The Essence of Nature Can be the Simplest (2)−− Self-Inhibition and Boundary Formation: Keeping a Safe Distance from Extracellular Fenton Reactions

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Abstract
The formation of boundaries, or inhibition zones, between neighboring colonies is a common phenomenon in nature, occurring not only between different species but also within the same species or even within a single colony. Our findings revealed that extracellular Fenton reactions play a crucial role in boundary formation both within and across strains and species. PKS-derived metabolites promote the formation of self-inhibition zones, while NRPS-derived metabolites inhibit these zones. This aligns with the fact that PKS-derived polycyclic aromatic metabolites initiate and enhance extracellular Fenton reactions, while NRPS-derived iron chelators or siderophores elevate extracellular Fenton reactions. The development of self-inhibition zones and boundaries can be influenced by modifying media components, such as proteins and starch, to regulate metabolic pathways. Experimental methods must be redesigned to study the true functions of compounds or metabolites—not merely to assess their antibiotic properties, but to determine whether they are involved in extracellular Fenton reactions and self-protection mechanisms.

Key words: Self-inhibition, boundary formation, extracellular Fenton reactions, PKS, NRPS, iron chelation, antibiotics

Introduction

The formation of boundaries, or inhibition zones, between neighboring colonies is a common phenomenon in nature, occurring not only between different species but also within the same species or even within a single colony.[1,2] This phenomenon is a key feature of inhibitory interactions both within and between species, and it has long been regarded as a fundamental and conserved mechanism for population control. Such interactions limit the growth of both non-self and self-colonies. Given that most microorganisms constantly compete for resources, interspecific inhibition is understandable. However, the reasons behind self-inhibition and the widespread occurrence of this mechanism remain intriguing.

It is now widely accepted that inhibition within and between strains and species results from multiple compounds, or from a single type of compounds produced by various organisms utilizing a shared biosynthetic pathway.[3,4] However, previous research has indicated that many boundary-forming bacteria, including *Escherichia coli*, one of the most extensively studied organisms, do not produce broad-spectrum antibiotic compounds. Instead, *Escherichia coli* produces bacteriocins and microcins, which have a narrow spectrum of activity.[3,4] To date, the specific compounds responsible for creating inhibition zones that affect a wide range of strains and species—including the host producing these compounds—remain unclear.

Rationale

Our recent paper reported that the Warburg effect, a hallmark of aerobic glycolysis, is a universal phenomenon in nature. Many organisms can carry out extracellular Fenton reactions that consume large amounts of oxygen, with minimal intracellular oxygen consumption during glycolysis. The development of extracellular Fenton reactions can be categorized into two phases: rapid eruption and slow formation.[5] The rapid extracellular Fenton reactions predominantly occur in microbes and plants containing key biosynthetic genes encoding polyketide synthase (PKS), nonribosomal peptide synthase (NRPS), and terpenoid synthase (TPS). PKS- or TPS-derived aromatic metabolites can initiate and regulate these reactions, while NRPS-derived siderophores sequester iron near the cell wall, protecting the host from the harmful effects of extracellular Fenton reactions. Because these reactions degrade organic matter indiscriminately, much like fire, it is likely an organism's instinctive self-protective response to maintain a safe distance from them. We hypothesize that extracellular Fenton reactions may play a role in the formation of self-inhibition zones and boundaries in organisms.

Results

1. Extracellular Fenton reactions inhibited fungal colony growth

Our research revealed that the thermophilic fungus *Thermomyces dupontii* accumulates anthraquinones under cold stress, which induce extracellular Fenton reactions. Intriguingly, the loss of anthraquinones in the mutant strain Δ*An* resulted in the loss of its ability to perform extracellular Fenton reactions.[6,7] On potato dextrose agar (PDA) medium, the Δ*An* mutant exhibited significantly larger colonies than the wild type (WT) (Fig. 1), suggesting that exogenous anthraquinones from the WT did not inhibit the colony growth of Δ*An*, and that the absence of anthraquinones enhanced fungal colony growth. This implies that the extracellular Fenton reactions in the WT slowed down the rate of fungal growth. Since *T. dupontii* possesses only five PKS-related genes—*An*, *PK12*, *PK18*, *PK25*, and *PK31*—we further compared the

colony growth of the other four mutants with that of WT (Fig. 2). Notably, these mutants also exhibited significantly larger colonies than WT. Transcriptional and metabolic analyses showed that the transcription of the *An* gene, responsible for anthraquinone synthesis, was significantly downregulated in all four mutants, and the content of the major anthraquinone was also notably reduced (Figs. 3A-3E). Our recent work demonstrated that *T. dupontii* utilizes NRPS-derived talathermophilins (TTPs) for iron chelation. The deletion of TTPs in *T. dupontii* led to a significant upregulation of *An* gene transcription in the mutant Δ*TTP*, resulting in a marked increase in anthraquinone levels (Figs. 3F-3G).[8,9] Comparison of the colony growth of Δ*TTP* and WT revealed that the Δ*TTP* mutant exhibited smaller colonies than the WT. Collectively, these results suggest that anthraquinone-mediated extracellular Fenton reactions inhibit fungal colony growth.

2. Extracellular Fenton reactions play a key role in boundary formation

Remarkably, a clear self-inhibition zone was observed between the WT colonies, whereas an obvious fusion zone, rather than a self-inhibition zone, appeared at the intersection of the Δ*An* mutant colonies (Fig. 1). Furthermore, the inhibition zones at the interaction between the Δ*An* mutant and WT colonies were significantly smaller than the self-inhibition zone of WT but larger than the self-inhibition zone of the Δ*An* mutant. Similarly, the other four mutants—Δ*PK12*, Δ*PK18*, Δ*PK25*, and Δ*PK31*, which produced fewer anthraquinones than the WT—displayed similar phenomena to those observed in the Δ*An* mutant (Fig. 2). In contrast, the Δ*TTP* mutant, which produced more anthraquinones than WT,[6,9] exhibited results opposite to those of the Δ*An* mutant (Fig. 2). Collectively, these results suggest that the extracellular Fenton reaction is responsible for self-inhibition zone and boundary formation, rather than the previously assumed unknown antibiotics. This finding provides an explanation for the widespread occurrence of boundary formation within and between strains and species in nature.

3. Boundary formation depended on different media

The results indicated that all *PKS* genes were involved in boundary formation, while the *NRPS* gene, responsible for iron chelation, played a role in inhibiting boundary formation. Previous studies have reported that starch and sugar preferentially induce the biosynthesis of PKS-derived metabolites, while amino acids and proteins promote the biosynthesis of NRPS-derived metabolites.[10,11] Two types of media, PDA and TYGA, were widely used to evaluate fungal colony growth. The TYGA medium consisted of 10 g/L tryptone, 5 g/L yeast extract, 5 g/L molasses, 10 g/L glucose, and 18 g/L agar. The common YMA medium, which lacked tryptone, contained 2 g/L yeast extract, 10 g/L malt extract, and 18 g/L agar. Given that disruption of TPP biosynthesis enhanced boundary formation in *T. dupontii* fungal colonies, four mutants—Δ*PT1*, Δ*PT2-c1*, Δ*PT2-hA*, and Δ*PT2-c3l*—each with a disruption in one of the two genes encoding prenyltransferases (PT1 and PT2) involved in TPP biosynthesis,[9] were evaluated for boundary formation on three different media: PDA, TYGA, and YMA. Notably, boundary formation was consistently visible on both PDA and YMA throughout the test period (Fig. 4), with the boundary on YMA being slightly larger than that on PDA. However, on TYGA, the boundary formation was significantly reduced compared to both PDA and YMA, eventually disappearing altogether (Fig. 4), indicating that tryptone in the medium inhibits boundary formation.

Perspectives

1. The major factors in self-inhibition and boundary formation

We found that all PKS-derived metabolites promote the formation of self-inhibition zones, while NRPS-derived metabolites inhibit these zones. This aligns with the fact that PKS-derived polycyclic aromatic metabolites initiate and enhance extracellular Fenton reactions,[5,12] while NRPS-derived iron chelators or siderophores elevate extracellular Fenton reactions.[5] The development of self-inhibition zones and boundaries can be influenced by modifying media components, such as proteins and starch, to regulate metabolic pathways. Additionally, cold stress is a key factor in inducing extracellular Fenton reactions. We hypothesize that cold stress enhances self-inhibition zone and boundary formation in organisms by increasing extracellular Fenton reactions, thereby slowing colony growth. This concept may provide new insights into the reduced colony growth of organisms under cold stress.

2. The functions of natural products in extracellular Fenton reactions

We propose that the self-inhibition zone in organisms serves as a phenotypic indicator of extracellular Fenton reactions, which helps explain the absence of commonly identified antibiotic products. In fact, extracellular Fenton reactions play a crucial role in boundary formation both within and across strains and species. Experimental methods must be redesigned to study the true functions of compounds or metabolites—not merely to assess their antibiotic properties, but to determine whether they are involved in extracellular Fenton reactions and self-protection mechanisms.

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Competing interests
The author declares no competing interests.

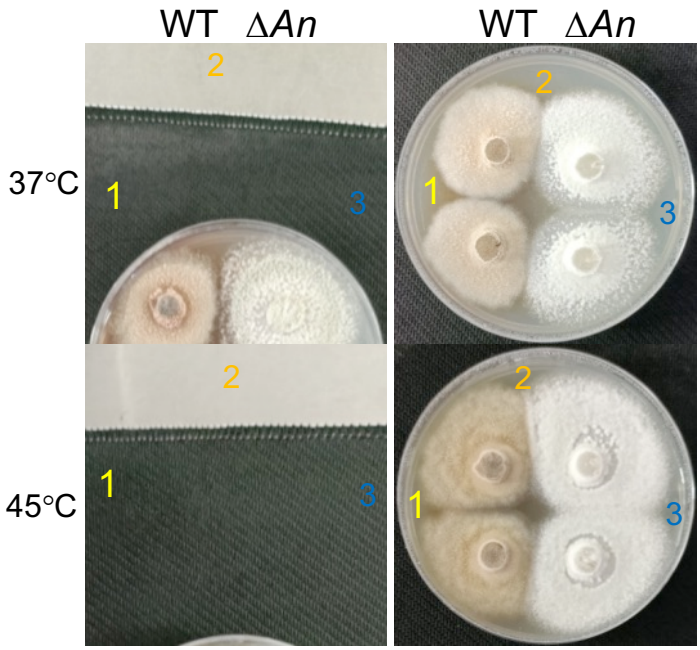

Figure 1. The formation of boundary (also known as inhibition zone) between neighboring colonies is due to their extracellular Fenton reactions, which inhibited the fungal colony growth of *Thermomyces dupontii* WT. The mutant *ΔAn* lost the ability to perform extracellular Fenton reactions while the WT strain can perform extracellular Fenton reactions. 1–3: Boundary formation between two WT colonies (1), between WT and *Δan* colonies (2); and between two *ΔAn* colonies (3). The width: 1>2>3.

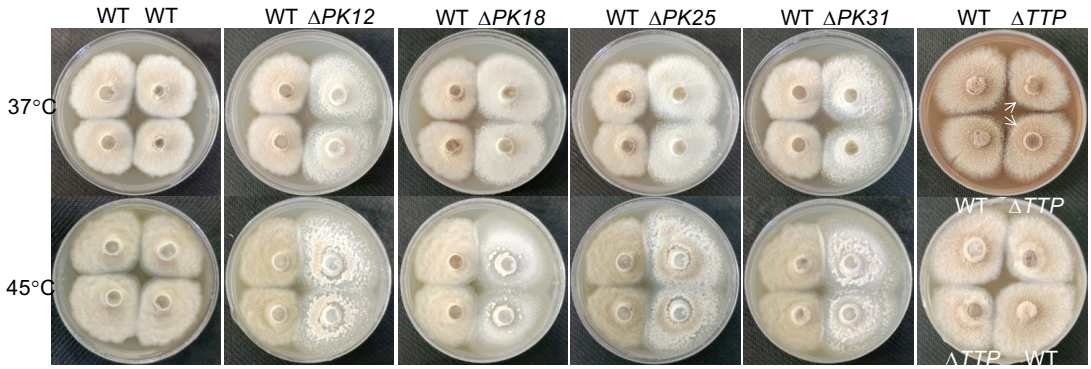

Figure 2. Comparison of the colony growths and self-inhibition zones of five mutants of *T. dupontii* with WT. Four mutants Δ*PK12*, Δ*PK18*, Δ*PK25*, and Δ*PK31*displayed significantly increased colonies compared with WT while mutant Δ*TPP* exhibited slightly decreased colony. In the other hand, four mutants Δ*PK12*, Δ*PK18*, Δ*PK25*, and Δ*PK31*displayed significantly decreased self-inhibition zones compared with WT while mutant Δ*TPP* exhibited slightly increased self-inhibition zones.

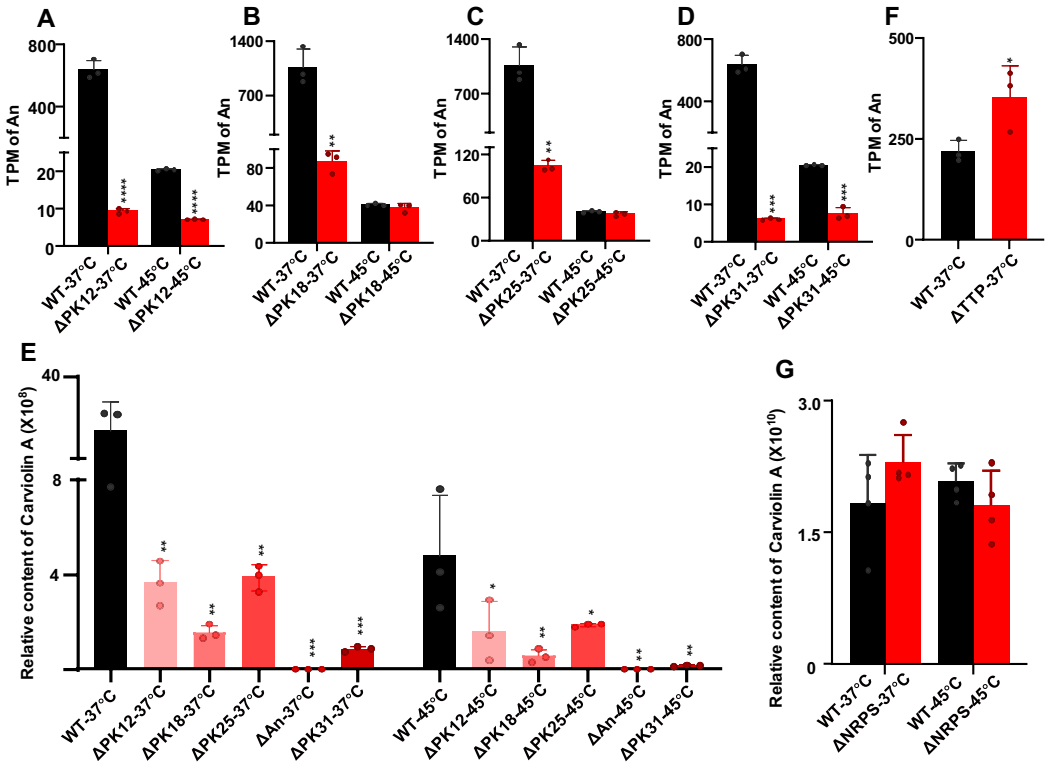

Figure 3. The effects of *PKS* genes and NRPS gene on the transcriptional level of the gene *an* and the contents of the anthraquinones in the fungus *T. dupontii*. in five mutants four mutants included Δ*PT1*, Δ*PT2-c1*, Δ*PT2-hA*, and Δ*PT2-c3l*, with disruption of two genes encoding two prenyltransferases (PT1 and PT2) responsible for TPP biosynthesis. The boundary formation on TYGA (in blue) is strongly decreased compared with those on PDA and YMA.

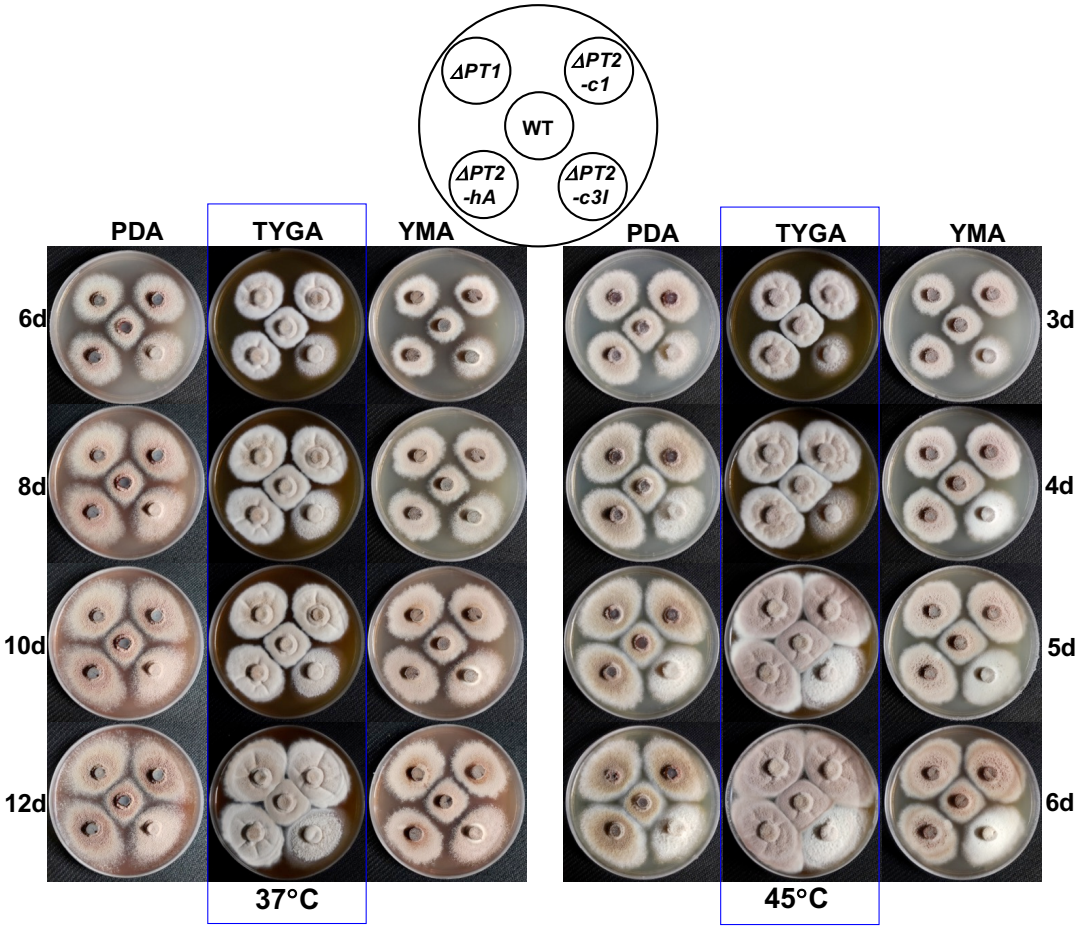

Figure 4. Comparison of the boundary formation of four mutants of *T. dupontii* with WT on three different media PDA, TYGA and YMA. Four mutants included Δ*PT1*, Δ*PT2-c1*, Δ*PT2-hA*, and Δ*PT2-c3l*, with disruption of two genes encoding two prenyltransferases (PT1 and PT2) responsible for TPP biosynthesis. The boundary formation on TYGA (in blue) is strongly decreased compared with those on PDA and YMA.