Commentary

Double agent indole-3-acetic acid: mechanistic analysis of indole-3-acetaldehyde dehydrogenase AldA that synthesizes IAA, an auxin that aids bacterial virulence

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The large diversity of organisms inhabiting various environmental niches on our planet are engaged in a lively exchange of biomolecules, including nutrients, hormones, and vitamins. In a quest to survive, organisms that we define as pathogens employ innovative methods to extract valuable resources from their host leading to an infection. One such instance is where plant-associated bacterial pathogens synthesize and deploy hormones or their molecular mimics to manipulate the physiology of the host plant. This commentary describes one such specific example—the mechanism of the enzyme AldA, an aldehyde dehydrogenase (ALDH) from the bacterial plant pathogen *Pseudomonas syringae* which produces the plant auxin hormone indole-3-acetic acid (IAA) by oxidizing the substrate indole-3-acetaldehyde (IAAld) using the cofactor nicotinamide adenine dinucleotide (NAD$^+$) (*Bioscience Reports* (2020) **40**(12), https://doi.org/10.1042/BSR20202959). Using mutagenesis, enzyme kinetics, and structural analysis, Zhang et al. established that the progress of the reaction hinges on the formation of two distinct conformations of NAD(H) during the reaction course. Additionally, a key mutation in the AldA active site 'aromatic box' changes the enzyme's preference for an aromatic substrate to an aliphatic one. Our commentary concludes that such molecular level investigations help to establish the nature of the dynamics of NAD(H) in ALDH-catalyzed reactions, and further show that the key active site residues control substrate specificity. We also contemplate that insights from the present study can be used to engineer novel ALDH enzymes for environmental, health, and industrial applications.

The immense diversity of life on earth is supported by an intricate give-and-take between organisms that inhabit various environmental niches. Most known ecosystems host multiple species that engage in an enduring exchange of biomolecules which are classified as positive or negative interactions (beneficial/neutral or detrimental to one or more of the species, respectively), and are precisely tailored to meet the nutritional and proliferative needs of the organisms [1,2]. Some of the most fascinating examples of interactions among species are where one organism is host to another which is a pathogen, that is, the pathogenic species acquires nutrients and proliferates, while the host suffers the consequences of the infection. For example, the relationship between *Phaeobacter gallaeciensis*, a marine bacterium and *Emiliania huxleyi*, a marine microalga is proposed to be a mutual exchange with the algae providing dimethylsulfoniopropionate (a carbon and sulfur source) to the bacteria and the bacteria providing growth hormones (phenylacetic acid) and antibiotics (tropodithietic acid) in return. In an intriguing turn

of events, once *E. huxleyi* begins to senesce, it releases *p*-coumaric acid which is sensed by *P. gallaeciensis*. *P. gallaeciensis* then synthesizes roseobacticides to kill the algae and scavenge nutrients from its victim for its growth [3]. In the natural world, there are several innovative means that pathogens use to gain entry into the host. Rabies virus, a member of the Rhabdoviridae family, enters into nerve ending-rich muscle and tissues through the bite of an animal and then proliferates and travels via nerve cells to the brain causing hydrophobia, hallucinations, and eventually death [4]. The blast disease of rice is caused by the fungus *Magnaporthe oryzae* which produces a flattened, hyphal cell from the germinating spore with an infection peg that penetrates the host causing brown diamond-shape lesions on the rice leaves [5]. Interestingly, many plant-associated microbes synthesize a plant hormone or its close mimic to gain entry and establish themselves by modulating the host plant's metabolism as well as their own virulence genes [6–8]. An extensively studied example of hormone-mediated plant–microbe pathogenesis is the crown gall disease caused by the Gram-negative soil bacterium, *Agrobacterium tumefaciens*. The bacterium senses chemoattractants such as phenolic compounds released from wounded regions on the plants and initiates transfer of bacterial DNA to the host chromosome which hijacks the metabolism in the infected plant cells [9]. *A. tumefaciens* harbors a specialized tumor-inducing (Ti) plasmid which encodes an array of genes that aid in its transfer, replication, and in overall plant–microbe pathogenesis [10]. The T-DNA region of the Ti plasmid is transferred to the plant nuclear DNA, which initiates the biosynthesis of plant hormones auxin and cytokinins via the *iaaM* and *iaaH*, and *ipt* pathways, respectively, which are absent from the host plant (Figure 1). This results in uncontrolled cell proliferation and subsequently crown gall tumors [10].

Another intriguing example is that of *Pseudomonas syringae*, a bacterial plant pathogen that manipulates hormone signaling as a means of breaking the host plant defense [8,11]. It gains entry into the tomato plant by producing the phytotoxin coronatine, a molecular mimic of the plant hormone jasmonic acid-isoleucine, resulting in the bacterial speck disease [7,12,13]. *P. syringae* also synthesizes the auxin indole-3-acetic acid (IAA), a common growth hormone synthesized by plants for cell enlargement, division, and differentiation and uses it to establish itself in its plant host [11,14]. IAA can be synthesized in bacteria from tryptophan as a precursor via five distinct routes, and also via tryptophan-independent pathways which mostly yet remain to be characterized in detail [15–18] (Figure 1).

Three of the tryptophan-dependent pathways result in the formation of a common intermediate indole-3-acetaldehyde (IAAld) which is oxidized by an aldehyde dehydrogenase (ALDH) to IAA. ALDHs are housekeeping enzymes that play a major role in the detoxification of reactive aldehydes by converting them into their corresponding carboxylic acid [20–23]. ALDHs are known to participate in important functions such as polyamine catabolism [24], ethanol metabolism [25,26], xenobiotic metabolism [27–29], and plant cell wall ester synthesis [30,31], yet, many ALDH-catalyzed reactions remain to be characterized in detail in bacteria.

In 2018, the Kunkel laboratory reported the identification of six putative ALDHs in the pathogenic *P. syringae* pv. tomato strain DC3000 [11]. Biochemical analysis of these six homologs revealed that AldA, AldB, and AldC were capable of producing IAA from IAAld. Further, a high-resolution crystal structure of wildtype AldA was obtained. Additionally, 42 classes of genes encoding putative ALDHs in various *Pseudomonas* species have been recently reported, expanding the scope of this family of enzymes in bacterial metabolism [23]. The role of AldA was established to be a nicotinamide adenine dinucleotide (NAD^+)-dependent IAAld dehydrogenase that produces IAA [11]. In 2020, biochemical and structural characterization of AldC by Lee et al. revealed it to be a long-chain aliphatic ALDH [32]. IAA produced by *P. syringae* has been reported to promote the virulence in *Arabidopsis thaliana* by two different mechanisms—one, it up-regulates the virulence gene expression in the bacterium, and second, it suppresses salicylic acid-mediated plant defenses by activating auxin signaling in the host plant (via the TIR1/AFB auxin co-receptor system) [8,11]. Studies with *tir1/afb* mutants show accumulation of IAA in *A. thaliana*, resulting in the deregulation of plant host signaling. This results in increased host susceptibility to the pathogen's entry [8] (Figure 2). Targeting the arsenal of enzymes that *P. syringae* uses for infection is one the strategies to suppress its pathogenicity. To this end, Zhang et al. have recently reported a detailed study of the enzyme AldA to provide a glimpse into its molecular mechanism of action [33].

The reaction mechanism of AldA can be broadly described in two parts. First, an active site catalytic residue Cys[302] forms a thiohemiacetal intermediate with the IAAld which is followed by a hydride transfer from the aldehyde to the NAD^+ cofactor resulting in a thioacyl-enzyme intermediate. Second, the active site residue Glu[267] activates a water molecule for the hydrolysis of the intermediate resulting in the release of the carboxylic acid product and the reduced cofactor NADH [11]. Zhang et al. created mutants of these two catalytic residues and other substrate-binding site residues to study the mechanism in detail using kinetic and structural tools [33].

The activity of the wildtype and mutants of AldA were tested with IAAld which is the physiological substrate. As expected, enzyme assays using the AldA catalytic residue Cys[302] and Glu[267] mutants (Cys[302]Ala, Glu[267]Gln, Glu[267]Ala) showed no detectable activity. Noticeably, mutations in the aliphatic and aromatic residues lining the
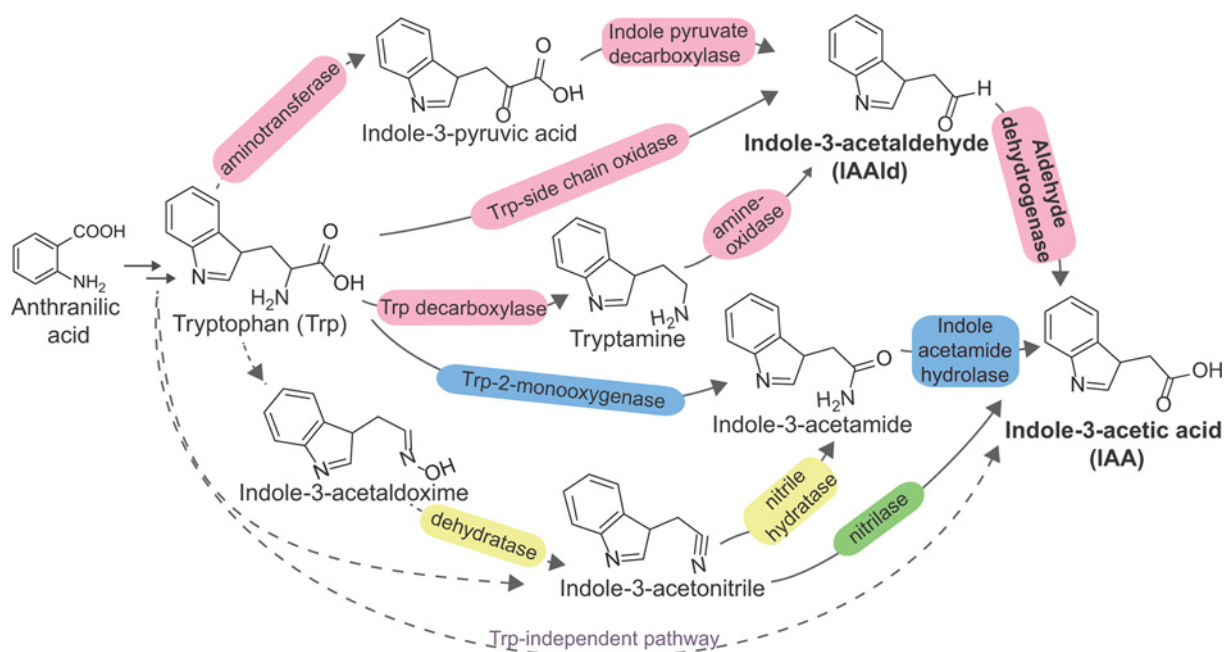
Figure 1. Bacterial biosynthesis of auxin

Certain plant-associated bacteria can produce auxin indole-3-acetic acid (IAA) from tryptophan (Trp) as a precursor using one or more than one of the routes shown here. Among the known Trp-dependent pathways, three routes involving (i) aminotransferase and indole-3-pyruvate decarboxylase, (ii) Trp-side chain oxidase, and (iii) Trp deacarboxylase and an amine-oxidase converge at indole-3-acetaldehyde (IAAld) which is then oxidized by aldehyde dehydrogenase (ALDH) to yield IAA *(enzymes shown in pink)*. Another prominent route involves the intermediate indole-3-acetamide which can be synthesized directly from tryptophan by tryptophan 2-monooxygenase (*iaaM*), and then converted into IAA via the enzyme indole acetamide hydrolase (*iaaH*) *(enzymes shown in blue)*. Alternately, indole-3-acetamide is synthesized via a two-step reaction involving a predicted dehydratase and nitrile hydratase *(enzymes shown in yellow)* followed by conversion into IAA as mentioned previously. Finally, some bacteria appear to use the lesser studied Trp-independent routes, though the precursors appear to be derived from Trp biosynthesis intermediates [16,17]. For example, indole-3-acetonitrile, derived from a precursor of Trp yield IAA in a single-step reaction catalyzed by the nitrilase enzyme *(enzyme shown in green)*. The reactions for which genetic and biochemical evidence of the enzymes involved are yet to discovered are shown with dashed arrows. *Pseudomonas syringae* DC3000 genome possesses genes for amine oxidase, nitrilase, indole acetamide hydrolase, ALDH, and a putative monooxygenase [19]. The enzymes shown with dashed arrows have some biochemical evidence however, the encoding genes are yet unknown. Figure adapted from Spaepen and Vanderleyden (2011) [15] and Duca et al. (2014) [18].

substrate-binding site of AldA were especially detrimental to the binding of IAA and lowered the catalytic efficiency significantly. These mutants were also tested for activity with octanal, the preferred substrate of AldC. Interestingly, the substitution of Phe^{169} with Trp (the corresponding residue found in AldC at that position) resulted in a higher turnover with octanal as compared with IAAld, thus leading to a reversal in the substrate preference and catalytic efficiency of the AldA Phe^{169}Trp mutant [33]. Such a shift in the preference for chemically distinct substrates arising from a single point mutation indicates that a small number of precise changes in the active site are likely responsible for the extensive biochemical scope of enzymes in the ALDH enzyme superfamily.

An interesting feature observed in the mechanism of ALDH enzymes is cofactor isomerization wherein after the hydride transfer, the nicotinamide ring of the NAD(H) cofactor tilts away from the catalytic cysteine and glutamate residues [35,36]. Zhang et al., captured the two orientations of the cofactor NAD$^+$ in their crystal structure analysis of AldA [33]. The 'extended' conformation where the nicotinamide half of the NAD$^+$ cofactor is buried in the active site, with the nicotinamide in close proximity to Cys^{302} and Glu^{267}, and the ribose part forming hydrogen bonds with another active site residue Glu^{401} was found in the wildtype AldA enzyme. In contrast, a 'contracted' conformation where the NAD$^+$ nicotinamide ring is pulled away from the catalytic site and the nicotinamide-ribose orients towards an active site residue Gln^{349} was found in the catalytically dead AldA Cys^{302}Ala mutant. This movement allows each half-reaction to occur efficiently, the hydride transfer part followed by the final hydrolysis of the enzyme–product
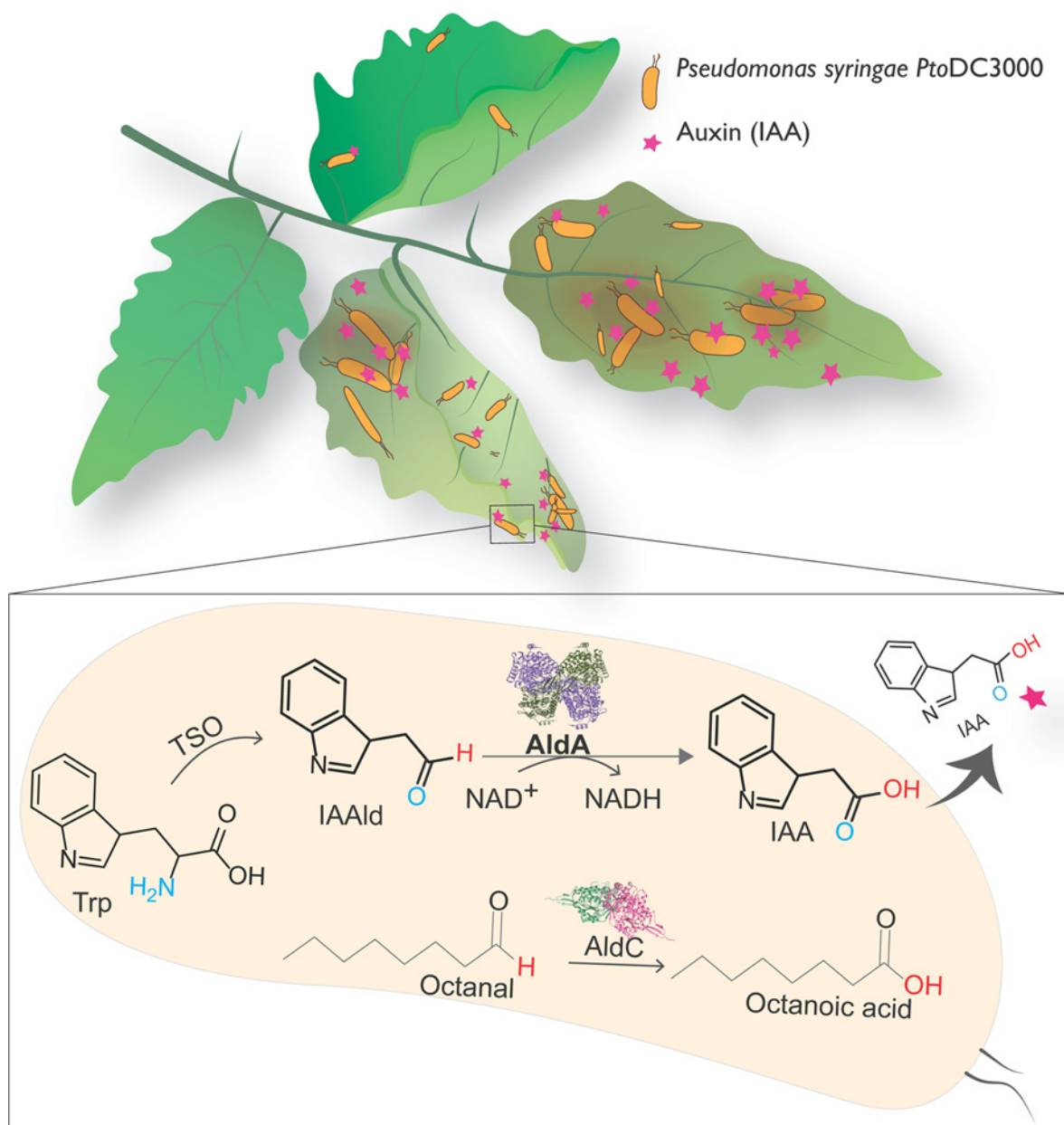
Figure 2. Plant pathogen *P. syringae pv. tomato strain* DC3000 synthesizes the auxin IAA, a plant growth hormone that facilitates its entry into the growing plant, and causing wilting and discoloration of the infected parts

AldA is an IAAld dehydrogenase in *P. syringae* DC3000 which catalyzes the oxidation of IAAld to IAA coupled with reduction of NAD$^+$ cofactor. Please refer to Figure 1from Zhang et al. (2020) [33] for the detailed mechanism. AldC, another ALDH found in *P. syringae* oxidizes a range of long-chain aliphatic aldehydes such as octanal to the corresponding acids. Such aliphatic molecules have been hypothesized to assist microbes in gaining entry into the host cell and also to act as nutrients for their proliferation [32,34].

complex which marks the completion of the reaction. A comparison of these two structures provides a glimpse into the dynamic interactions of the AldA active site residues with NAD(H) through the course of reaction, and highlights the molecular significance of these interactions in the mechanism of ALDH.

This series of investigations from the Kunkel and Jez labs on *P. syringae* ALDHs have opened several avenues in the study of bacterial IAA biosynthesis and their role in plant–microbe interactions. For example, the three characterized ALDH homologs AldA, AldB, and AldC were found via bioinformatics-based searches and share sufficient sequence

identity [11]. However, their biochemical characterization show that they have different substrate preferences which would lead to them playing distinct functional roles in the cells. Obtaining a molecular level picture of the differences between the ALDH homologs via detailed biochemical characterization with the enzymes and their mutants would contribute to a bigger picture understanding of their function and physiological roles in *P. syringae* pathogenesis. Further, in addition to ALDHs that synthesize IAA from IAAld, the *P. synringae* DC3000 genome also possesses genes to convert tryptamine, indole-3-acetamide, and indole-3-acetonitrile into IAA [19] (Figure 1). Additionally, a homolog of lysine monooxygenase has been hypothesized to synthesize indole-3-acetamide from tryptophan [11]. Functional characterization of these gene products are required to understand the flux of tryptophan to produce IAA and validate all the operating pathways for auxin biosynthesis in the bacterium. Given the agro-biotechnological and ecological importance of *P. syringae* [37,38], mechanistic enzymology studies such as these are important steps for in-depth understanding of host–microbe interaction and to keep its pathogenicity in check.

Conclusion

IAA plays a vital role in plant growth, and doubles up as an agent that regulates bacterial virulence, thus aiding the entry of *P. syringae* and other microbial plant pathogens into plants. Specifically inhibiting IAA biosynthesis in pathogenic bacteria may therefore act as an effective strategy to reduce widespread microbial infections in plants. Through the characterization and detailed mechanistic study of *P. syringae* AldA, the Jez and Kunkel laboratories took a step in this direction [11,33]. The unique NAD(H) cofactor isomerization that occurs in ALDH enzymes (and also seen in the *P. syringae* AldA structures in this work) is especially attractive—analyzing this step not only broadens our basic understanding of how NAD(H) functions, but also provides a prospective target for inhibiting the reaction at the halfway point and inactivating the enzyme. Finally, the ALDH family appears to accommodate a variety of aldehyde substrates [21–23,25,39–41]. Analysis of the active site 'aromatic box' in AldA allowed for a single amino acid substitution that altered the substrate preference from an indole-based substrate (aromatic) to an octanal (aliphatic) with higher catalytic efficiency [33]. This opens up the possibility of directed evolution and enzyme engineering of the ALDH superfamily members to design enzymes that can aid in the detoxification of a wide range of aldehyde substrates in environmental, health, and industrial applications.

Abbreviations
ALDH, aldehyde dehydrogenase; IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; NAD^+, nicotinamide adenine dinucleotide; T-DNA, transfer-DNA; Ti, tumor-inducing.

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