Point of VIEW

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Secondary Metabolomics: Natural Products Mass Spectrometry Goes Global

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ABSTRACT A global LC–MS metabolite analysis of wild-type *Pseudomonas auerigunosa* and mutants targeting the natural product pyochelin revealed the production of previously unknown metabolites, the 2-alkyl-4,5-dihydrothiazole-4-carboxylates.

In a Letter in this issue of *ACS Chemical Biology*, Vinayavekhin and Saghatelian report adaptations of two metabolomic tools developed in the Suizdak and Cravatt laboratories to investigate global secondary metabolic changes in the organic soluble fraction of the wild-type (WT) *Pseudomonas aeruginosa* as compared to mutants of the biosynthetic gene cluster of the siderophore pyochelin (*pch*) (*1*). The first adapted tool is "discovery metabolic profiling" (DMP) (*2*). DMP is an untargeted LC–MS method for the detection of global metabolic differences between the WT form and an enzyme-inactivated form of an organism. The second tool is the XCMS algorithm (*3*), which can compare two or more LC–MS runs to identify hundreds of metabolites and quantify their relative amounts among all the samples. Saghatelian's "secondary metabolomics" approach can be applied in a versatile way to tackle current challenges in natural product research such as (i) the discovery of new natural products, (ii) the dissection of natural product biosynthesis, and (iii) the investigation of biosynthetic pathway cross-talk.

Utilizing their untargeted metabolomic approach, Vinayavekhin and Saghatelian detected 176 metabolites that are more abundant in the WT *P. aeruginosa* strains and 22 metabolites that were more abundant in the *pch* mutants, highlighting the true complexity of the metabolic effects caused by the *pch* mutations. Among the 176 elevated metabolites of the WT were pyochelin and three biosynthetic intermediates of the pyochelin pathway (Figure 1, panel A). The release of the pyochelin intermediates is mediated by the editing thioesterase PchC (*4*). The observed upregulation of intermediates in the WT and not in the *pch* mutants is due to the positive feedback regulation of all pyochelin biosynthetic enzymes by the end-product pyochelin. Furthermore, two additional metabolites were identified, characterized as 2-alkyl-4,5-dihydrothiazole-4-carboxylates (ATCs), whose levels were similarly regulated by iron and pyochelin. Vinayavekhin and Saghatelian ably demonstrated, *via* complementation and single *pch* gene overexpression studies with pyochelin mutants, that the ATCs are products of the pyochelin pathway.

Despite this interpretation that the ATCs are new functional (*i.e.*, iron-binding) natural products produced by the pyochelin gene cluster, it has not been shown that they are biologically functional. Because modern mass spectrometers have very high sensitivity, it is possible that Saghatelian captured off-pathway products without an essential cellular function. In normal pyochelin biosynthesis, the acyl-adenylate of salicylate is loaded onto the first thiolation domain of PchE. The resulting salicyl-*S*-PchE(T_1) then condenses with a cysteine loaded onto the second thiolation domain and then proceeds down the pyochelin biosynthetic pathway (Figure 1, panel B). However, one of the reasons many secondary

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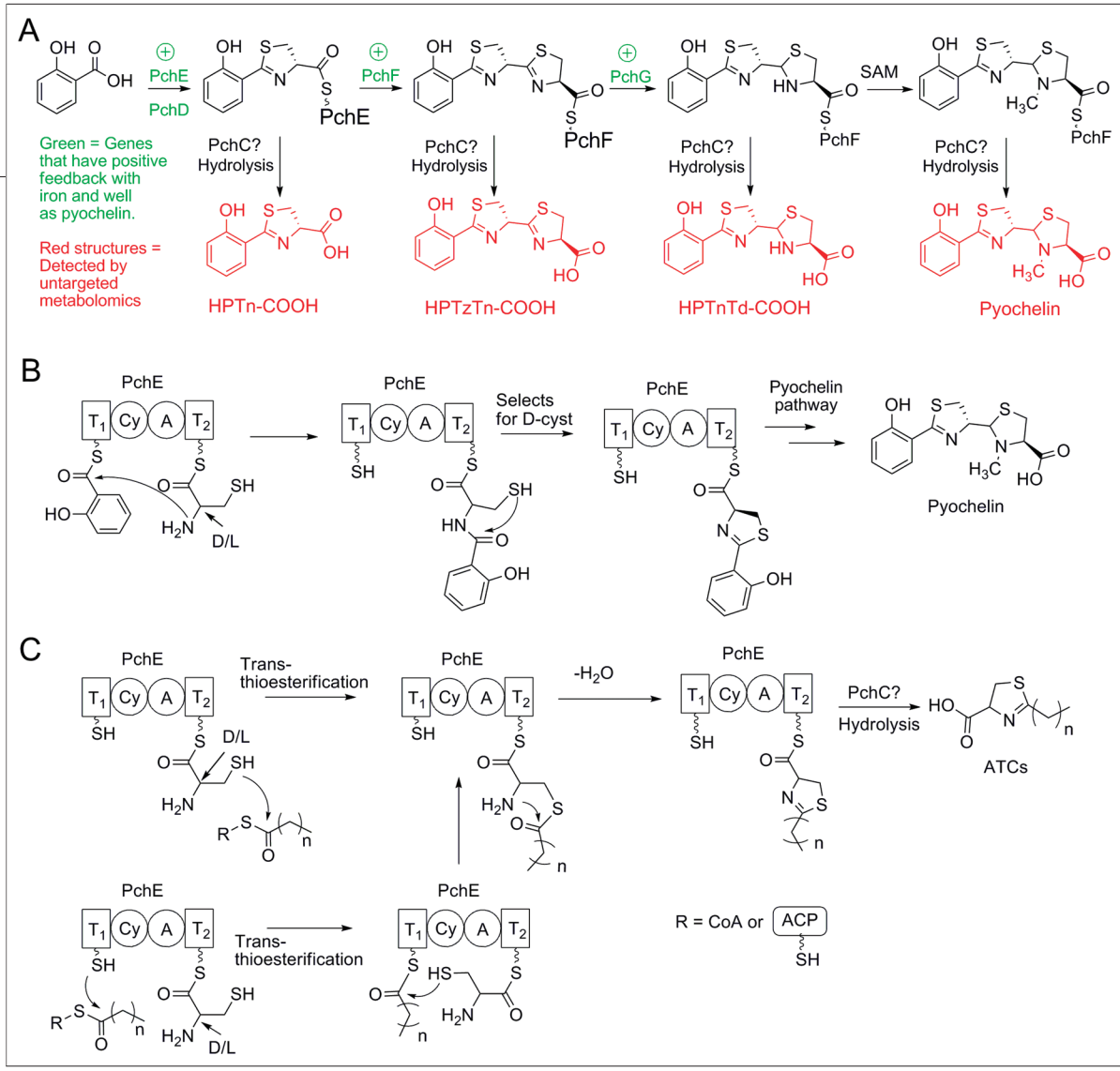

Figure 1. A) Pyochelin biosynthetic pathway. Annotated metabolites (red) and proteins regulated by positive feedback mechanisms (green) were characterized by untargeted metabolomics. Detected intermediates are putatively released by editing thioesterase PchC or hydrolysis. B) PchE biochemistry in pyochelin biosynthesis. Condensation of salicyl-*S*-PchE(T_1) with cysteinyl-*S*-PchE(T_2) and subsequent thiazoline formation yields pyochelin intermediate HPTn-*S*-PchE(T_2). C) The proposed role of PchE in the cross-talk with activated fatty acids in the biosynthesis of ACTs. Trans-thioesterification of an activated fatty acid with cysteinyl-*S*-PchE(T_2) side chain thiol and subsequent thiazoline formation yields ATC-*S*-PchE(T_2). Trans-thioesterification could occur directly to the cysteine thiol group or indirectly *via* PchE(T_1) PPant-thiol. ATCs are released from PchE *via* hydrolysis, a reaction that may be mediated by the editing thioesterase PchC.

biosynthetic pathways have editing thioesterase domains is to remove off-pathway reaction products. Therefore, ATCs may result from trans-thioesterification of activated fatty acids (*e.g.*, ACP- or CoA-bound) and cyclized with cysteine while attached to PchE. The release of the ATC would then be mediated by the editing function of the thioesterase PchC (Figure 1, panel C). Using a loose definition of the wording, the formation of a product between the fatty acid biosynthetic pathway and an activated cysteine on the pyochelin pathway may be considered a form of cross-talk between these two different pathways. To test this hypothesis, one could incubate phosphopantetheinylated-PchE with cysteine, ATP, and acyl-CoAs or acyl-*S*-ACPs, all of which may be present inside a cell at concentrations as high as 1 mM, and observe the formation of ATCs. Because of the sensitivity of Saghatelian's method to identify various types of unknown metabolites such as intermediates, pathway products, and pathway side-products, it is important to carefully analyze and confirm observed gene cluster-regulated compounds as active biosynthetic end-products. If ATCs are true biologically relevant iron binding molecules, they would, together with citrate, pyochelin, and pyoverdine, represent yet another metabolite secreted by *P. aeruginosa* to scavenge iron.

As shown in the paper by Saghatelian, the characterization of biosynthetic intermediates and products depends on the regulation of the pathway. When the pathway is regulated by a positive feedback mechanism, such as the pyochelin pathway in *P.*

aeruginosa, the biosynthetic intermediates can be characterized by gene overexpression and WT/KO metabolic analysis in a mutant strain with abolished end-product formation. By this approach, the ATCs from the pyochelin pathway were identified as products of the *pchE* gene product. When PchE was overexpressed in a *pchD* KO, ATC production was observed and its production was no longer subject to the positive feedback exerted by pyochelin. This finding supports the possibility that ATCs are off-pathway cross-talk products of cysteinyl-*S*-PchE and an activated fatty acid.

Comparative liquid chromatography–mass spectrometry (LC–MS) has been extensively used in natural product biosynthetic research. Most researchers apply this standard method for either the detection of known natural products, intermediates, and off-pathway products (*5*) or the discovery of new secondary metabolites produced by "orphan" or "cryptic" gene clusters from metabolic extracts of bacteria, fungi or other organisms (*6*). In a general comparative LC–MS experiment, the metabolic extract of a gene knockout (KO) is compared to the extract obtained from the WT organism. The comparison of LC or LC–MS traces from WT *versus* KO then enables the identification of the natural products produced by the gene cluster as was done with the recently characterized thiopeptide family of natural products (*7*–*9*). Herein, the metabolites regulated by the target gene cluster are identified as an abolished peak in the LC trace of the KO. However the natural biosynthetic scientist does not usually look at the changes of all the metabolites they can detect, especially when many of the metabolites that change are present in low concentrations.

Comprehensive mass spectrometry that quantifies, in a relative fashion, the metabolites observed in LC–MS traces can be a valuable method for researchers interested in natural product biosynthesis. Obvious applications of the global detection of secondary metabolic changes as a consequence to the generation of knockouts are the discovery of new natural products produced by known gene clusters, the discovery of natural products produced by orphan gene clusters, and the discovery of putative off-pathway reactions (*10*). Although Saghatelian's method has yet to be applied on an orphan gene cluster, a comparative LC–MS-based genome mining approach revealed three new natural products from an orphan type III PKS gene cluster from *Streptomyces coelicolor* (*6*).

However, perhaps just as important is the fact that a global view of metabolites may enable a deeper understanding of the physiological function of these metabolites. In addition, due to the very large number of metabolites that are compared, the secondary metabolomics approach may also aid in the dissection of secondary metabolic networks and provide deeper understanding of their global regulatory mechanisms *in vivo*. For example, the approach could be applied to the identification of global regulator genes and the engineering of strains with increased production of particular natural products. While the discovery of ATCs is one example, ultimately, a global view of the secondary metabolome, as described by Saghatelian, will yield valuable information for the fundamental understanding of global secondary metabolite production and secondary metabolite regulation in the genomic and proteomic context of bacteria or fungi.

REFERENCES

1. Vinayavekhin, N., and Saghatelian, A. (2009) Regulation of alkyl-dihydrothiazole-carboxylates (ATCs) by iron and the pyochelin gene cluster in *Pseudomonas aeruginosa*, *ACS Chem. Biol. 4*, 617–623.
2. Saghatelian, A., Trauger, S. A., Want, E. J., Hawkins, E. G., Siuzdak, G., and Cravatt, B. F. (2004) Assignment of endogenous substrates to enzymes by global metabolite profiling, *Biochemistry 43*, (45), 14332–14339.
3. Smith, C. A., Want, E. J., O'Maille, G., Abagyan, R., and Siuzdak, G. (2006) XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification, *Anal. Chem. 78*, (3), 779–787.
4. Reimmann, C., Patel, H. M., Walsh, C. T., and Haas, D. (2004) PchC thioesterase optimizes nonribosomal biosynthesis of the peptide siderophore pyochelin in *Pseudomonas aeruginosa*, *J. Bacteriol. 186*, (19), 6367–6373.
5. Li, X. B., Qiao, B., and Yuan, Y. J. (2006) Differential analysis of secondary metabolites by LC-MS following strain improvement of *Streptomyces lydicus* AS 4.2501, *Biotechnol. Appl. Biochem. 45*, (Pt 3), 107–118.
6. Song, L., Barona-Gomez, F., Corre, C., Xiang, L., Udwary, D. W., Austin, M. B., Noel, J. P., Moore, B. S., and Challis, G. L. (2006) Type III polyketide synthase β-ketoacyl-ACP starter unit and ethylmalonyl-CoA extender unit selectivity discovered by *Streptomyces coelicolor* genome mining, *J. Am. Chem. Soc. 128*, (46), 14754–14755.
7. Liao, R., Duan, L., Lei, C., Pan, H., Ding, Y., Zhang, Q., Chen, D., Shen, B., Yu, Y., and Liu, W. (2009) Thiopeptide biosynthesis featuring ribosomally synthesized precursor peptides and conserved post-translational modifications, *Chem. Biol. 16*, 141–147.
8. Wieland Brown, L. C., Acker, M. G., Clardy, J., Walsh, C. T., and Fischbach, M. A. (2009) Thirteen posttranslational modifications convert a 14-residue peptide into the antibiotic thiocillin, *Proc. Natl. Acad. Sci. U.S.A. 106*, (8), 2549–2553.
9. Kelly, W. L., Pan, L., and Li, C. (2009) Thiostrepton biosynthesis: prototype for a new family of bacteriocins, *J. Am. Chem. Soc. 131*, (12), 4327–4334.
10. Moldenhauer, J., Chen, X. H., Borriss, R., and Piel, J. (2007) Biosynthesis of the antibiotic bacillaene, the product of a giant polyketide synthase complex of the trans-AT family, *Angew. Chem., Int. Ed. 46*, (43), 8195–8197.