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Antibacterial particles and predatory bacteria as alternatives to antibacterial chemicals in the era of antibiotic resistance

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Abstract

This review is focused on the subset of antibacterial agents whose action involves one-on-one targeting of infecting bacteria. These agents target individual bacteria and their efficacy is based on particle numbers in contrast to chemical agents such as antibiotics, whose efficacy is based on minimal inhibitory concentrations. Four extant members of this class are predatory bacteria, functional (plaque-forming) phages, and engineered particulate systems, phagemids (plasmids that contain a phage packaging signal) and antibacterial drones (ABDs) that package chromosomal island DNA carrying antibacterial genes. We differentiate the natural predators, phages and predatory bacteria, from the engineered delivery vehicles, phagemids and ABDs, because the latter are much more versatile and can largely bypass the historical warfare that informs the predator-prey interactions.

Introduction

In the throes of the antimicrobial resistance crisis, considerable funding has engendered a large array of research projects, ranging from the search for scarcer and scarcer new antibiotics, through the development of antibacterial peptides, bacteriocins, antisense RNAs, to novel small molecules targeting essential bacterial functions [1]. In this review, we focus on an additional and somewhat distinct class of anti-bacterials, which target individual bacteria rather than depending on the achievement of a body-wide MIC, and we single them out for general evaluation and comparison. Included are predatory bacteria, functional (plaque-forming) phages, and two types of engineered particulate systems, phagemids (plasmids that contain a phage packaging signal) and antibacterial drones (ABDs) that package chromosomal island DNA carrying antibacterial genes. They have important advantages over antibacterial chemicals and have several common and distinguishing features: Their efficacy is measured by their efficiency of killing or disabling target bacteria rather than by minimal inhibitory concentrations; they cause no dysbiosis of the microbiome, there are probably few if any pre-existing allergies, they have no known toxic side effects and are not themselves toxic and they can, in principle, be engineered to avoid resistance. But they are nowhere near the medical quick-fix of antibiotics in their heyday. And it is ironic that one of their great advantages is their narrow host range, which is

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actually too narrow since the ideal would be to target all strains of at least one species – clearly the most critical objective for this class of anti-bacterials.

Predatory bacteria

Predatory bacteria, of which the prototype is *Bdellovibrio bacteriovorus,* were discovered nearly 60 years ago [2], giving rise to a broad field of inquiry in which a large and diverse group of organisms was discovered and many individual species characterized [3]. These fall into three main groups on the basis of their mode of predation: endobiotic, where the predatory organism invades and multiplies within its prey, epibiotic, where the predatory organism attaches to and kills its prey from the outside, and transbiotic or group predators, where the predator secretes lytic proteins and feeds on the released cellular contents of its prey [4]. The endobiotic prototype is *Bdellovibrio bacteriovorus,* whose biology has been analyzed in great detail [5] and which has been tested in a variety of animal models. From the clinical point of view, the epibiotic prototype is *Micavibrio aeruginosavorus,* which has also been studied in considerable detail [6]. Transbiotic predators are not of clinical interest per se, although some of their products are of major importance. *B. bacteriovorus* and *M. aeruginosavorus* are the only two that have thus far been considered for clinical utility.

Endobiotic predation: B. bacteriovorus

Predation.: *B. bacteriovorus* is a small Gram-negative motile rod with a polar flagellum responsible for motility. It is an obligatory endoparasite of most Gram-negative bacteria including many major pathogens, (see fig. 1B). Its predatory activity involves an attack phase (AP) in which the free-living organism locates its prey, responds to a specific signal in the cell wall, and enters a growth phase (GP). It attaches to the cell wall, apparently by means of a type IV pilus, and in response to a second cellular signal, secretes hydrolytic enzymes that open a channel in the cell wall through which the organism enters the periplasm [7]. It then causes the cell to form a round ‘bdelloplast’ and grows into a multi-cell filament by metabolizing the prey cell’s contents, then septates and lyses the cell.

Safety; side effects.: *B. bacteriovorus* is non-toxic for mammals and is cleared from a mouse in about 3 weeks following parenteral administration [8]. It can survive in macrophages, apparently without visible ill effects on the cell [6]. It stimulates modest production of cytokines, especially IL-13 [9] and has no significant effect on the colonic microflora or intestinal histology in rats [8, 9].

In vitro antibacterial activity.: *B. bacteriovorus* was recognized early as a potential therapeutic antibacterial, and its activity analyzed extensively in vitro. In a study with *Klebsiella pneumoniae*, it was found to reduce the bacterial population by about 10^6-fold, but the surviving bacteria became phenotypically resistant and shortly regrew [10]. A possible solution for this appeared serendipitously when *B. bacteriovorus* was isolated from wastewater in association with a lytic bacteriophage. This association survived filtration and dilution and presumably represented physical attachment of the phage to the *B. bacteriovorus* cell, perhaps similarly to the recently observed attachment of phage particles to *Bacillus subtilis* flagellae [11]. Treatment with this lytic phage along with

B. bacteriovorus [12], resulted in eradication of the prey organism. In other studies, *B. bacteriovorus* was observed to penetrate and disrupt biofims [13], and to be active against biofilm-forming oral pathogens [14]. Interestingly, a residual population of prey organisms persists in a biofilm despite prolonged incubation and the continued presence of a greater number of *B. bacteriovorus* [13]. In this latter study *B. bacteriovorus* reduced the level of the same prey organisms in the planktonic state to undetectable. The overall view of in vitro predation studies with *B. bacteriovorus* is that under many conditions a considerable number of prey organisms survive *B. bacteriovorus* predation, possibly owing to interfering substances such as cyanide and indole produced by the prey organism [15] but that under some conditions it can completely eliminate its prey – an unappealing prospect for an obligatory parasite.

In vivo antibacterial activity.: In one series of experiments with a *Klebsiella pneumoniae* lung infection in rats, *B. bacteriovorus* reduced the intrapulmonary bacterial titer by about 10^3-fold [10]. However, it did not reduce the bacterial burden in various organs in rats infected IV with *K. pneumoniae;* inexplicably, the *B. bacteriovorus*-treated rats seemed healthy compared to the untreated controls, which become moribund after 36 h [16]. In other in vivo studies, *B. bacteriovorus* attenuated *Salmonella* infections in chicks [17], cured *K. pneumoniae* infections by cooperative action with immune cells in zebrafish larvae, and rescued mice infected by aerosol with *Yersinia pestis* [18].

Epibiotic predation: *B. bacteriovorus and B. exovorus*—The closely related *B. exovorus* is an epibiotic predator as shown in Fig. 1 C & D, and, somewhat surprisingly, *B. bacteriovorus* can attack *S. aureus* epibiotically, both in planktonic suspensions [19, 20] and in biofilms which it can disrupt [21]. Electron microscopy shows *B. bacteriovorus* attached externally to partially disrupted *S. aureus,* suggesting that it destroys the cell from without and extracts the contents in the typical epibiotic manner.

Micavibrio aeruginosovorus.: *M. aeruginosovorus i*s an epibiotic predator whose predatory behavior has been studied extensively in comparison with that of the endobiotic *B. bacteriovorus*, both for its interesting biology as an epibiotic prototype and for its clinical potential [22]. It is very effective against biofilms in vitro [23] and has been tested for safety in animals [8], where it induces a modest cytokine response but has no detectable toxic effects.

Given all of the results described above, and many more in the literature, plus the frequent isolation of *B. bacteriovorus* from human stools, predatory bacteria have been very widely touted as potential therapeutics for human infections with Gram-negative bacteria. See, for example [24], among many other publications. It would seem that an obvious place to start would be with external or intestinal infections. Nevertheless, it does not appear ever to have been used as such, perhaps because the idea of eating, smearing, or injecting live bacteria to chase other live bacteria in or on the body may simply seem too outlandish.

Bacteriophages

The great expectations for phage therapy that followed the discovery of phages were not met, owing to a profound lack of understanding of phage biology, so that phage therapy (PT) was largely sidelined by the discovery of antibiotics in the late ‘30’s. It has limped along, especially in Eastern Europe, where, largely owing to ease and inexpensiveness of production, it continues to be a cottage industry, largely discredited in the West owing to a chronic lack of rigorous supporting clinical data. If an individual with an infection recovers after anecdotal treatment with a cocktail of 15 or 20 different phages, that seems to be sufficient support for the cocktail strategy, and if the individual does not recover, *trop mal (*or even, *tant pis*). In the era of antimicrobial resistance (AMR), phage therapy has had a re-awakening in the West, but rather than simply adopting the rather unsatisfying Eastern European cocktail strategy, research in the West has proceeded by detailed investigations of the biological principles that would have to inform any rational therapeutics. Many of these biological principles were well-established, inasmuch as the study of phages has been a cornerstone of modern molecular biology since the 1950’s. Pre-clinically, simple studies in which animals infected with laboratory strains are treated with phages to which they are known to be susceptible, always result in dramatic cures, serving as ample proof of principle and showing that phages are non-toxic. But in the extrapolation to naturally-occurring strains, a major feature of phage biology reared its ugly head - Phages had been at war with their bacterial hosts since both appeared on the planet, and the bacteria have developed a vast armamentarium of resistances starting with blockage of adsorption, DNA destruction by restriction enzymes and CRISPRs, and extending to every aspect of the phage life cycle with which the host could theoretically interfere [25]. So bacteria, unlike the US capitol, were well prepared for the onslaught of their enemies. Consequently, the problem of limited phage host range has thus far been surmounted only in individual cases in which, with a bacterial diagnosis in hand, an extensive search of phage collections would uncover one to which the infecting organism was sensitive; and in a few cases, anecdotal compassionate use of such phages has saved patients dying from untreatable AMR bacteria [26] [27]. Bacteria recovered from such patients, after successful phage treatment, are, however, generally resistant to the phage [26]. Though highly satisfying, this paradigm hardly represents a general therapeutic platform and is not likely to lead to one. Nor is it likely that the problems of ancient natural or recently acquired phage resistance will easily be surmounted. Instead, the Eastern European cocktail concept has been adopted, after all, but more rationally. For a detailed and thoughtful review see [28]. Well-developed examples include: combinations of several broad-host range phages that can collectively surmount most resistance mechanisms or selection for phage mutants or variants resistant to host defense mechanisms [29] [30] [31]. These strategies can potentially cover 90% or more of strains of a given species; specific bacterial diagnosis, however, will probably always be necessary (though Chan, e*t al*. [28] envision the possibility that carefully constructed cocktails could be used presumptively). A more direct means of broadening specificity and enhancing effectiveness involves engineering of individual phages using the recently described “rebooting” method involving transfection of bacterial L-forms with intact phage genomic DNA [32]. This method can be used to develop individual phages with any combination of features [33] such as modified tails to broaden adsorption specificity [34], resistance to restriction enzymes by incorporating methylases and to CRISPR by

incorporating anti-CRISPR genes. An additional strategy, for which a clinical trial is currently underway by Locus Pharmaceuticals, is engineering phages to deliver bactericidal CRISPRs [35]. The advantage of this over phages is not obvious. Ongoing theoretical problems with PT are the potential mobilization of virulence or resistance genes in the target population and release of toxic bacterial components by phage-induced lysis. PT is likely to have an important role in bacterial infection control in the AMR era, but that role will have to be evaluated by double-blind, placebo-controlled trials, which are only just beginning [36] [37].

Phagemids and antibacterial drones (ABDs)

The promising idea has recently emerged of packaging antibacterial genes in phage-like capsids. This avoids some of the consequences of predator-prey warfare and enables precise engineering and limitless versatility of antibacterial cargos, with two different incarnations being phagemids and ABDs.

Phagemids

Phagemids are hybrid plasmids containing a phage packaging module plus added genes of interest, which are packaged in phage particles by the cognate helper phage [38] [39] [40]. In the first published paper to report the potential therapeutic use of a phagemid [38], the intergenic region of filamentous phage f1, which directs f1 packaging, was cloned to a plasmid containing *gef* or *chpBK,* each encoding the toxin component of a TA system. The plasmid was transferred into a host cell containing the packaging-defective f1 helper phage, R408. This cell packages and extrudes large numbers of f1-like particles containing the modified plasmid, and only a few ordinary f1 particles. After a demonstration of the ability of these particles to kill F+ bacteria in vitro, they were administered to mice that had been infected with F+ *E. coli* and they reduced the number of infecting bacteria by about 1000-fold, demonstrating the therapeutic potential of the phagemid. In a second report, Bikard et al [40] cloned the ΦNM1 terminase and *rinA* genes to the rolling circle staphylococcal plasmid pC194, followed by a CRISPR/cas9 module with a spacer targeting the *aphA* (kanamycin resistance) gene. In this scheme, phage lysates contained only about 3% phagemid particles (~3×10^5/ml) and the phagemid was structurally unstable, as is typically the case for rolling circle plasmids with large inserts. These particles were shown to eliminate a kanamycin resistance (KmR) plasmid and to kill cells in which the *aphA* (KmR) gene was chromosomal, in both cases via Cas9-induced DNA cleavage of the *aphA* protospacer. This preparation was used to treat a mouse staphylococcal skin infection, causing a decrease (but only a five-fold decrease) in the number of infecting KmR staphylococci, as measured with GFP-tagged cells. In a third phagemid paper, Krom *et al.* [39] used the same f1/m13 system as Westwater *et al* [38], but developed a much more elaborate and sophisticated set of constructs to accomplish several different outcomes, successfully targeting F+ *E. coli* cells in vitro and in vivo.

Although the bactericidal effectiveness of phagemids based on coliphage f1/m15 has been convincingly demonstrated, the host range of these phagemids among natural *E. coli* populations must be extremely narrow, as only F+ or HFR strains are susceptible and F is a rare, mating-derepressed plasmid; the only possible way to extend this range

has been to modify the phage to recognize mating pili of other plasmids [41], which, incidentally, are normally mating-repressed and do not express their mating pili. A much better phagemid strategy is that reported by Bikard, *et al.* [40], in which the helper phage is a typical staphylococcal siphovirus. Although its host range would be limited by restriction enzymes etc., as is the case for the ABDs, effective remedies for both can be envisioned. In the configuration described by Bikard *et al.,* much improvement could be achieved by eliminating phage maturation and improving plasmid stability and particle yield.

ABDs

The ABDs, developed in and for *S. aureus*, are based upon the highly mobile, phage-inducible pathogenicity islands known as SaPIs [42] [43], which are ideal for conversion to antibacterial agents. SaPIs are ~15 kb chromosomal elements, long ago derived from prophages; while retaining several key prophage functions, including an integrase, a master repressor, a replicon, and a SaPI-specific terminase small subunit (TerS), they have evolved into distinct genetic elements with a unique lifestyle, including repressors that are not SOS induced, pathogenic cargos, and an important role for their bacterial cells. They are induced to excise and replicate by helper phage-encoded anti-repressors [44] [45], are packaged in small phage-like particles assembled from phage virion proteins [46], and released in very high numbers (~10^9/ml) [47], disseminating their cargo of superantigen and other virulence genes. Their recent conversion from disease-causing pathogenicity islands to disease curing ABDs involved i) deletion of virulence genes, ii) expansion of cargo capacity by deletion of genes responsible for small capsid formation, and iii) incorporation of antibacterial cargos. In addition, the ABD helper prophage was modified to prevent phage production by deletion of its specific TerS gene. The helper prophage replicates upon induction with mitomycin C but its DNA is not packaged. Its anti-repressor induces the resident ABD, which is packaged in full-sized phage particles, using its specific TerS, with some 30 kb of cloning space available for cargo genes. Initial studies have used ABDs containing *tetM* or *cadA* for selection and scoring, and CRISPR/*cas9,* armed with spacers targeting highly conserved but non-essential genes such as *agrA* or *fnbA.* Upon entry into a susceptible cell, Cas9::spacer is produced, binds to and induces a double strand cut in the *agrA* or *fnbA* protospacer, which is lethal owing to a lack of non-homologous end joining capability [48] [42]. A similar system using CRISPR/cas3a, which targets RNA nonspecifically, has recently been developed by Kiga, *et al.* [49].

Either ABD (Fig. 2) caused a 10^4-fold decrease in viability, with survivors being CRISPR-resistant mutants. An ABD containing the non-nucleolytic CRISPR/*dcas9* armed with a spacer targeting the P_2P_3 regulatory region of the virulence-controlling *agr* locus (Fig. 3) totally blocked *agr* expression. These two ABDs were tested in murine subcutaneous abscess [50] and peritoneal lethality [51] models, sharply attenuating the abscesses and rescuing mice infected intraperitoneally with a lethal dose of staphylococci [52]. These results were obtained with strains susceptible to the ABD, including the clinically important methicillin-resistant (MRSA) strain USA300. Some other strains, which were resistant to both the ABD helper phage (80α) and to the broad host range phage K, were nevertheless susceptible to ABD lethality [52]. This was assumed to reflect the much simpler requirement for ABD cargo expression than for phage sensitivity, which requires execution of the entire phage

lytic cycle. Nevertheless, many clinical *S. aureus* isolates are only weakly susceptible to the ABDs. This remains a major problem facing the clinical applicability of the ABD system, as is also the case for any other phage particle-based antibacterial strategy. In the case of the ABDs, since adsorption/injection is largely non-specific in staphylococci, and CRISPRs are very rare, the most serious impediment is almost certainly restriction enzyme degradation; possible solutions are under development in the author's laboratory.

Conclusion

The discovery of antibiotics was followed by the tremendously exciting and extremely rapid development of antimicrobial therapeutics – so much so that the medical world was lulled into a state of complacency – indeed "spoiled" by the power and efficacy of these miracle drugs. The predicted AMR crisis, born in <20 years, has steadily worsened until it has finally occasioned an intense effort to remedy it by any means possible. This effort has generated a wide field of endeavor that has been increasing for at least 40 years but has yet to come up with anything remotely as effective as the antibiotic quick-fix for infections. In this review, we have addressed a specific sub-field in this endeavor, the development of four single-unit systems that attack individual bacteria, one-by-one, in contrast to soluble chemicals, and report that each of them has great promise but is as yet very far from ameliorating the AMR crisis. Three of these use phage particles for delivery of antibacterial agents – phages themselves, phagemids, and ABDs. Each is plagued by pre-existing resistances and host range limitations, the overcoming of which is the key to clinical utility but is highly challenging, to say the least. The use of intact phages has progressed the furthest but remains limited to the rescue of patients fatally infected by pan-resistant bacteria, using pre-tested personalized phages. The fourth, using live predatory bacteria, is highly promising, especially in particular situations, but seems plagued by the distasteful prospect of using live bacteria to chase and attack other live bacteria within or on the human body. On the whole, each of these one-on-one targeting systems has a potential place in antibacterial bacterial infection control in the AMR era, but each has a long way to go.

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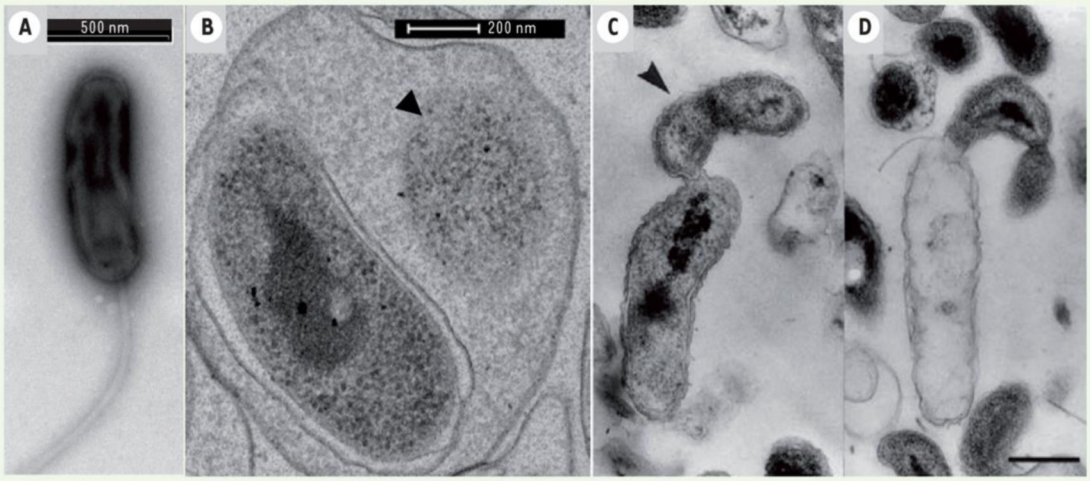

Fig. 1. Transmission electron microscopic observation of endobiotic predation by *B. bacteriovorus* H100 (B) and epibiotic predation by *B. exovorus* (C & D.

B. bacteriovorus is shown within the periplasm of an *E. coli* cell (B) and *B. exovorus* attached externally to the organism (C & D). One can observe the reduced cytoplasm of the prey organism (B arrow), the extracellular growth and division of the epibiotic predator (C arrow), and the emptied body of the prey organism (D). Reproduced from [3] with permission

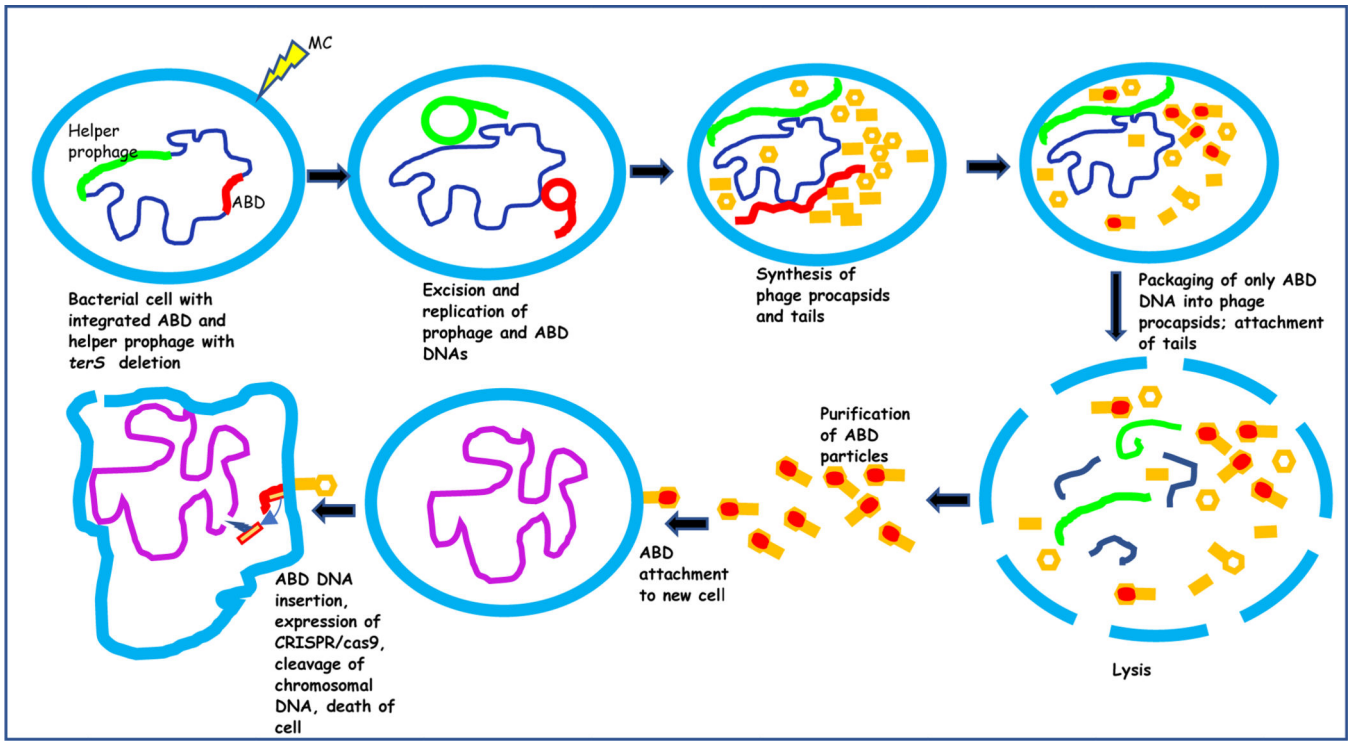

Fig. 2. Production and activity of ABD2003, carrying CRISPR/cas9:: fnbA.

Integrated ABD (red) and helper prophage (green) are induced by mitomycin C (MC) to excise and replicate, followed by procapsid production and packaging of ABD DNA (only), followed by lysis, release and purification of ABD particles. These attach to and infect target cells. ABD DNA is injected, cas9 is expressed and cleaves the *agrA* protospacer,

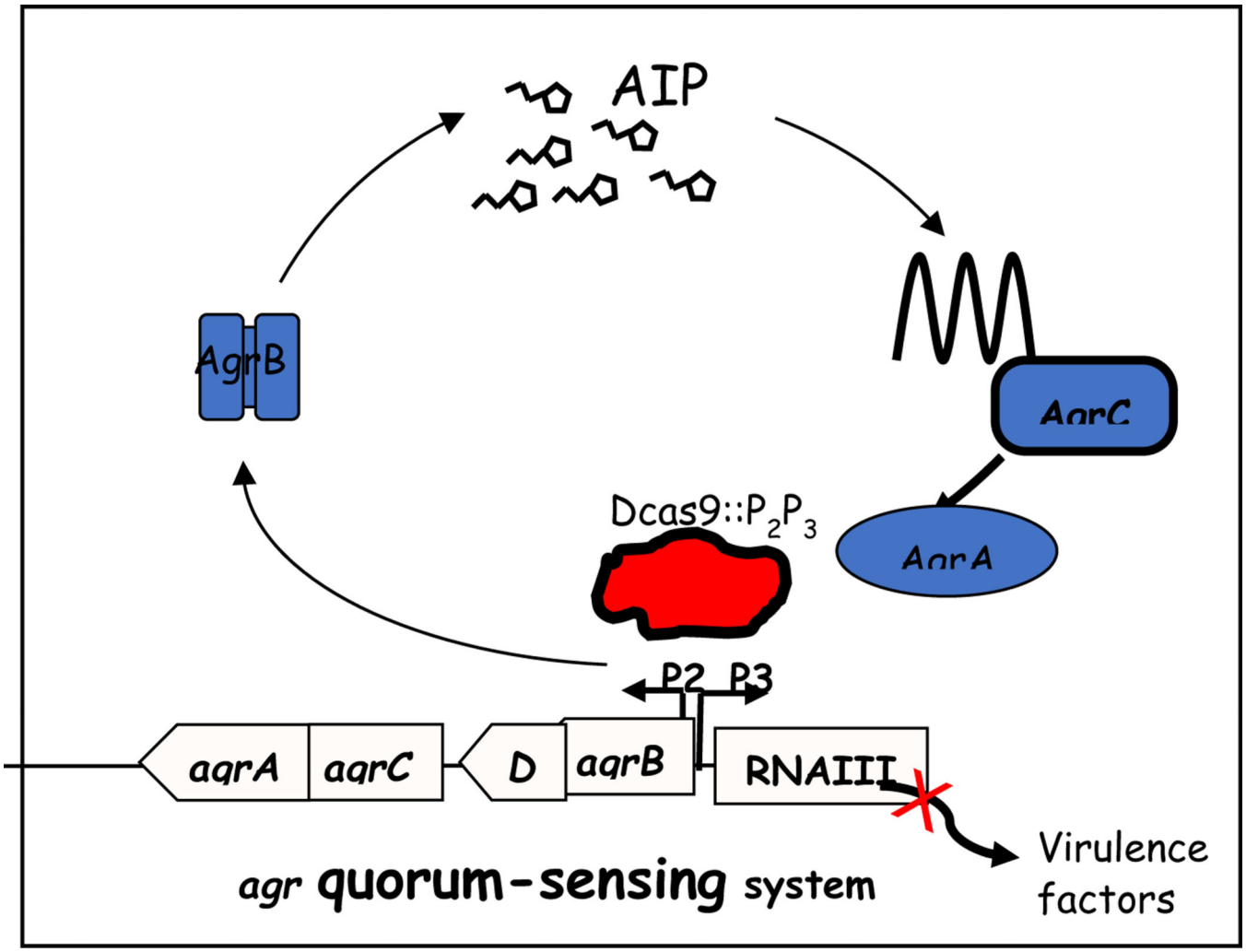

Fig. 3. Blockage of agr expression by ABD2006 containing CRISPR/dcas9::agrP_2P_3.

Agr is an autocatalytic 2-component signal transduction system consisting of a transmembrane receptor, AgrC, which senses and is activated by a cyclic thiolactone peptide, the AIP, processed from a precursor, AgrD by AgrB, which is also required for its secretion. Activated AgrC transphosphorylates AgrA, which activates the two regulatory promoters, P_2 and P_3, thus autoactivating the P_2 operon as well as the P_3 transcript, a regulatory RNA known as RNAIII, which regulates the virulon. dCas9::P_2P_3 binds to the promoter region, shutting down the regulon