that will ensure that the single-cell community continues to grow and thrive.

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Forum

Specific Antivirulence Activity, A New Concept for Reliable Screening of Virulence Inhibitors

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Antivirulence therapy, disarming pathogens rather than killing them, is emerging as a novel strategy for disease control. Reporter strains expressing an easily measurable and quantifiable phenotype enable easy screening of virulence inhibitors. In this paper I propose a novel concept to exclude false positives in this type of screening.

Targeting Virulence: A Novel Approach To Controlling Bacterial Infections

Disease caused by antibiotic-resistant pathogens is becoming a serious problem, both in human and veterinary medicine. According to the WHO, antibiotic-resistant pathogens are currently the second leading cause of death worldwide [1]. An alternative strategy to the use of antibiotics to combat bacterial infections is the specific inhibition of functions that pathogens require to cause infection, thereby preventing them from attacking their host. This novel strategy has been termed antivirulence therapy, and it can be applied to controlling bacterial disease in both humans and animals [2,3]. Major advantages of targeting virulence rather than viability are that there will probably be a lower tendency to develop resistance (although this possibility cannot be excluded [4]), and that there will be less-severe side effects towards the neutral and beneficial bacteria associated with a host under treatment (which are also inactivated by traditional antimicrobials). One of the most intensively studied strategies for antivirulence therapy is disrupting quorum sensing, bacterial cell-to-cell communication with small signal molecules. Various types of quorum-sensing systems and signals have been described; more are still being discovered, and many compounds have been claimed to be able to inhibit quorum sensing in various pathogens (see [5] for recent review).

Identification of Quorum-Sensing Inhibitors Based on Signal Molecule Reporter Strains

One of the factors that has resulted in an expansion of quorum-sensing research is the development of signal molecule reporter strains, which demonstrate a phenotype that can easily be monitored and quantified (e.g., *gfp*, *lacZ*, or luminescence genes) in response to the presence of quorum-sensing signal molecules [6]. However, an important limitation of the use of such reporter strains is the fact that the quorum sensing-regulated reporter phenotypes are often co-dependent on other factors and/or depend on the metabolic activity of the cells. For instance, bioluminescence is dependent on metabolic activity because the reaction requires large amounts of energy [7]. The same is true for reporters based on other phenotypes such as β-galactosidase activity (through *lacZ*) and the production of green fluorescent protein (GFP) [8].

As a consequence, the identification of quorum-sensing inhibitors based on the inhibition of the reporter phenotype is prone to bias if no adequate control experiments are included. As a control, many studies in the field have verified that there is no effect on viability in nutrient-rich growth media by confirming that the quorum-sensing inhibitory effects occur at concentrations below the minimal inhibitory concentration (MIC) or by confirming that the quorum-sensing inhibitory concentration of the compound has no impact on growth kinetics or final density after a given incubation period. However, we found that this type of control experiment might miss toxic effects that have a significant impact on a reporter phenotype, without affecting growth [9]. We recently argued that even the most sensitive growth test (the determination of the impact on growth kinetics) might miss significant toxic effects [8]. The most straightforward control experiment to exclude false positives consists of verifying the impact of a putative inhibitor in a control strain in which the same reporter phenotype is independent of quorum sensing (by placing the reporter gene under the control of a constitutive or inducible promoter). However, in most cases, even true quorum-sensing inhibitors will start to show off-target effects on such a control strain as well at elevated concentrations (Figure 1), and a new concept is therefore necessary to interpret the data obtained in this type of experiment.

Specific Quorum Sensing-Disrupting Activity A_{QSI}

We recently proposed a new parameter, A_{QSI} (specific quorum sensing-disrupting activity) as an elegant way to interpret data

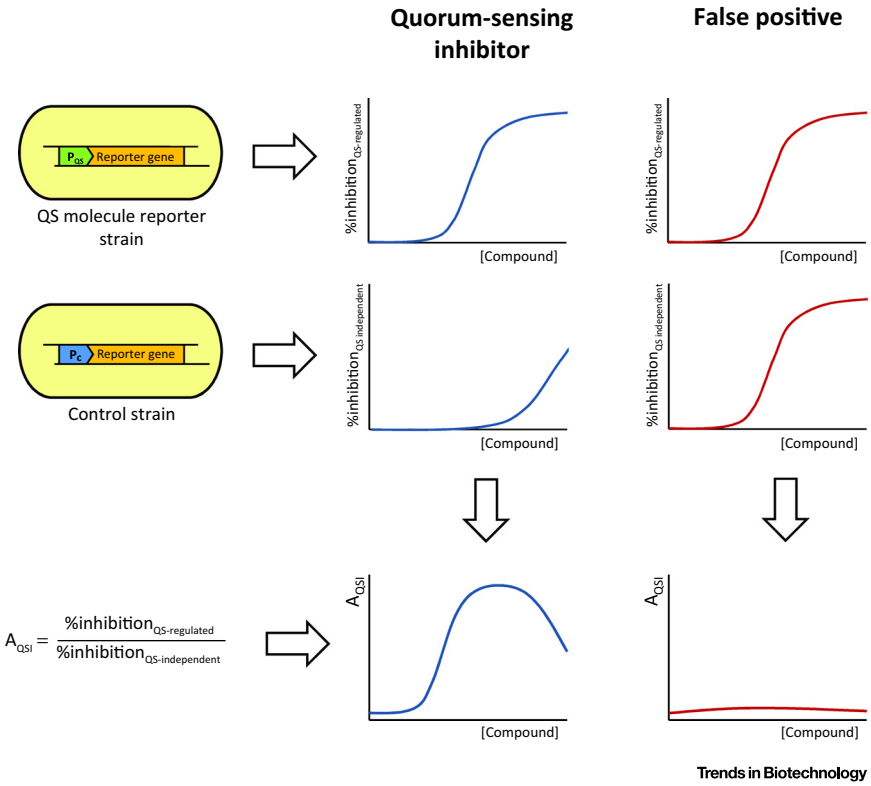

Figure 1. Schematic Representation of the A_{QSI} Concept. The impact of putative inhibitors on the expression of a reporter phenotype under control of a quorum sensing-regulated promoter (P_{QS}) is determined (top row). Similarly, the impact on the same reporter phenotype under control of a constitutive (or inducible) promoter (P_C) is determined (middle row). For each concentration tested, A_{QSI} is determined as the ratio between the two (bottom row). For a true quorum-sensing inhibitor, A_{QSI} will increase with increasing concentrations, reach a maximum, and might then decrease again because the compound might show off-target effects at elevated concentrations. The higher the maximum A_{QSI} value, the more specific the quorum-sensing inhibitor will be. For a false positive, A_{QSI} will not increase above 2 because the impact on the reporter phenotype is entirely due to off-target effects at all concentrations. Abbreviations: A_{QSI}, specific quorum sensing-disrupting activity; QS, quorum sensing.

on the impact of putative quorum-sensing inhibitors on a quorum-sensing reporter and corresponding control strain [10]. A_{QSI} is defined as:

$$A_{QSI} = \frac{\%\text{Inhibition}_{\text{QS-regulated}}}{\%\text{Inhibition}_{\text{QS-independent}}}$$

where $\%\text{Inhibition}_{\text{QS-regulated}}$ is the percent inhibition of the reporter phenotype when controlled by quorum sensing at a given concentration of the putative inhibitor, and $\%\text{Inhibition}_{\text{QS-independent}}$ is the percent inhibition of the same phenotype in the control strain at the same concentration of the putative inhibitor. This ratio can be determined for different concentrations of the putative inhibitor to determine the optimal active quorum-sensing inhibitory concentration (Figure 1). We considered compounds to be quorum-sensing inhibitors if they significantly inhibited the quorum sensing-regulated reporter phenotype and if A_{QSI} was higher than 2 for at least one of the concentrations tested [10]. Compounds with A_{QSI} values higher than 10 were considered to be specific quorum-sensing inhibitors.

This concept was applied to a set of thiophenones, structural analogs of brominated furanones, the best-known quorum-sensing inhibitors [11]. We found that, of the 20 compounds tested, 17 significantly inhibited bioluminescence controlled by quorum-sensing in the model bacterium *Vibrio harveyi* [10]. Five of these were considered to be false positives because A_{QSI} was lower than 2 at all concentrations tested. Six compounds were found to be specific quorum-sensing inhibitors because they showed A_{QSI} values higher than 10. Interestingly, there was a positive correlation between A_{QSI} of the thiophenones and their capability to protect a host from infection in a gnotobiotic model system.

It should be noted that compounds could be identified as specific inhibitors ($A_{QSI} > 10$) even if they have a relatively weak quorum-sensing inhibitory activity, in other words if they have a low impact on the quorum sensing-regulated reporter phenotype and an even lower (or no) impact on the same phenotype in the control strain. Hence, A_{QSI} values need to be interpreted alongside the actual impact on the quorum-sensing reporter, and the best candidates for drug development should show both high inhibition of the quorum-sensing reporter and a high A_{QSI} value (implying that they are both strong and specific inhibitors).

Specific Antivirulence activity A_{AV}: A Novel Concept To Identify Virulence Inhibitors Based on Screening Assays with Reporter Strains

In addition to quorum sensing, several other mechanisms are required for infection by pathogenic bacteria. These mechanisms include specific virulence factors such as adhesion molecules and secretion systems [12], as well as other regulatory mechanisms such as the sensing of compounds released by the host (e.g., catecholamines) [13]. These mechanisms can therefore also serve as targets for the development of novel antivirulence therapies, and some compounds able to interfere with them have been identified [12,13]. Assays similar to those used for the identification of quorum-sensing inhibitors can be developed to screen for inhibitors of these other mechanisms, in other words by constructing a reporter strain (in which a promoter that controls the