The medical treatment of infectious diseases often requires combination therapies that blend two molecules to enhance drug efficacy. Nature does the same. In a new article, Mrak et al. identify and functionally characterize natural products from *Streptomyces rapamycinicus* that show synergistic antifungal activity with the well-known immunosuppressant metabolite rapamycin, produced by the same strain. The genomic co-association of the two biosynthetic gene clusters paves the way toward new strategies to discover synergistic pairs of antibiotics through large-scale genome mining.

Combination therapy is a promising solution for treating immunocompromised patients, resistant infections, and cancer. Particularly interesting are compounds that act synergistically—having efficacy greater than the sum of their individual contributions. Synergism can occur directly, by binding to the same target, or indirectly, by causing cascading effects in a targeted pathway. The utility of synergism is evident in several well-documented streptogramin antibiotics, such as Synercid. Considering the potency of such combinations, it is perhaps unsurprising that similar examples of synergistic compounds are found in nature. Several studies have identified the coordinated production of totally distinct compounds from adjacent biosynthetic gene clusters (BGCs) that work together. The article by Mrak and colleagues (1) adds a new example of such a “supercluster” for the production of rapamycin and the actinoplanic acids (APLs). This successful discovery of synergistic activity also outlines a potentially impactful strategy for prioritization of unknown BGCs, and thus possible combinatorial drug treatments, via synergistic cluster mining.

The synergistic pairs found in nature thus far are quite diverse (Fig. 1). For example, *Streptomyces rochei* harbors a plasmid with two adjacent BGCs that encode the coordinated production of lankamycin and lankacidin, compounds shown to cooperatively attack the 50S ribosomal subunit (2). Likewise, the biosynthesis of members of several synergistic streptogramin antibiotic pairs, such as griseoviridin/viridogrisein, virginiamycin M and S, and pristinamycin I and II (3), is encoded in intertwined superclusters that produce each pair. Similarly, the BGC for the β-lactam antibiotic cephapramycin C is co-localized with the gene cluster encoding the biosynthesis of clavulanic acid, an inhibitor of β-lactamases that could otherwise confer resistance to this antibiotic (4). Examples also occur outside actinomycetes: A supercluster in *Bacillus* encodes the biosynthesis of the synergistic lipopeptides fengycin and bacillomycin D (5), one in *Pseudomonas* encodes the synergistic antibiotics muirinocin and jessenipeptin (6), and one in the fungus *Aspergillus* encodes the production of both fumagillin and pseurotin, although proof of synergistic action for this example is still pending (7).

The work of Mrak et al. (1) began by revisiting the compounds synthesized by the rapamycin producer *Streptomyces rapamycinicus*. The authors noticed two previously undetected compounds; isolation and structural elucidation indicated they were medically relevant APLs. Subsequently, genome re-sequencing and analysis led to the identification of a polyketide synthase (PKS) gene cluster with a domain architecture complementary to this structure. Experimental validation was carried out with in-frame deletions of several genes in the BGC, which revealed a single nonribosomal peptide synthetase (NRPS) module to be responsible for the incorporation of a rare tricarballylate moiety; comparison to the only other synthetic route to this moiety suggests an interesting case of convergent evolution. Further interrogation uncovered promiscuous domains and post-processing steps that resulted in a proposed complete biosynthetic pathway—the first for APLs.

The APL gene cluster was also found consistently co-localized with the rapamycin BGC in the genomes of *Streptomyces iranensis* and *Actinoplanes sp.* MA7066, the only other genome-sequenced species that are known to make rapamycin. This suggested to the authors a possible synergistic relationship between the compounds. To test for possible effects, an antifungal assay was chosen based on the known activities of rapamycin and actinoplanic acids against the eukaryotic TOR complex and Ras farnesyltransferase, respectively. The results showed strong synergism against three fungal genera using concentrations within the production capacity of *S. rapamycinicus*. Clear results from *Rhizopus oryzae* showed growth inhibition at 1 nM rapamycin with 2 μM APL, in contrast to only slight inhibition at 25 nM and no activity alone, respectively. Given that the TOR complex is integral to basic cellular processes and farnesylated GTPases of the Ras family are often a part of the upstream signaling pathways, it was concluded that total arrest in growth is most likely achieved by APL-enhanced rapamycin activity.

The authors noted that this example of co-associated products with synergistic potency highlights a new avenue that has
yet to be exploited in genome-mining efforts. Computational analysis of microbial genomes has led to the identification of thousands of BGCs of unknown function, which beg for meaningful ways of prioritization to exploit them for drug discovery. Indeed, prioritizing predicted BGCs for the identification of synergistic product pairs is likely to yield useful antibiotics, as the synergy also poses a hurdle for resistance development. Although existing tools for identifying BGCs and grouping them into families across species (8) could be exploited to systematically identify BGC co-associations, it will be important to discriminate possible synergies from co-association “by chance,” e.g., due to recent common ancestry of closely related strains.

Based on observations in known superclusters such as the rapamycin-APL case, we propose three criteria that could be leveraged. First, co-conservation across larger evolutionary time scales would distinguish real associations from associations due to recent common ancestry; this could be assessed through a statistical framework that takes into account the species phylogeny, and which offsets the degree of neutral sequence divergence and gene rearrangements against the level of co-association of pairs of BGCs within the same genomes (with a “bonus” for being located adjacently). Second, co-regulation of pairs of BGCs could be either predicted (through computational identification of transcription factor–binding sites) or assessed experimentally using transcriptome analysis. Third, the fact that synergistic molecules tackle the same target or related targets could be leveraged by employing approaches such as the antibiotic-resistant target seeker (ARTS) (9) pipeline to identify resistance genes co-associated with both BGCs. The latter would even make it possible to specifically pinpoint pairs that are likely to have novel mechanisms of action. Given the abundance of gene cluster families of unknown function that lie waiting in the riches of genomic data, this presents a promising new avenue for genome-based drug discovery.

Figure 1. Examples of co-localized BGCs that produce synergistic compounds. Mode of action/synergy are in italics with red arrows indicating core biosynthetic genes: griseoviridin-viridogrisein and lankacidin-lankamycin (a), clavulanic acid–aiding cephapycin C (b), lipopeptide jessenipeptin and mupirocin (c), and actinoplanic acid and rapamycin (d).

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