

A vast collection of microbial genes that are toxic to bacteria

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Abstract

In the process of clone-based genome sequencing, initial assemblies frequently contain cloning gaps that can be resolved using cloning-independent methods, but the reason for their occurrence is largely unknown. By analyzing 9,328,693 sequencing clones from 393 microbial genomes we systematically mapped more than 15,000 genes residing in cloning gaps and experimentally showed that their expression products are toxic to the *Escherichia coli* host. A subset of these toxic sequences was further evaluated through a series of functional assays exploring the mechanisms of their toxicity. Among these genes our assays revealed novel toxins and restriction enzymes, and new classes of small non-coding toxic RNAs that reproducibly inhibit *E. coli* growth. Further analyses also revealed abundant, short toxic DNA fragments that were predicted to suppress *E. coli* growth by interacting with the replication initiator *dnaA*. Our results show that cloning gaps, once considered the result of technical problems, actually serve as a rich source for the discovery of biotechnologically valuable functions, and suggest new modes of antimicrobial interventions.

Introduction

Since 1995 whole genomes of microbial species are being sequenced at an ever increasing pace (Kyrpides 2009). Thousands of bacterial and archaeal genomes have already been sequenced to completion, with thousands of additional genomes being sequenced every year (Liolios et al. 2010). The availability of sequenced microbial genomes provides access to the huge wealth of biotechnologically beneficial functions encoded in microbial genes and indeed, numerous examples for the discovery and utilization of such functions were described. Genome analyses have lead to the discovery of new antimicrobial drug targets (Payne et al. 2007), as well as to the finding of new, clinically successful vaccines (Rappuoli 2007). The study of microbial genes of unknown functions was the basis for the discovery of the CRISPR system (Jansen et al. 2002; Makarova et al. 2002), which was subsequently shown to provide adaptive immunity against phage in bacteria (Barrangou et al. 2007), and is now being used to protect beneficial lactic acid bacteria in the dairy industry against common phages (Horvath and Barrangou 2010; Sorek et al. 2008). Cellulose-degrading enzymes with biofuel implications (Warnecke et al. 2007) and new restriction enzymes (Zheng et al. 2009) are additional examples for industrially valuable functions encoded in microbial genomes. Of obvious specific interest are new genes and genomic elements that have potential implications for discovery of antimicrobials.

Despite the substantial progress in understanding the arsenal of useful functions encoded within microbial genomes, many additional functions are probably still obscure. Indeed, some 30-50% of all genes in every sequenced microbial genome are of unknown function (Markowitz et al. 2010). Summarized across all currently sequenced genomes, the number of such enigmatic genes exceeds 1.2 million, highlighting the great potential for biotechnologically and clinically useful functions yet to be discovered.

Here we identified thousands of microbial genes that are toxic to *E. coli*. Our approach is based on a by-product of the Sanger whole genome shotgun (WGS) sequencing method, where multiple copies of the organism's genome are randomly

sheared into overlapping fragments of DNA, and plasmids containing the cloned fragments are transformed into an *E. coli* cell (JGI 2007). The ends of the cloned fragments are then sequenced, and overlapping sequences are used for genome assembly (Fig S1). However, in the course of many genome sequencing projects, a small fraction of the genome fails to be cloned into *E. coli*, resulting in sequence gaps. We have previously shown that genes toxic to the *E. coli* host can cause these gaps, and that many of these toxic genes are protein constituents of the bacterial ribosome (Sorek et al. 2007). The toxicity of such genes was hypothesized to stem from their incompatibility with the *E. coli* molecular machinery, or from intolerance of the host to increased dosage of the ribosomal protein gene in addition to the endogenous homolog (Sorek et al. 2007).

In contrast to this defined inhibitory action of common, ribosomal protein genes, numerous cloning gaps harbor "hypothetical" genes that have no functional annotation, or do not span protein coding genes at all, making the reason for their occurrence obscure. In this study we experimentally demonstrate that such gaps encode a vast array of toxic products, including novel toxins, restriction enzymes, toxic small RNAs and toxic DNA motifs. We compiled PanDaTox, the Pan Genomic Database of genes Toxic to Bacteria, which contains detailed information on thousands of genes and non-coding elements predicted to be toxic to bacteria based on our gap analysis (<http://www.weizmann.ac.il/pandatox>).

Results

Pan-genomic discovery of toxic genes of unknown functions. To explore the functional toxicity repertoire of cloning-resistant genes, we analyzed 393 microbial genomes (360 bacteria, 28 archaea and 5 eukaryotes) for which the raw sequencing data was accessible and that had sufficient clone coverage (Methods). We used the original sequencing data to map the clone positions on these genomes, and detected genes that were not fully covered by any single clone (Fig S2). Since the probability of a given gene to be fully covered by a single clone depends on its size and on the sequencing library (e.g., a 4kb gene will never be fully covered in a library of 3kb clones), we developed a statistical framework to assign a p-value for unclonability, based on multiple random coverage simulations for each gene (Supplementary Methods). Genes with $p < 0.01$ (corrected for multiple testing) were further analyzed as cloning-resistant.

Our analysis retrieved 15,927 unclonable genes, as well as additional 25,894 genes with coverage significantly reduced compared to the coverage expected by chance (Table S1). Unclonable genes with COG functional annotations largely followed the pattern we previously observed, belonging to a very narrow set of gene families, i.e., the same orthologous gene could not be cloned into *E. coli* from many different genomes (Sorek et al. 2007) (Fig S3). However, 10,509 uncharacterized genes, many of them species-specific, were also found to be unclonable or with significantly reduced coverage ($p < 0.01$) (Fig S4). These genes are of potential biotechnological interest, as they might represent novel functional modules inhibiting the growth of bacteria.

To examine whether gap-residing genes of unknown function are toxic to *E. coli*, we attempted to clone 56 such protein coding genes from 25 different genomes (chosen based on availability of genomic DNA material to amplify from) into an inducible expression system that strongly suppresses the expression of the cloned gene in the absence of the expression inducer (IPTG) (Table S2). We successfully cloned 55 of the 56 selected genes. Our inability to clone the remaining gene implies that it is highly toxic, so that even a minute leakage of its expression was lethal to the host cells.

We next tested whether the protein products of the 55 genes we cloned in the inducible expression system are toxic to *E. coli*. For this, we activated their expression within *E. coli* using increasing amounts of IPTG as described in (Sorek et al. 2007). Forty four of the genes (80%) inhibited the growth of *E. coli* following induction of their expression, indicating that the products of these genes are toxic to the host (Table S2). These results verify our cloning-analysis method in detecting thousands of microbial genes that are potentially toxic to *E. coli*.

New restriction enzymes. We set out to study the repertoire of toxicity mechanisms in the toxic genes set. It was previously shown that restriction endonucleases, when cloned into an *E. coli* sequencing host without the presence of their companion DNA methyltransferase, can kill the host due to cleavage of its DNA (Zheng et al. 2009). This concept was used to identify restriction enzymes in 5 bacterial genomes based on shotgun sequencing data (Zheng et al. 2009). Indeed, 64 genes annotated as belonging to restriction/modification systems were found in our data to be unclonable or had statistically significant decrease in their clone coverage (Table S3).

Because restriction enzymes evolve rapidly (Stern and Sorek 2011), they frequently lack homology to known sequences and are thus annotated as uncharacterized genes. We therefore hypothesized that some of the uncharacterized unclonable genes in our set are in fact new restriction endonucleases. To test this hypothesis, we used *in-vitro* transcription/translation to synthesize protein products for the 44 genes whose toxicity to cells was verified (above), and tested their ability to cleave DNA by incubating them with lambda-phage genomic DNA. One of these proteins was able to cleave DNA into a fragment pattern typical for restriction enzymes (Fig 1A). This gene is annotated as "hypothetical protein" in the genome of *Synechococcus elongatus* PCC 7942 (locus tag Synpcc7942_2459), and has very few homologs in other microbial genomes, none of which is functionally characterized. It is adjacent to a DNA-cytosine methyltransferase in that genome, characteristic of *bona fide* restriction enzymes. The methyltransferase is predicted to modify the recognition sequence CCWGG (Roberts et al. 2010), further suggesting that the new restriction enzyme we found cleaves at this sequence motif. These results demonstrate that our set of toxic genes is a potent source for discovery of previously uncharacterized restriction enzymes.

New toxin-antitoxin systems. Toxin-antitoxin (TA) systems are comprised of a stable toxic protein and an unstable antitoxin that neutralizes it. Although originally hypothesized to be responsible mainly for plasmid stabilization (Jensen and Gerdes 1995; Van Melderen and Saavedra De Bast 2009), these systems are now known to be involved also in anti-phage defense (Fineran et al. 2009; Hazan and Engelberg-Kulka 2004; Koga et al. 2011; Pecota and Wood 1996), antibiotics resistance via a dormant, 'persistence' behavior (Lewis 2010), and altruistic cell suicide in bacterial communities (Amitai et al. 2009; Hazan et al. 2004), and are therefore attracting growing biotechnological attention (Gerdes et al. 2005). Type II toxin-antitoxin systems, where both toxin and antitoxin are proteins, are the most widespread across bacteria, and are classified into 11 families based on protein sequence similarity (Shao et al. 2011).

We hypothesized that our set of toxic genes might contain novel families of toxin-antitoxin systems. To search for novel TA systems, we searched, among the 44 genes whose toxicity to cells was verified, for those genes that consistently appeared in a bi-gene operon and showed a high rate of horizontal gene transfer, which is typical of known TA systems (Pandey and Gerdes 2005). One such gene was identified and selected for further functional assays (Figure 1B). This gene, found in the genome of *Nitrobacter winogradskyi* Nb-255 (locus tag Nwi_0828), is preceded by a small protein (the putative antitoxin) that has a predicted DNA binding domain, which is a common characteristic of antitoxins. We found 9 homologs of this bi-gene operon in various microbial genomes, and in 3 of the 4 cases for which we had clone coverage data, the putative toxin showed unclonability or statistically significant reduction in clone coverage. We denote these putative toxin/antitoxin pair shosT and shosA, respectively (identified based on SHOTgun Sequencing).

To test whether shosT/shosA indeed form a new TA system, we further cloned shosT from *Novosphingobium aromaticivorans* DSM 12444 in an expression vector under the control of IPTG-responsive promoter, and the cognate shosA in a second expression vector under the control of arabinose-responsive promoter. *E. coli* cells holding both these vectors were unable to grow when the expression of shosT alone was induced, but thrived when both shosT and shosA were expressed simultaneously (Figure 1C). Furthermore, induction of shosA expression 2.5 hours after shosT

induction resulted in cell growth recovery, indicating that the toxin has a bacteriostatic effect (Figure 1D). Since neither *shosT* nor *shosA* are homologous to any component of previously identified TA systems, the system we discovered forms a completely new family of type II TA system. The mechanism by which *shosT* inhibits growth is yet to be determined.

Toxic small RNAs: Whereas the vast majority of cloning gaps in the 393 genomes analyzed could be explained by the presence of toxic protein-coding genes, some gaps did not span any gene at all. We recorded 873 instances of uncloned intergenic regions, where the two genes flanking the region were covered by at least one clone each (Table S4, Methods). Of these, 46 intergenic gaps probably harbor previously unannotated short toxic protein coding genes based on the presence of a conserved ORF (Table S4; File S1). Gene annotation software frequently fail to identify short protein coding genes in microbial genomes (Overbeek et al. 2007).

However, the lack of obvious conserved ORFs in the vast majority of intergenic gaps prompted us to hypothesize that some of these gaps might contain non-coding RNA (ncRNA) species that inhibit bacterial growth. To test this hypothesis, we first searched these uncloned intergenic regions for sequence signatures of a sigma 70/38 promoter and a downstream rho-independent transcriptional terminator as evidence for the presence of a transcribed ncRNA (Methods). We then clustered these putative ncRNAs into groups based on sequence similarity, and looked for orthology groups in which the same intergenic region was reproducibly unclonable in two or more genomes (Fig 2A; Table S5). As additional evidence for ncRNAs, we searched for conservation patterns indicative of RNA secondary structures (Fig 2B). Altogether, we were able to cluster 69 of the intergenic toxic regions into 17 orthology groups, each of these putatively representing a toxic small RNA (tsRNA) species (Table S4-S5).

To verify that unclonable intergenic gaps are indeed transcribed into short RNAs in their genome of origin, we selected 3 such putative tsRNAs for further validation. These putative tsRNAs were conserved in the lineage of Betaproteobacteria, and specifically occurred in intergenic gaps in the bacterium *Burkholderia cenocepacia*, an opportunistic pathogen that can lead to necrotizing pneumonia or death in cystic

fibrosis or immunocompromised patients (LiPuma 2003). Using Northern blot analysis with total RNA derived from four *Burkholderia* species, we showed that these tsRNAs are indeed expressed in all four species tested (Fig 2C). To further examine whether the expression of these tsRNAs is toxic to *E. coli*, we cloned them under the control of an IPTG-inducible promoter, and activated their expression within the *E. coli* cell. Indeed, bacterial growth was completely abolished following induction of tsRNA expression, confirming that these non-coding RNA species are capable of inhibiting *E. coli* growth (Fig 2D).

Growth of *E. coli* was not affected when *in-vitro* expressed tsRNAs were added to its growth medium, indicating that these tsRNAs cannot penetrate the bacterial cell from the outside (Methods). We were also unable to isolate tsRNAs from the growth media when *Burkholderia cenocepacia* was grown in the presence of *P. aeruginosa* or *E. coli*, indicating that tsRNAs are not secreted by *Burkholderia cenocepacia* in the experimental conditions tested (Methods).

To further explore the biological roles of these tsRNAs in Betaproteobacteria, we searched for conserved sequence signatures within each of the 3 tsRNAs under study. In all 3 cases, a highly conserved sequence motif was found in a region predicted to be structurally open (Fig S5). These conserved motifs, sized 11 bp, harbored a sequence that was complementary to the consensus ribosomal binding site (RBS) in *Burkholderia* (Fig S5). RBS targeting motif in a structurally open domain of a short RNA is a strong characteristic of regulatory small RNAs (sRNAs) in bacteria, which are known to regulate gene expression by short base pairings with the 5' untranslated region (5' UTR) in the mRNA of the regulated genes (Waters and Storz 2009). Such sRNAs, in complex with the bacterial protein Hfq, usually inhibit the translation of the targeted mRNA and/or lead it to degradation (Waters and Storz 2009). Therefore, the tsRNAs we detected presumably hold a conserved function in gene regulation in *Burkholderia*. The reason for their toxicity in *E. coli* is yet to be determined, although it could be hypothesized that they might target and mis-regulate essential genes within the *E. coli* cell. Together, these results show that investigation of cloning gaps in intergenic regions can lead to discovery of novel non-coding RNA genes.

Toxic DNA binding motifs: To investigate whether some features of the cloned DNA itself, regardless of expression, can be toxic to the *E. coli* sequencing host, we performed a motif enrichment search in those intergenic gaps in which no sRNAs or short ORFs were predicted. The most prominent motif found was the 9-mer TTATCCACA, which was enriched by 64 fold over what was expected by chance ($p < 4 \times 10^{-35}$, Fisher's Exact test). This motif is identical to the *E. coli* DnaA box, a short DNA element crucial for replication initiation (Crooke et al. 1993). Multiple DnaA box sequences are found at the origin of replication (oriC), and serve as the binding sites for the DNA replication initiator protein DnaA (Fuller et al. 1984; Samitt et al. 1989) (Figure 3). This binding initiates a chain of structural changes at the oriC, and this, along with recruitment of additional proteins, leads to an eventual formation of the replication fork (reviewed in (Katayama et al. 2010)).

Since the level of DnaA in the cell is tightly controlled to prevent premature replication initiation, addition of high affinity DnaA boxes on a multi-copy plasmid is expected to titrate available DnaA proteins from the chromosomal oriC, leading to replication inhibition (Christensen et al. 1999) (Fig 3). We hypothesize this to be the mechanism of toxicity leading to the observed unclonability of the DnaA box sequences in our set. Indeed, unclonability was positively correlated with the number of cloned DnaA boxes (Fig 3B), and was more pronounced when high affinity DnaA boxes were present (Fig 3C). Previous attempts to directly clone DNA sequences that contain multiple DnaA boxes were successful only when the DnaA box was mutated (van den Berg et al. 1985).

One of the recurrently unclonable DnaA-box containing regions was an intergenic region occurring next to the *orn* gene in *E. coli* (Fig 3A). This region was conserved in 13 *Enterobacteria*, and unclonable in 9 (69%) of them. Intriguingly, this region was previously identified as *datA*, a regulatory locus for replication, which titrates exceptionally large amounts of the DnaA protein to prevent over-initiation and ensure a single replication per cell cycle (Kitagawa et al. 1998; Ogawa et al. 2002). Indeed, it was previously shown that increasing the copy number of *datA* in *E. coli* results in dramatic reduction in oriC-dependent replication (Morigen et al. 2001), supporting our hypothesis that increased amounts of DnaA boxes in the cell have a growth inhibiting effect due to replication deficiency.

If the interaction between DnaA and the DnaA-boxes is so crucial for regulation of DNA replication, then one would expect that increasing the amount of the DnaA protein in the cell will also have toxic effects, as this will cause multiple premature replication initiation events (Fig 3D, right panel). We indeed found that *dnaA* is one of the most highly unclonable genes across bacteria (Table S1, Sorek et al. 2007). Moreover, we and others have previously shown that overexpression of DnaA in *E. coli* results in growth inhibition (Grigorian et al. 2003; Sorek et al. 2007). Together, our results imply that de-regulation of the interaction between DnaA and the DnaA-box results in growth inhibition in *E. coli*.

The Pan-genomic Database of genes Toxic to bacteria (PanDaTox): The diversity of functions found in our vast collection of genes toxic to *E. coli* dictates that efforts of a single lab cannot expose the full functional repertoire encoded within these toxic genes, and calls for a community-based research effort. To provide the scientific community with data-rich access to these genes we developed the web-based resource PanDaTox – the Pan genomic Database of genes and genomic elements Toxic to bacteria (<http://www.weizmann.ac.il/pandatox>). Using PanDaTox, researchers can search for toxic genes and toxic non-coding elements in their genome of interest; find the homologs of these toxic genes and query whether these homologs are toxic as well; use keywords to search for genes answering specific criteria; and perform sequence-based searches (blast) for genes in the database that are similar to a query sequence of choice. The toxicity information for each gene is presented in both numerical and graphical manners, and details on each gene, including its DNA sequence, protein sequence, and various links to external web sources are shown.

Discussion

Hundreds of bacterial genomes were sequenced for over 15 years using the clone-based, Sanger sequencing method. In this study we show that sequencing gaps, which used to be considered a technical nuisance, are actually a biotechnological goldmine, containing extensive information on thousands of genes toxic to *E. coli* cells. We further demonstrated that these highly diverse toxic sequences hold various functions

relevant for biotechnological applications. Still, our studies merely scratched the surface of the potential, producing functional assignment for only a minority of the toxic genes we detected; further studies are needed in order to reveal other beneficial functions, not tested in the current study, using directed assays on these toxic genes. We expect that PanDaTox will be an important enabling tool for such future studies.

Although microbial genomes are no longer routinely sequenced by clone-based methods, the concept of cloning gaps as reporters of gene toxicity can be further expanded to any genome of choice. It is relatively straightforward to prepare a clone library of any sequenced genome within *E. coli* or any bacterium of choice, and then use next generation sequencing of the cloned fragments to detect genes absent from the clone library and hence toxic to the receiving host. We envision that such additional clonability analyses would be especially informative when applied on fungal genomes, based on the expectation that such genomes would contain an arsenal of antimicrobial genes.

Our experiments on 55 protein-coding genes suggest a true-positive predictive rate of about 80%, i.e., 80% of genes predicted to be toxic based on clonability information were found to inhibit the growth of bacteria experimentally. While the reasons for the 20% false positives observed in our experiments are currently unclear, several explanations are possible. First, the predicted unclonability of these genes might stem from a nearby intergenic element (tsRNAs or toxic binding site) that affects the clonability of the neighboring gene ("hitchhiker" effect, see Supplementary Methods). Second, the toxicity of the gene might be caused by a very strong promoter, since such promoters (e.g. the rRNA operon promoter) are known to limit vector propagation when cloned into plasmids (Boros et al. 1983). As our experiments involve replacing the native promoter by an inducible promoter, the toxic promoter effect in these cases may be abolished.

Since *E. coli* was used as the host in all sequencing projects analyzed, the genes we identified are primarily those which are toxic to *E. coli*. Our dataset is therefore biased against genes that are not functional in this organism, or those genes whose targets are not present in this organism.

Our results pinpoint the interaction between DnaA and the DnaA-box as a favorable axis for antimicrobial intervention. Compounds that inhibit this interaction, either by binding to the protein or by sequestering the DNA sequence motif, are expected to form potent antibiotics. Since both the protein and the sequence motif are highly conserved (Fujita et al. 1992) antibiotics that target the protein-DNA interface might have broad range of function. Moreover, it will be difficult for bacteria to escape the effect of such a putative compound by mutating the DnaA box and the interfacing binding domain in DnaA, because the DnaA protein must bind multiple DnaA box sequences to allow replication initiation.

There is an undisputable urgent need for new antimicrobial compounds in the clinic. The study of the vast and diverse set of toxic elements presented in this study might suggest multiple ways by which growth of bacteria can be inhibited, as implied in the case of the DnaA/DnaA-box interactions. Moreover, it is not unlikely that the set of toxic genes we identified also includes antimicrobial peptides and other toxins produced by specific bacteria to gain selective advantage over competing microbes. Our collection of toxic genes can therefore serve as a lead for future discoveries of antimicrobials having clinical implications.

Methods

Mapping of raw sequencing data to finished genomes was performed as previously described (Sorek et al. 2007), and toxic genes were identified using a statistical framework developed to assess gene clonability (Supplementary Methods). Initial experimental evaluation of gene toxicity was also performed as described (Sorek et al. 2007; Supplementary Methods).

Nuclease activity assay: toxic genes were transcribed and translated *in-vitro* using RTS 100 E. coli KY kit (Roche cat # 03 186 156 001) in a total reaction volume of 10ul, in 30C for 6 hours according to manufacturer's instructions. Then, 1ul of the reaction volume was added to 1ug of unmethylated Lambda phage DNA (Promega), in the presence of one of the buffers 1, 2, 3 or 4 (New England BioLabs) in a total reaction volume of 20ul. This 20ul reaction was incubated for 2 hrs in 37C, and was

then run on 0.7% LE agarose gel to test for band pattern indicative of nuclease activity. As negative control, the same procedure was repeated without addition of DNA template of the toxic gene into the RTS 100 *E.coli* KY reaction (+IVT in Fig 1A), or without incubating Lambda DNA with the RTS 100 *E.coli* KY reaction at all (-IVT in Fig 1A).

Toxin-antitoxin experiments: The *shosA* antitoxin gene (locus tag *saro_2199*) and the *shosT* toxin gene (locus tag *saro_2200*) were amplified from *Novosphingobium aromaticivorans* DSM 12444 chromosomal DNA. The toxin was then directionally ligated into the pRSFDuet-1 vector (Novagen) and the antitoxin ligated into the pBAD/HisA plasmid (Invitrogen). Since transformation of the toxin gene alone resulted in mutations in the toxin due to toxicity, the two plasmids (carrying the toxin and antitoxin) were co-transformed into *E. coli* BL21(DE3)pLysS (Stratagene) in the presence of 0.3% arabinose to induce the antitoxin. The clones were screened and verified by direct sequencing with primers on the pRSFduet-1 and pBAD/HisA vectors.

For the toxicity assay on plates, clones were cultured in LB medium with 100 µg/ml ampicillin, 50 µg/ml kanamycin, 34 µg/ml chloramphenicol and 0.3% arabinose overnight. The next day, a portion of each overnight culture was inoculated into fresh medium (10-fold dilution) and 10 µl were spotted on LB plates supplemented with 100 µg/ml ampicillin, 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. Toxin, antitoxin or both were induced by 100 µM IPTG and 0.3% arabinose, respectively, as indicated in Fig 1C.

For the kinetics experiment (Fig 1D), three different colonies of *E. coli* BL21(DE3)pLysS containing both the toxin and antitoxin were cultured overnight in 5ml liquid LB with 100 µg/ml ampicillin, 50 µg/ml kanamycin, 34 µg/ml chloramphenicol and 0.3% arabinose. In the next day, cells were diluted into 175 µl LB with ampicillin, kanamycin and chloramphenicol (same concentrations) in a final OD₆₀₀ of about 0.05 and placed in a 96 well plate (6 wells for each of the 3 colonies) that was inserted in a plate reader (Infinite M200). To avoid liquid evaporation during kinetic measurements, 35 µl mineral oil was supplemented to each well on top of the LB. Cells were grown and shaken in 37°C, and OD₆₀₀ was measured every 7 mins for

22 hours. For the “only antitoxin” curve in Fig 1D, 0.3% arabinose was added in T=0 in technical duplicate for each of the 3 colonies. For the “only toxin” curve in Fig 1D, 100 μ M IPTG was added after ~4 hours (between cycles 35 and 36). For the “toxin and antitoxin” curve in Fig 1D, 100 μ M IPTG was added after ~4 hours (between cycles 35 and 36) and 0.3% arabinose was then added ~2.5 hours later (between cycles 57 and 58).

Toxic small RNAs: Computational analysis of toxic small RNAs and dnaA boxes within uncloned intergenic regions was performed as described in the Supplementary Methods. For the experiments with *Burkholderia* tsRNAs, four *Burkholderia cenocepacia* strains (AU1054, HI2424, J2315, MC0-3) were grown to mid-log phase as follows. Cultures were grown in 10 ml LB overnight at 37°C. One mL of overnight culture was used to inoculate 100 ml of LB media (1: 100 dilution) and shaken at 200 rpm at 37°C until OD₆₀₀ neared 1.0. Cultures were spun in 50 ml sterile centrifuge tubes at 4°C at 4,000 rpm for 15 minutes in an Eppendorf 5810R centrifuge. Supernatants were disposed and cell pellets were resuspended in 6 mL phosphate buffered saline [PBS (7.95g Na₂HPO₄, 1.44 g KH₂PO₄, 90 g NaCl, per liter)] and mixed with 1 ml RNAlater solution (Ambion, Catalog #AM7021). Samples were stored at -20°C. RNA was then extracted using the hot phenol method (Masse et al., 2003).

The tsRNAs were amplified from the genome of *Burkholderia cenocepacia* HI2424 and labeled by Ready-To-Go DNA Labeling Beads (Amersham, cat # 27-9240-01) as instructed by the manufacturer. For Northern blot analyses, the NortherMax kit (Ambion) was then used for all gel running, blotting and hybridization procedures, using 10ug of RNA per lane.

To test the toxicity of tsRNAs to *E. coli*, amplified tsRNAs were cloned into PCR-SMART vector (Lucigen) and transformed into *E. coli* BL21- Gold(DE3)pLysS cells (Stratagene). Toxicity was then tested using increasing concentrations of IPTG as indicated above for toxic protein coding genes. As negative control, each tsRNA was similarly cloned in the reverse orientation, so that it was expressed (upon IPTG-mediated activation of expression) from its antisense strand. To test whether tsRNAs can act when administered in the growth media, clones holding tsRNAs were grown

to OD₆₀₀ in 10ml LB medium, and IPTG was then added to a final concentration of 1mM. After 4 hours of incubation with IPTG cells were spun down at 4000 rpm (4C for 15 mins) and total RNA was extracted using Trizol reagent. Expression of sRNAs was verified by gel electrophoresis of the extracted RNA. 10ul of RNA at a concentration of 1ug/ul was then added to *E. coli* BL21- Gold(DE3)pLysS cells growing in 15ml of LB medium, with a starting OD₆₀₀ of 0.1 . Growth of cells was monitored for 6 hours with and without the addition of RNA.

For the co-culturing experiments, *Escherichia coli* TW8211 or *P. aeruginosa* were grown overnight in LB medium, diluted 1:100 in 100 mL fresh LB and grown to OD₆₀₀ ~1.0. Bacterial cells were collected by centrifugation and resuspended in 2 mL LB. One ml of this was mixed with either *B. cenocepacia* AU1054 or HI2424 cultures grown to the same OD in parallel. The mixtures were then grown for 2 hours with shaking at 37°C. In parallel, individual cultures were also grown and similarly treated. Cells were harvested and RNA was extracted and purified from either pelleted cells or supernatants using the hot phenol method (Masse et al., 2003).

Data Access

Access to PanDaTox is available at <http://www.weizmann.ac.il/pandatox> . Sequences of tsRNAs and new annotations of protein coding genes were deposited in Genbank (accessions: JQ317270-JQ317274 and JQ323150-JQ323152).

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Figure legends

Figure 1: Functional interrogation of toxic genes. (A) Endonuclease activity of a gap-residing gene. ORF #2459 in *Synechococcus elongatus* PCC 7942 was translated *in-vitro*, and the translated product was incubated for 2 hours with 1 μ g of phage Lambda genomic DNA (48.5 kbp long). Band pattern on agarose gel indicates digestion of the Lambda DNA. Negative control lanes: Lambda DNA incubated with in-vitro translation reaction only (+IVT) or not incubated (-IVT) (B) *shosA/shosT* is a bi-gene operon that undergoes extensive horizontal transfer. The genomic context surrounding the gene pair in 7 genomes is shown to be different for every genome, indicative of horizontal gene transfer. Genes are depicted as block arrows. Blue and red filled arrows represent the predicted antitoxin (*shosA*) and toxin (*shosT*), respectively. Genes marked by asterisk are transposase and integrase genes, which are hallmarks of horizontal gene transfer. (C) *ShosA/ShosT* is a toxin-antitoxin system. Predicted toxin and antitoxin were co-transformed into *E. coli* BL21(DE3)pLysS on compatible plasmids: the toxin cloned under the control of IPTG responsive promoter, and antitoxin under the control of arabinose.-responsive promoter. Induction of toxin expression (100 μ M IPTG), and induction of antitoxin (0.3% arabinose) was performed. Cells do not grow when toxin is induced (IPTG+) unless antitoxin is co-induced (ara+). Cells without any inducer also do not grow, most probably due to toxin leakage from the IPTG-inducible promoter. (D) *ShosT* has a bacteriostatic effect. Bacterial OD₆₀₀ was measured over time when only antitoxin was induced (dark blue); only toxin induced (red); and when antitoxin expression was induced 2.5 hrs after toxin induction (grey). Each experiment was performed in six repetitions (biological triplicate and technical duplicate).

Figure 2: Toxic small RNAs reside in intergenic gaps. (A) Reproducibility of cloning deficiency of an intergenic region in *Burkholderia*. Coverage plots of a syntenic 6kb region in chromosome 1 of two *Burkholderia* species. Colored rectangles represent genes. This region harbors toxic small RNA #1 (tsRNA #1) (B) Multiple alignment of the cloning-deficient intergenic region. The orthologous region to those shown in panel A was aligned between 5 closely related organisms. Arrows depict deletions that are not divisible by 3, indicating that this region does not code for protein. Compensatory, stem-preserving mutations are colored red/blue, indicative of conserved RNA structure. (C) Northern blot analysis of 3 tsRNAs found in intergenic gaps in *Burkholderia*. Radio-labeled probes for each of the tsRNAs were hybridized to the total RNA of four *Burkholderia* species: *B. cenocepacia* AU1054; *B. cenocepacia* HI2424; *B. sp.* J2315; and *B. sp.* MC0-3. Predicted secondary structures of small RNAs were calculated using RNAfold (Hofacker et al. 1994). Coordinates are given relative to the *B. cenocepacia* HI2424 genome. Two bands of tsRNA #1 probably indicate post-transcriptional sRNA processing of the long form (94 bases) into a shorter form (~60 bases) (D) Toxicity of tsRNA #1 in *E. coli*. tsRNA #1 from *B. cenocepacia* HI2424 was cloned into *E. coli* under the control of IPTG-inducible promoter. Top left, colonies of *E. coli* grow when the expression of the tsRNA is suppressed; Top right, growth inhibition is observed following activation of tsRNA expression. Bottom, growth inhibition is not observed when the antisense of the small RNA is expressed in the same system.

Figure 3: Toxic DNA binding sites. (A) Reproducibility of an intergenic gap in Enterobacteria. Coverage plots of a syntenic 5kb in *E. coli* (refseq: NC_009800) and *S. enterica* (refseq: NC_011080) are presented. Colored rectangles represent genes, with homologous genes sharing the same color. DnaA boxes with up to one mismatch relative to the consensus sequence are marked by red arrows. Upper and lower arrows denote DnaA boxes on the forward and reverse strands, respectively. This region harbors the DnaA-titrating *datA* locus (Kitagawa et al. 1998). (B) Unclonability increases when multiple clustered DnaA boxes exist in a cloned DNA fragment. DnaA boxes distant up to 20 bp apart were clustered together. Horizontal axis denotes the minimal cluster size. (C) Unclonability increased with predicted affinity of DnaA box to DnaA. Consensus DnaA boxes (TTATCCACA) are more unclonable than boxes with one or two mismatches. Data is shown for clusters containing two or more DnaA boxes. (D) A model for *dnaA*/DnaA-box toxicity. In normal conditions (left) DnaA is produced from a single locus and binds to the *oriC* to initiate replication. When DnaA box clusters are cloned on a high copy plasmid (middle), they titrate cellular DnaA protein and inhibit replication. When additional copies of the *dnaA* gene are cloned (right), replication over-initiation occurs.