Targeting the Replication Initiator of the Second Vibrio Chromosome: Towards Generation of Vibrionaceae-Specific Antimicrobial Agents

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Abstract

The Vibrionaceae is comprised of numerous aquatic species and includes several human pathogens, such as Vibrio cholerae, the cause of cholera. All organisms in this family have two chromosomes, and replication of the smaller one depends on rctB, a gene that is restricted to the Vibrionaceae. Given the increasing prevalence of multi-drug resistance in pathogenic vibrios, there is a need for new targets and drugs to combat these pathogens. Here, we carried out a high throughput cell-based screen to find small molecule inhibitors of RctB. We identified a compound that blocked growth of an E. coli strain bearing an rctB-dependent plasmid but did not influence growth of E. coli lacking this plasmid. This compound, designated vibrepin, had potent cidal activity against V. cholerae and inhibited the growth of all vibrio species tested. Vibrepin blocked RctB oriCII unwinding, apparently by promoting formation of large non-functional RctB complexes. Although vibrepin also appears to have targets other than RctB, our findings suggest that RctB is an attractive target for generation of novel antibiotics that only block growth of vibrios. Vibrio-specific agents, unlike antibiotics currently used in clinical practice, will not engender resistance in the normal human flora or in non-vibrio environmental microorganisms.

Introduction

The Vibrionaceae are a diverse family of bacteria that includes more than 80 species [1,2]. Vibrios are gram-negative rods that usually inhabit aquatic habitats, often in association with eukaryotes. This large family includes important human pathogens such as V. cholerae, V. parahamolyticus and V. vulnificus, which can cause gastrointestinal disorders and other illnesses [2,3]. Vibrio species are also pathogenic for several economically important marine organisms; for example farmed shrimp are harmed by V. harveyi and V. nigripulchritudo [2,4,5].

One notable attribute of the Vibrionaceae is their bipartite genomes. The genomes of most other γ-proteobacteria (such as E. coli) consist of single circular chromosomes, whereas the genomes of vibrios are comprised of two circular chromosomes [6]. Studies of V. cholerae, the agent of cholera, have revealed that different proteins initiate replication of its two chromosomes. Initiator proteins bind to and melt origins of replication and also recruit components of the replisome to the origin [7]. DnaA, a conserved AAA+ ATPase, is thought to be the initiator of chromosome DNA replication in most eubacteria [8,9], and several observations support the idea that DnaA serves as the initiator of replication of the large V. cholerae chromosome (chrI) as well. The origin of replication of chrI, oriClI, is similar in sequence to oriC, the origin of replication of the E. coli chromosome [10], and both contain several binding sites for DnaA. Replication of oriClI-dependent minichromosomes is DnaA-dependent [10], and overexpression of DnaA leads to overinitiation of oriClI [11]. Finally, DnaA cannot unwind oriClI but can’t unwind oriClII, the origin of replication of the small V. cholerae chromosome (chrII) [12].

Several observations suggest that RctB, a 658 amino acid protein that lacks any known motifs or similarity to characterized initiators, is the initiator of chrII replication. First, RctB binds to several sites within oriClII [10]. Second, overproduction of RctB in V. cholerae promotes overinitiation of chrII and not chrI [11]. Third, RctB is necessary and sufficient to enable replication of oriClII-based minichromosomes in E. coli, and the copy number of such minichromosomes increases as the level of RctB is raised [12,13]. Finally, RctB cannot unwind oriClII and not oriClII [12].

rctB homologues are encoded by all vibrios that have been tested but are not found outside the Vibrionaceae [10]. As a conserved and essential gene product restricted to the Vibrionaceae, RctB might be an attractive target for new vibrio-specific antibiotics. Given the increasing prevalence of multi drug resistance in pathogenic vibrios [14–18], there is a growing need for new drugs to combat these organisms. Here, we carried out a high throughput cell-based screen to find small molecule inhibitors of RctB. We identified a compound that blocks growth of an E. coli strain bearing an rctB-dependent plasmid but does not influence growth of E. coli lacking this plasmid. This compound, designated...
Results

A candidate RctB inhibitor identified with a high throughput cell-based screen

We developed a high throughput cell-based screen to identify small molecule inhibitors of RctB. This assay relied on pYB289, a small plasmid that contains only oriCII, rctB and a gene (aph) that confers resistance to kanamycin (Figure 1A). This plasmid can replicate in E. coli, since expression of rctB in E. coli is sufficient to enable replication of oriCII-dependent plasmids in this heterologous host [12, 13]. E. coli harboring pYB289 exhibit kanamycin resistance, and we used kanamycin resistance as a marker of this plasmid’s replication in our screen. We screened a library of ~138,000 small molecules for compounds that inhibited growth of E. coli Mach1 harboring pYB289 in the presence of kanamycin but did not inhibit growth of Mach1 without the plasmid. Several candidate RctB inhibitors were identified, and one - 3-(3,4-dichlorophenyl)cyclopropane-1,1,2,2-tetracarbonitrile (Figure 1B), designated here as vibrepin (for vibrio replication inhibitor) - was selected for further study, since its predicted pharmacologic properties were superior to the others.

Genetic evidence that vibrepin targets RctB

Vibrepin had no effect on the growth (indicated by increased OD600 nm in cultures) of E. coli strain DH5α (Figure 1C). In addition, it did not influence growth in the presence of kanamycin of DH5α harboring pWSK129 or pYB190, plasmids with distinct non-RctB dependent origins of replication (pSC101 and pUC, respectively) that contain aph cassettes, and so does not appear to interfere with establishment of kanamycin resistance (Figure 1G and data not shown). However, 16 μg/ml vibrepin completely inhibited growth of E. coli DH5α harboring pYB289 (wt RctB), pYB340 (encoding RctB[P516Q]) or pWSK129 (a non-oriCII-based plasmid). Thick black lines represent growth in the presence of vibrepin (16 μg/ml) and gray lines represent growth in the presence of DMSO. Representative growth curves (average of triplicate wells from a plate reader) from 3 or more independent experiments are presented. The higher initial optical density of the cultures containing vibrepin is due to the incomplete solubility of this compound in LB media at 16 μg/ml. This is apparent in H) which shows the optical density generated by vibrepin (thick line), or DMSO (thin gray line) without the addition of cells.

Figure 1. Identification of a small molecule that inhibits RctB-dependent replication. A) Schematic of the RctB-dependent oriCII-bearing plasmid, pYB289, used in the high throughput screen for small molecule inhibitors of RctB. B) Structure of vibrepin, one of the compounds identified in the screen. C–G) growth curves of E. coli DH5α harboring no plasmid, pYB289 (encoding wt RctB), pYB340 (encoding RctB[P516Q]) and pWSK129 (a non-oriCII-based plasmid). Thick black lines represent growth in the presence of vibrepin (16 μg/ml) and gray lines represent growth in the presence of DMSO. Representative growth curves (average of triplicate wells from a plate reader) from 3 or more independent experiments are presented. The higher initial optical density of the cultures containing vibrepin is due to the incomplete solubility of this compound in LB media at 16 μg/ml. This is apparent in H) which shows the optical density generated by vibrepin (thick line), or DMSO (thin gray line) without the addition of cells.

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growth in the presence of kanamycin of DH5α containing the RctB-dependent plasmid pYB289 for 6 hr (Figure 1D). Some growth of this strain was detectable after 6 hr, probably because the drug was no longer present, since these cells did not appear to be resistant to vibrepin if tested in fresh media.

Although vibrepin-resistant DH5α/pYB289 did not arise in these assays, growth of DH5α containing one of several derivatives of pYB289 (pYB340 or pYB344) that encode variants of RctB with single amino acid substitutions (RctB L363I and P516Q, respectively, which were identified in an unrelated study, see materials and methods) was not impaired by vibrepin (Figure 1E and F). These strains grew as well in the presence of vibrepin as did DH5α lacking a plasmid. Since the only differences between strains DH5α/pYB289, DH5α/pYB340 and DH5α/pYB344 are single amino acid differences in RctB, these observations strongly support the idea that vibrepin targets RctB.

We also assessed the compound’s effect on growth of DH5α/pYB289 in the absence of kanamycin. We anticipated that vibrepin would not inhibit bacterial growth under these conditions, since RctB activity should not be required in the absence of kanamycin. Unexpectedly, we found that vibrepin impaired the growth of DH5α/pYB289 even when plasmid replication was not required. Addition of 16 μg/ml vibrepin to cultures of this strain lacking kanamycin prevented an increase in OD₆₀₀ nm for ~2 hr and caused a decrease in the number of viable cells (Figure 2B) but did not influence growth of DH5α lacking this plasmid (Figure 2A). Vibrepin also stimulated the loss of pYB289 from DH5α in the absence of kanamycin selection (Figure 2D). Vibrepin even had a mild inhibitory effect on the growth of DH5α harboring pYB376, a pSC101-based vector containing rctB (Figure 2C). Collectively, these results suggest that production of wild type RctB in the presence of vibrepin may have toxic effects, at least in E. coli, and that such effects may contribute to the growth inhibition observed in the assay used above. However, since vibrepin was a less potent inhibitor of DH5α/pYB289 growth in the absence of kanamycin than in its presence, it is likely that vibrepin inhibits growth of this strain by more than one mechanism.

Biochemical evidence that vibrepin targets RctB

To assess vibrepin’s influence on RctB’s activity as a replication inhibitor, we tested the compound’s effects on unwinding of oriCII by RctB, using a P1 nuclelease-based assay. In this assay, the single-strand specific P1 endonuclease cleaves a plasmid containing oriCII if it becomes unwound; linearized plasmid is then detected by agarose gel electrophoresis [12]. As seen in Figure 3A, unwinding of oriCII by RctB was markedly inhibited by vibrepin. DMSO, the solvent used to dissolve vibrepin, did not influence RctB unwinding activity (data not shown). These observations are consistent with the hypothesis that vibrepin interferes with RctB function as an initiator of replication by blocking its ability to unwind oriCII.

We also explored whether vibrepin influences the oligomeric state of RctB. In our ongoing studies of the mechanism of action of RctB, we found that purified RctB does not pellet after centrifugation at 20,000 × g for 30 min in the absence of DNA. However, in the presence of 10 μg/ml of vibrepin, ~50% of RctB was found in the pellet fraction after centrifugation; when higher amounts of vibrepin were added, most of the RctB added to the assay pelleted (Figure 3B). In contrast, RctB [P516Q], which appeared to be resistant to in vivo inhibition by vibrepin (Figure 1F), was less susceptible than wild type RctB to vibrepin-induced aggregation in the pelleting assay (Figure 3C). Furthermore, vibrepin did not promote the aggregation of either CpxR [19] (Figure 3D) or ParA2 (data not shown), DNA-binding proteins unrelated to RctB. Thus, vibrepin does not indiscriminately aggregate proteins. The vibrepin solvent DMSO also did not promote the pelleting of RctB. Together, these findings suggest

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**Figure 2. Influence of vibrepin on an E. coli strain bearing an RctB-dependent plasmid in the absence of kanamycin.** A–C) DH5α (A), DH5α/pYB289 (B), or DH5α/pYB376 (C) were incubated in LB media in test tubes with either vibrepin (16 μg/ml, thick black lines) or DMSO (gray lines). OD₆₀₀ nm and colony forming units (CFU) were determined at the indicated times. Representative growth curves from 3 or more independent experiments are presented. D) The relative amount of the oriCII-based plasmid pYB289 in DH5α after 4 hr of treatment with vibrepin (16 μg/ml) or DMSO. The amount of pYB289 relative to chromosomal DNA was determined before (t = 0) and 4 hrs after treatment (t = 4) using Southern hybridization. The relative amount of pYB289 at t = 0 was set as 1; the mean and standard deviations after 4 hr were calculated from 3 independent experiments.

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that vibrepin leads to the formation of high molecular weight complexes of RctB that are no longer soluble. Consistent with these observations, we found that vibrepin increased the apparent radius of RctB complexes approximately 4-fold in dynamic light scattering (DLS) assays (from 153 to 588, Figure 3E). The range of radii of the RctB complexes after addition of vibrepin was narrow, suggesting that vibrepin promotes the formation of RctB complexes of a particular stoichiometry rather than random aggregation of this protein. Although the apparent radius of RctB [P516Q] was greater than that of wild type RctB, vibrepin only had a minor effect (from 257 to 370, Figure 3F). Collectively these observations suggest that vibrepin may interfere with RctB oriCII unwinding by promoting the formation of non-functional RctB complexes.

Diverse vibrios are susceptible to vibrepin

RctB is required for replication of V. cholerae chrII and is hypothesized to govern chrII replication initiation in all other vibrio species as well, since it is highly conserved. We therefore assessed whether vibrepin could inhibit the growth of vibrio species. Vibrepin prevented growth of V. cholerae at doses as low as 1.0 μg/ml (Figure 4A). Used at this concentration, vibrepin induced stasis; however, at higher doses vibrepin had cidal activity. At concentrations of 4 μg/ml, vibrepin reduced the numbers of N16961 colony forming units (CFU) by more than 5 orders of magnitude within 30 minutes (Figure 4A).

Vibrepin also prevented growth of all additional vibrio species tested (Table 1). Drug concentrations ranging from 0.4 to 0.8 μg/ml were sufficient to inhibit the growth of the three major human vibrio pathogens V. cholerae, V. parahaemolyticus and V. vulnificus, as well as the shrimp pathogen V. nigripulchritudo (Table 1). In contrast, vibrepin concentrations <13 μg/ml did not inhibit growth of any of the E. coli strains we tested, including several pathogenic strains, and most E. coli strains were resistant to at least 16 μg/ml of vibrepin (Table 1). Together these observations are consistent with the idea that vibrepin targets RctB, a vibrio-specific essential protein. However, we also observed that vibrepin inhibited growth of Bacillus subtilis and Staphylococcus aureus (Table 1), two Gram-positive species that lack RctB homologues. Thus, it appears that some organisms contain vibrepin targets other than RctB. The vibrepin target(s) in these Gram-positive bacteria has yet to be defined.

Several approaches were taken to confirm the target of vibrepin in V. cholerae. First, we repeatedly screened for V. cholerae mutants that had spontaneously acquired resistance to vibrepin; however, resistant colonies were never obtained. We were not able to introduce point mutations in the chromosomal copy of rctB. However, we transformed V. cholerae with plasmids encoding alleles of RctB that were resistant to vibrepin in E. coli (pYB303 and pYB343, encoding RctB L365I and P516Q, respectively), and assessed whether they conferred resistance. Exogenous production of RctB from these plasmids (which did not alter V. cholerae’s growth rate) did not render cells resistant (data not shown), suggesting that the presence of wt RctB results in dominant sensitivity, as might be expected given vibrepin’s toxicity in DH5α/pYB289 even in the absence of plasmid selection. Alternatively, V. cholerae may contain vibrepin targets in addition to RctB, which confer sensitivity even in the presence of resistant rctB alleles. Such targets may be related to the non-RctB targets that must exist in Gram-positive organisms.

Vibrepin promotes RctB aggregation in vivo

We used an RctB-GFP fusion protein to explore whether vibrepin altered the subcellular distribution of RctB in V. cholerae. We constructed a strain where rctB-gfp fusion is expressed from the native rctB promoter by introducing a gene encoding GFP in-frame at the 3’ end of rctB. Since this rctB-gfp fusion is the only copy of rctB in the cell and this strain had wild type growth (data not shown), the
RctB-GFP fusion protein must be functional. In this strain, the distribution of RctB-GFP was generally diffuse, though small foci, usually near mid cell, were occasionally observed (Figure 4B). One hour after addition of 1 μg/ml of vibrepin, the diffuse pattern of RctB-GFP fluorescence was no longer observed; instead, large puncta of RctB-GFP were seen (Figure 4C). In control experiments, we found that vibrepin treatment did not alter the pattern of untagged GFP fluorescence (Figure 4D and E), consistent with our observation that vibrepin does not lead to indiscriminate aggregation of proteins in vitro. These data suggest that the RctB complexes induced by vibrepin in vitro may be a reflection of its mode of action in vivo, and that vibrepin’s toxicity for V. cholerae may result, at least in part, from induction of RctB aggregation.

Comparative analysis of vibrepin and a structural analog

Different vibrepin targets could recognize distinct moieties in this compound. In an initial structure activity study of vibrepin, we identified a compound [3-[3-dimethylamino-phenyl]-cyclopropane-1,1,2,2-tetracarbomitrile] [referred to as C2], that is similar in structure to vibrepin and that contains the same highly substituted cyclopropane moiety linked to a phenyl group (compare Figures 5A and 1B). However, C2 did not inhibit growth of DH5α/pYB289 (Figure 5C) or V. cholerae (Figure 5D). Since C2 lacks the chlorine substitutions on the phenyl group that are present in vibrepin, these observations may suggest that the chlorines in vibrepin are important for its targeting/inhibition of RctB. However, C2 is not void of antibiotic activity. This compound inhibited growth of B. subtilis (Figure 5E) and S. aureus (Figure 5F), albeit with lower potency than vibrepin. Together, these observations raise the possibility that the additional (non-RctB) targets of vibrepin may interact with moieties of this compound that are chemically distinguishable from the parts of the molecule that inhibit RctB.

Table 1. Minimal inhibitory concentrations (μg/ml)\(^1\) of vibrepin for various bacteria.

| Organism                  | Concentration (μg/ml) |
|---------------------------|-----------------------|
| V. cholerae N16961        | 1.5                   |
| V. vulnificus ATCC 27562  | 0.4                   |
| V. nigrilucitudo          | 0.8                   |
| E. coli DH5α              | >16                   |
| E. coli MG1655            | >16                   |
| EPEC 2348/69              | >16                   |
| EHEC EDL933               | 13                    |
| B. subtilis PY79          | 1.0                   |
| S. aureus MW2             | 2.0                   |

\(^1\)Concentrations of vibrepin that blocked increases in OD\(600\) nm for at least 6 hours. The average values derived from at least 3 experiments are shown. When 16 μg/ml did not inhibit growth, a value of >16 is shown.

Discussion

The development of new agents to combat emerging multidrug resistant pathogens is a critical challenge for infectious disease research [20,21]. In recent years, multidrug resistance in V. cholerae, V. vulnificus, and V. parahaemolyticus, important human pathogens [14–18], and in vibrio species that damage shellfish and other marine organisms raised in aquaculture facilities [22] has been reported. Since RctB is required for replication of the vibrio second chromosome and conserved among the Vibrionaceae, we reasoned that RctB could be a target for development of antibiotics that specifically target vibrios. Our cell-based screen for small molecules that inhibited growth of an E. coli strain containing an oriCII- and RctB-dependent plasmid yielded vibrepin. Evidence that this compound targets RctB includes the observations that vibrepin did not inhibit growth of E. coli lacking the RctB-dependent plasmid or E. coli strains bearing nearly identical oriCII-dependent plasmids that contained single amino acid substitutions in RctB. Furthermore, vibrepin blocked unwinding of oriCII by RctB, apparently by promoting the formation of non-functional RctB complexes. Finally, vibrepin inhibited the growth of all vibrio species tested and had potent cidal activity against V. cholerae. Although vibrepin also appears to have targets other than RctB, our findings suggest that RctB is an attractive target for generation of antibiotics that only block growth of vibrios.

One mechanism by which vibrepin appears to inhibit RctB function is by promoting formation of RctB complexes. Vibrepin induction of RctB complexes is relatively specific, as the compound hardly affected the oligomeric state of RctB[P516Q].
and did not induce multimerization of two DNA-binding proteins unrelated to RctB, CpxR and ParA2. There may be several consequences of RctB aggregation. First, since vibrepin inhibited RctB-mediated unwinding of oriCII, RctB complexes may be unable to initiate chrII replication. Second, since vibrepin impaired growth of DH5α/pYB289 in the absence of kanamycin, formation of RctB complexes may be toxic to cells even when RctB-dependent replication is not required. To date, the mechanism underlying such toxicity is unknown. Vibrepin also has targets other than RctB, since this compound inhibited the growth of bacterial species that do not encode RctB orthologues. The multiple effects of vibrepin on RctB as well as the possible existence of a non-RctB vibrepin target(s) in V. cholerae likely explains our inability to isolate vibrepin-resistant V. cholerae mutants.

Bacteria-specific mediators of DNA replication might be expected to be attractive targets for antimicrobial agents. Recently, O’Donnell and colleagues identified a small molecule that inhibits the interaction of the E. coli β-clamp with DNA polymerases using an in vitro biochemical screen [23]. This compound did not inhibit the interaction of the yeast clamp with polymerase and hence should not target eukaryotes; however, it is not known whether this compound inhibits bacterial growth. Inhibitors of DnaA, the initiator of chromosome DNA replication in almost all eubacteria, might also have potential as broad spectrum antibiotics and Skarstad and colleagues reported the development of a high throughput cell-based assay to identify such inhibitors [24]. However, no antibiotics are currently in use that directly target bacterial chromosome replication.

We found that RctB activity can be inhibited and thus showed that this initiator of replication of the second vibrio chromosome is a reasonable target for development of new Vibrionaceae-specific antimicrobial agents. Even though vibrepin has targets besides RctB, we anticipate that it will be possible to modify vibrepin or find new compounds that only target RctB. All antibiotics used in the clinic today are relatively broad spectrum and target highly conserved cellular processes. Therefore, these compounds inevitably select for resistant organisms in the normal human flora as well as in environmental microorganisms after antibiotics are shed into the environment. Genes that confer resistance can then be horizontally transmitted from bystander bacteria to pathogens. Vibrionaceae-specific antibiotics will not engender resistance in the normal human flora or in non-vibrio environmental microorganisms. Thus, in principle, genes mediating resistance to these compounds will not arise in and be transferred from non-vibrios to vibrios, perhaps postponing the development of resistance. Vibrio-specific agents may be useful as new agents for aquaculture and in the prevention and treatment of human vibrioses.

Materials and Methods

High throughput screen for RctB inhibitors

High throughput screening for small molecule inhibitors of growth of YBA685 in the presence of kanamycin was carried out at the NSRB screening facility at Harvard Medical School. YBA685 is E. coli strain Mach1 containing pYB289, an rctB-dependent vector (Figure 1A). Mach1 (Invitrogen), which grows faster than most laboratory E. coli strains, was used for the screening phase of our study to minimize the time required to detect growth inhibition. For screening, an overnight culture of YBA685 was inoculated at a 1:500 dilution into LB broth containing kanamycin (50 μg/ml); the culture was grown at 37°C until reaching an OD600 nm of ~0.1; then, 30 μl aliquots of the culture were transferred into 384-well plates. The compound library (100 nl of 5 mg/ml in DMSO, final concentration 16.7 μg/ml) was pin-transferred to the plates in duplicate. After the plates were incubated at 37°C for 3 hours, the OD600 nm of the wells was measured. We used Z-scores [25] to evaluate the growth.
inhibition of the compounds tested. The mean and standard deviation of the OD_{600 nm} values from the experimental wells in each plate were obtained; then Z-scores for each well were calculated as the difference between the OD_{600 nm} values in each well and the average OD_{600 nm} divided by the standard deviation. Compounds with Z-scores below -3 in duplicate plates were considered positive hits. There were 149 positive hits among 137,694 compounds screened. These compounds were counter-screened to exclude molecules that inhibited the growth of Mach1 in the absence of pYB289. Ultimately, we identified four compounds that inhibited growth of YBA605 in the presence of kanamycin but did not inhibit Mach1 growth. Vibrepin (CID 2803695) and C2 were purchased in milligram quantities from Maybridge (Tintagel, UK) and Sigma-Aldrich (St. Louis, MO), respectively. All compounds were dissolved at 50 mg/ml concentration in DMSO and stored at −20°C.

Strains and plasmids

The strains and plasmids used in this study are listed in Table 2 and 3, respectively.

| Strain | Relevant characteristics | Sources/References |
|--------|-------------------------|--------------------|
| mach1  | invิตrogen              |                    |
| YBA601 | DH5α/pYB289             | This study         |
| YBA685 | mach1/pYB289            | This study         |
| YBA796 | DH5α/pYB340             | This study         |
| YBA803 | DH5α/pYB344             | This study         |
| EHEC EDL933 |                     | [27]              |
| EPEC 2348/69 |                    | [28]              |
| N16961 |                         | [29]              |
| YBB182 | N16961 lacZ::gfp         | This study         |
| YBB697 | N16961/pYB303           | This study         |
| YBB815 | N16961/pYB345           | This study         |
| YBB874 | N16961 rctB::gfp         | This study         |
| V. nigripulchritudo |             | [5]               |
| V. parahaemolyticus VP47 |             | [30]              |
| V. vulnificus ATCC 27562 |            | [31]              |
| V. fluvialis H-08942 |               | [32]              |
| B. subtilis PY79 |               | [33]              |
| S. aureus MW2 |              | [34]              |

table: Table 2. Strains used in this study.

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Table 3. Plasmids used in this study.

| Plasmid | Relevant characteristics | Sources/References |
|---------|-------------------------|--------------------|
| A-52    | rctB L365I oriCII rctA aph | This study         |
| A-57    | rctB P516Q oriCII rctA aph | This study         |
| pCVD442 | Allele exchange vector, Amp\(^\beta\) sacB mob\(^\ast\) | [35]              |
| pET28b  | IPTG inducible expression vector | Novagen         |
| pHET-RctB |                   | [12]              |
| pGZ119EH | IPTG inducible expression vector | [36]              |
| pJZ111  | pCVD442 derivative to construct lacZ::gfp | [37]              |
| pORIL1  |                         | [12]              |
| pWSK129 | pSC101 ori aph          | [38]              |
| pYB190  | pUC ori aph             | [26]              |
| pYB289  | rctB oriCII aph         | [12]              |
| pYB303  | pGZ119 rctB [L365I]     | This study         |
| pYB340  | rctB L365I oriCII aph   | This study         |
| pYB344  | rctB P516Q oriCII aph   | This study         |
| pYB345  | pGZ119 rctB [P516Q]     | This study         |
| pYB346  | pET28b rctB [P516Q]     | This study         |
| pYB364  | pCVD442 derivative to construct rctB::gfp chromosomal fusion | This study |
| pYB376  |                         | [38]              |

table: Table 3. Plasmids used in this study.

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DH5α bearing any of these 3 plasmids were indistinguishable. To create rctB [L365I] or rctB [P516Q] expression vectors, the relevant rctB variant was PCR amplified and then inserted into pGZ119EH as previously described [12]. rctB [P516Q] was inserted into pET28b (Novagen) (yielding pYB346), for high level expression of C-terminal His tagged RctB [P516Q]. All the relevant DNA sequences of all vectors used in this study were determined. The sequences of the PCR primers used in this study are available upon request.

A V. cholerae strain containing a rctB::gfp translational fusion in the rctB locus and under the control of the native rctB promoter (YBB874) and a strain harboring gfp gene inserted in the lacZ locus under the control of the Plc promoter (YBB192) were constructed by allele exchange techniques using pCVD442-based plasmids (pYB364 and pJZ111, respectively) as described [26].

Growth curves

A SynergyHT microplate reader (BioTek, Winooski, VT) was used to determine the growth kinetics shown in Figures 1 and 5. In these experiments, overnight cultures were diluted 1:200 and then incubated for 9 hours. For the growth curves shown in Figures 2 and 4A, overnight cultures were diluted into fresh media in test tubes and OD_{600 nm} and CFU were determined at the indicated time points. All cultures were grown in LB media at 37°C except Tryptic Soy Broth (BD) was used for S. aureus cultures and LB containing 0.5 M NaCl was used for V. nigripulchritudo cultures which were grown at 30°C.

Quantification of oriCII plasmid

The copy numbers of oriCII-based plasmids pYB340 and pYB344 relative to pYB289 were measured by quantitative PCR as described previously [12]. For Figure 2D, Southern hybridization of total genomic DNA was used to quantify pYB289 plasmid
DNA (relative to *E. coli* chromosomal DNA) in vibrepin treated and untreated cells. A — 600 bp DNA fragment of *rctB* was used as a probe for pYB289 and a similar sized *narW* fragment was used as a probe for the *E. coli* chromosome. Probe preparation and detection were carried out with the ECL Direct nucleic acid labeling kit and detection system (GE Healthcare) according to the manufacture’s instructions. Bands were quantified using a Fujifilm FLA-5100 imager.

**P1 nuclease cleavage assay**

The P1 nuclease-based assays for *RctB* unwinding of *oriC*-containing plasmid substrates were performed as described previously [12]. Briefly, different concentrations of C-terminal His-tagged versions of *RctB* was mixed with 150 fmol of pOrII in 50 μl of a solution composed of 10 mM Heps-KOH (pH 7.6), 8 mM magnesium acetate, 30% glycerol and 320 μg/ml BSA. After 10 min at 37°C, 1.2 units of P1 nuclease was added to each reaction for 30 seconds; the reactions were stopped with 40 μl of stop buffer (25 mM EDTA, 1% SDS). For quantification of the linearized fraction of the pOrII substrate DNA, an aliquot of the reaction was electrophoresed on a 0.8% agarose gel and then stained with ethidium bromide; the proportion of the plasmid DNA linearized by P1 nuclease was determined using densitometry.

**Sedimentation assay**

Purified C-terminal His-tagged *RctB* or *RctB*[P516Q] (10 μM) was incubated in 20 μl buffer F (30 mM Tris pH 8, 5 mM MgSO₄, 100 mM KCl, 2 mM DTT) in the absence or presence of compounds for 10 min at 30°C. Reactions were then centrifuged for 30 min at 4°C to 20,000×g in a refrigerated table top centrifuge. Centrufuged supernatants were collected and filtered through a 0.1 μm ultrafilter (Millipore filter). In each assay, 300 ng of protein was added to a 12 μl reaction in sedimentation buffer F in a quartz cuvette in presence or absence of compounds. The amplitude plotted on the y-axis in Figure 3E and F is reflective of the intensity measurements generated in these experiments; intensity is proportional to the size and concentration of the scattering particles. Amplitude values were calculated using Omniscan 3.0 software. Each curve is representative of at least 5 measurements.

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### Author Contributions

Conceived and designed the experiments: YY SD EAS MKW. Performed the experiments: YY SD EAS. Analyzed the data: YY SD EAS MKW. Contributed reagents/materials/analysis tools: YY SD. Wrote the paper: YY SD MKW.

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