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Divergent functional roles of D-amino acids secreted by *Vibrio cholerae*

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Summary. The L-forms of amino acids are used in all kingdoms of life to synthesize proteins. However, the bacterium *Vibrio cholerae*, the causative agent of cholera, produces D-amino acids which are released to the environment at millimolar concentrations. We baptized these D-amino acids as non-canonical D-amino acids (NCDAAs) since they are different from those (i.e. D-alanine and D-glutamate) normally present in the bacterial cell wall. In *V. cholerae*, production of NCDAAs relies on the BsrV enzyme, a periplasmic broad spectrum racemase. BsrV multispecific activity, produces of a wide range of distinct D-amino acids. Using a combination of genetics and molecular physiology approaches we have demonstrated that NCDAAs target different cellular processes which may function as part of a cooperative strategy in vibrio communities to protect non-producing members from competing bacteria. Because NCDAA production is widespread in bacteria, we anticipate that NCDAAs are relevant modulators of microbial subpopulations in diverse ecosystems.

Keywords: *Vibrio cholerae* · D-amino acids · cell wall · D-methionine · D-arginine

Bacteria are terrific colonizers that can survive and progress in a wide range of environments due to their highly creative metabolisms and their rapid capacity to expand their populations. However, living in most niches requires their ability to socialize in complex communities both with friendly and not so friendly neighbours that range from animals, plants, fungi, protists, viruses to archaeas. In these environments, production of extracellular effectors has been largely appreciated as one of the classic strategies devised by bacteria to modulate the surrounding biodiversity by interfering with the growth and/or viability of nearby organisms [7]. One of such bacterial effectors only recognized in the last decade are D-amino acids, the enantiomeric forms of the (L)-amino acids that are the basic constituents of proteins in all kingdoms of life. Since the discovery that many phylogenetically unrelated bacteria secrete high levels of D-amino acids to the environment [6], many laboratories around the world have been intrigued by the biological and ecological impact of these effectors. Today we are just beginning to uncover a few of the seemingly many regulatory roles of D-amino acids in distinct aspects of bacterial physiology such as cell wall biogenesis, biofilm integrity, and spore germination [3].

Although scientists have appreciated D-amino acids for a long time, their major importance in biology has come fundamentally associated to their role as main constituents of the peptidoglycan cell wall, a bacteria specific structure that envelopes the cell and provides the morphology and resistance to the internal turgor pressure [8]. With very few exceptions, cell wall D-amino acids are normally D-alanine and D-glutamate, both produced by cytoplasmic highly specific racemases [5]. However, in 2009, we discovered that *Vibrio cholerae*, the etiological agent of the diarrheal disease cholera, produced millimolar concentrations of mainly D-methionine and D-leucine to the extracellular media [6]. Given that these D-amino acids were different from D-Ala and D-Glu, we called them non canonical D-amino acids (NCDAAs). Interestingly, both D-Met and D-Leu are produced by a single enzyme, a broad spectrum racemase (Bsr), which in *V. cholerae* is expressed

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under the control of the stress sigma factor RpoS [2, 6]. According to this, NCDAAs production occurs in stationary phase and is followed by their efficient incorporation into the peptidoglycan structure replacing the terminal D-Ala of the peptide moieties. Such NCDAAs editing slows down peptidoglycan biosynthesis thereby permitting coordination between cell wall synthesis and *V. cholerae* population expansion when resources scarce [2, 6].

A key moment in our research was when we characterized the biochemical and structural properties of BsrV [4]. We found that BsrV can racemize a great variety (>10) of amino acids, which was not consistent with the limited types of D-amino acids we had detected in the *V. cholerae* extracellular media (i.e. mainly D-Met and D-Leu). As often happens in science, the discovery of the release of D-Met and D-Leu was somehow fortuitous. D-Met and D-Leu were identified in stationary-phase supernatant active fractions that induced a rod-to-sphere morphological transition of a cell wall-sensitive mutant (i.e., *mrcA*) [6]. As expected from BsrV *in vitro* multispecificity, a non-biased chemical analysis revealed the accumulation of other D-amino acids (such as e.g. D-Arg) at high concentrations in the extracellular medium of *V. cholerae* [1]. Given that D-Arg was not detected in the fractions that induced *mrcA* to turn from rod-to-spherical shape, it raised a fundamental question: Do all D-amino acids have the same biological role?. In principle, this made very little sense in our opinion: why *V. cholerae* should be making different D-amino acids if all share a unique function?

To address these questions, we performed genetic screenings to uncover *V. cholerae* (naturally resistant to D-amino acids) mutants sensitive to D-Met or D-Arg and found that several mutants on cell wall associated genes were synthetically lethal in the presence of D-Met while, no conditional lethality was observed in the presence of D-Arg [1]. Despite D-Arg is totally innocuous to *V. cholerae*, this D-amino acid was a potent bactericide for many bacterial species. We used this as an advantage to obtain deeper insights on the potentially different mechanism of action of D-Arg. Suppressor mutations against D-Arg lethality in two independent bacterial species (*Caulobacter crescentus* and *Agrobacterium tumefaciens*) targeted the phosphate uptake system, buttressing the idea that D-amino acids must be considered as functionally non redundant environmental effectors [1].

D-Arg bactericidal activity on a great diversity of species makes this D-amino acid a powerful chemical weapon for *V. cholerae* against microbes inhabiting the same niche. However, production of NCDAAs is not widely conserved in the genus. The fact that virtually all vibrios are resistant to D-amino acids suggests that D-Arg could be used as an inter-species altruistic cooperation strategy to promote the expansion of vibrios population within challenging polymicrobial environments [1]. Indeed, vibrio species co-inhabit diverse marine and fresh water niches and thus can certainly benefit from the production of D-Arg.

The ability of some bacteria to efficiently generate suppressor mutations to overcome the deleterious effects of D-Arg might, at least to some extent, explain the production of distinct sets of NCDAAs to target different cellular processes and minimize the emergence of competing microbes. Whether the bactericidal activities of certain NCDAAs can be applied in combinatory antimicrobial therapies remains to be determined.

As there are diverse Bsr-producing bacterial species other than vibrios, we anticipate that D-Arg could govern additional microbial social interactions in other environments. The relative abundance of certain L-amino acids in a particular niche would also affect the final composition and amount of the secreted D-amino acids. Further research into the role of NCDAAs in other processes, such as signaling, development and metabolic interference would provide valuable mechanistic insights on the evolution of microbial ecosystems.

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