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Peer Review

Review of: "Multinuclear Non-Heme Iron-Dependent Oxidative Enzymes: Landscape of Their Substrates, Partner Proteins"

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The article is titled **Multinuclear Non-Heme Iron-Dependent Oxidative Enzymes: Landscape of Their Substrates, Partner Proteins"** and is a study on the subject of the MNIO enzyme superfamily and its associated ribosomally-synthesized post-translationally modified peptides (RiPPs). It considerably increases the repertoire of MNIO substrates and partner proteins, and biosynthetic gene clusters (BGCs) are suggested. Novel types of precursor families and mechanistic associations between the presence of signal peptides and partner protein subtype are proposed. The article is well-analytical, highly methodologically rigorous, and highly novel in the research of natural product biosynthesis. It is an important asset for further functional and biochemical research of metal-responsive systems of peptides.

1. Large-Scale Genome Mining and Dataset Integration

One of the most rigorous aspects of the methodology is the **comprehensive genome mining strategy** applied across multiple databases (NCBI nr protein database, assembled bacterial genomes, RefSeq, GenBank, and WGS datasets). The inclusion of tens of thousands of genomes and approximately 14,000 MNIO enzyme sequences substantially strengthens statistical reliability and evolutionary inference.
This large dataset minimizes sampling bias and allows the authors to identify conserved biosynthetic gene cluster architectures with high confidence.

2. Multi-Layered Bioinformatic Validation Strategy

The study employs several complementary computational approaches, including:

Sequence Similarity Network (SSN) analysis

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Hidden Markov Model (HMM) construction and refinement

RODEO-type gene cluster mining

Domain architecture profiling

Co-occurrence genomic analysis

The integration of multiple orthogonal methods increases the robustness of precursor identification and reduces false-positive functional assignments. The iterative process of building new HMM signatures and validating them through SSN clustering is particularly rigorous.

3. Structural Prediction Integration Using AlphaFold and HHpred

The use of structural prediction tools adds an important mechanistic dimension to the study. By combining AlphaFold2 structural modeling with HHpred and DALI structural homology analyses, the authors provide structural support for functional hypotheses, particularly regarding partner protein classification and substrate recognition mechanisms.

This approach significantly enhances confidence in the proposed classification of new MNIO partner protein families.

4. Systematic Biosynthetic Gene Cluster Contextualization

The detailed examination of gene organization surrounding MNIO enzymes represents another strong methodological component. By analyzing conserved gene order, operon structure, and domain co-occurrence, the authors provide convincing genomic evidence linking MNIO enzymes with specific precursor families and partner proteins.

This context-based validation is critical for accurate RiPP pathway prediction.

Most Beneficial Novel Insights for Future Research

1. Discovery of New MNIO Substrate Diversity

The identification of multiple previously uncharacterized precursor families, including potential protein-sized substrates, significantly expands the known scope of MNIO enzyme activity. This suggests that MNIO-dependent modification systems may function beyond classical RiPP biosynthesis, opening new avenues for natural product discovery and enzymology research.

2. Identification of PME-Type 2 Partner Proteins

The discovery of DUF2063-like partner proteins lacking canonical domain signatures is particularly impactful. This finding demonstrates that MNIO enzyme systems rely on a broader spectrum of accessory proteins than previously recognized.

This insight may reshape the understanding of enzyme-partner specificity and could guide future experimental work on substrate recognition and catalytic regulation.

3. Correlation Between Signal Peptide Presence and Partner Protein Type

The observation that MNIO enzymes associated with Sec signal peptide-containing precursors preferentially utilize PME-type 1 partners, while signal peptide-lacking substrates tend to associate with PME-type 2 partners, provides a novel mechanistic hypothesis.

This relationship may reveal previously unrecognized regulatory mechanisms controlling precursor processing and export timing.

4. Expansion of Metal-Stress Response Paradigms

The study provides strong genomic evidence suggesting that MNIO-modified peptides may participate in broader metal homeostasis beyond copper, including potential roles in mercury, calcium, oxidative, and sulfur stress responses.

This expands the ecological and physiological relevance of MNIO systems and suggests potential applications in antimicrobial and environmental microbiology research.

5. Establishment of a Bioinformatic Framework for Future RiPP Discovery

The combination of genome mining, HMM refinement, SSN clustering, and structural modeling creates a reproducible and scalable workflow. This framework can be applied to identify additional RiPP families and oxidative enzyme systems across diverse microbial taxa.

Declarations

Potential competing interests: No potential competing interests to declare.