. 70, 1076–1093
15. Han, K., Kim, K. S., Bak, G., Park, H., and Lee, Y. (2010) Nucleic Acids Res. 38, 5851–5866
16. Wassarman, K. M., Repolla, F., Rosenow, C., Storz, G., and Gottesman, S. (2001) Genes Dev. 15, 1637–1651
17. Calamia, J., and Manoil, C. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 4937–4941
18. Gurevka, R., and Langosch, D. (2001) J. Biol. Chem. 276, 45580–45587
19. Krishnakumar, S. S., and London, E. (2007) J. Mol. Biol. 374, 671–687
20. Boulaiz, H., Prados, J., Melguizo, C., Marchal, J. A., Carrillo, E., Peran, M., Rodriguez-Serrano, F., Martinez-Amat, A., Caba, O., Hita, F., Concha, A., and Aráñez, A. (2008) Br. J. Dermatol. 159, 370–378
21. Boulaiz, H., Prados, J., Melguizo, C., García, A. M., Marchal, J. A., Ramos, I. L., Carrillo, E., Vélez, C., and Aranega, A. (2003) Br. J. Cancer. 89, 192–198
22. Prados, J., Melguizo, C., Rama, A., Ortiz, R., Boulaiz, H., Rodriguez-Serrano, F., Caba, O., Rodriguez-Herva, J. I., Ramos, J. L., and Aranega, A. (2008) Int. J. Oncol. 33, 121–127
23. Dörr, T., Vulić, M., and Lewis, K. (2010) PLoS Biol. 8, e1000317
24. Lee, S., Hinz, A., Bauerle, E., Angermeyer, A., Juhászova, K., Kaneko, Y., Singh, P. K., and Manoil, C. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 14570–14575