The FEBS Journal

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COMMENTARY

Exploring a potential Achilles heel of *Mycobacterium tuberculosis*: defining the ClpC1 interactome

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Keywords
AAA+ protease complex; adaptor proteins; antibiotic target; ClpCP; *Mycobacterium tuberculosis*; N-degrons; proteolysis

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(Received 27 April 2020, accepted 20 May 2020)

doi:10.1111/febs.15430

Comment on: https://doi.org/10.1111/febs.15335

Introduction

Protein degradation plays a vital role in the correct maintenance of a cell, not only under normal physiological conditions but also in response to stress. In bacteria, this crucial cellular task is performed by a dedicated group of ATP-dependent machines, termed ATPase associated with diverse cellular activities (AAA+) proteases. These machines are invariably composed of a hexameric AAA+ unfoldase (e.g. ClpC1) and a barrel-shaped peptidase (e.g. ClpP). The functional association of both complexes facilitates the ATP-dependent translocation of an unfolded substrate directly into the central cavity of the peptidase, where the translocated polypeptide is shredded into the short peptides [1].

These machines are not only important for the general removal of damaged proteins that accumulate on stress, but they can also directly influence the regulation of developmental and stress response pathways by controlling the activity or stability of key regulators. In many cases, they also control the equilibrium of cellular TA systems [2]. The complex and intricate involvement of these machines in various important and sometimes essential cellular processes is often dependent on the modulation of substrate recognition by the AAA+ protease, which is commonly mediated by specific adaptor proteins through docking to specialized accessory domains within the AAA+ protein [3,4]. In *Escherichia coli*, the adaptor protein ClpS recognizes N-degron (previously termed N-end rule) substrates and through docking to the N-terminal domain of ClpA targets them to ClpP for degradation [5,6]. In addition, ClpS also inhibits the turnover of selected substrates, including proteins labelled the SsrA tag as well as ClpA itself [7].

Consistent with a central role in bacterial physiology, the dysregulation of these machines by novel antibiotics [8–10] or hyperactivating mutations [11] is toxic to bacteria. This is acutely evident in the human pathogen *Mtb*, as ClpC1 is the target of four different anti-mycobacterial compounds [9,12,13] (Fig. 1). The precise mode of action, however, of these novel antibiotics remains poorly understood, and given each component of the ClpC1 protease (ClpC1, ClpP1 and ClpP2) is essential, the key *in vivo* and *in vitro*

Abbreviations
AAA+, ATPase associated with diverse cellular activities; BACTH, bacterial adenylate cyclase two-hybrid; Clp, caseinolytic protease; *E. coli*, *Escherichia coli*; *Mtb*, *Mycobacterium tuberculosis*; TA, toxin–antitoxin.

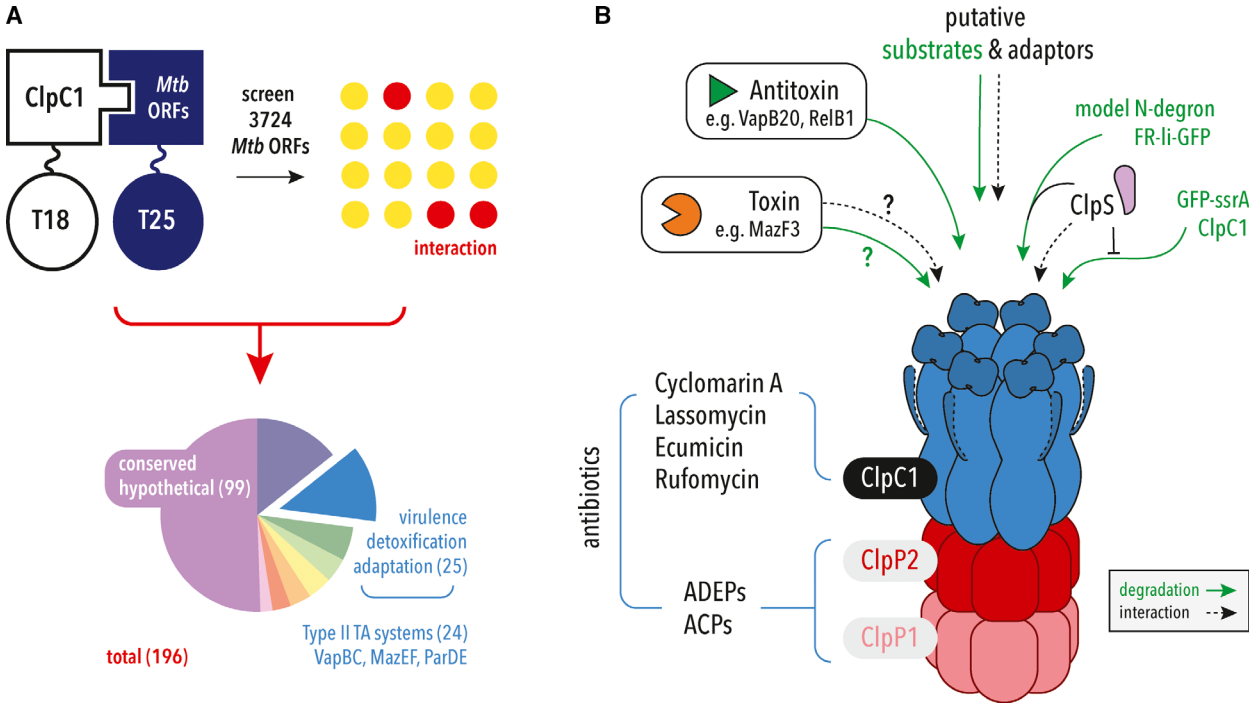

Fig. 1. (A) ClpC1-interacting proteins were identified using a BACTH screen, which is based on the co-expression of two subdomains (T18 and T25) of adenylate cyclase from *Bordetella pertussis*, one of which is fused to *Mtb* ClpC1 and the other to a library of 3724 *Mtb* gene products (representing ~ 90 % of annotated *Mtb* ORFs). Of the 196 ORFs identified to interact (in red) with ClpC1, 99 were conserved hypothetical proteins and 24 belonged to Type II TA systems. (B) The *Mtb* ClpCP protease (ClpC1/ClpP1P2) is a novel antibiotic target. Antibiotics that target either the ClpP1P2 peptidase (ADEPs and ACPs) or the ClpC1 unfoldase (Cyclomarin A, lassomycin, ecumicin and rufomycin) are known to dysregulate ClpCP activity. ClpC1 interacts both with putative substrates (green) and adaptors (black). In the absence of the adaptor ClpS, the ClpCP protease is responsible for the turnover (solid green line) of antitoxins (e.g. VapB20 and RelB1) and GFP-ssrA. The addition of ClpS inhibits the ClpCP-mediated turnover of GFP-ssrA but facilitates the degradation of a model N-degron substrate (FR-li-GFP). The consequence of toxin (i.e. MazF3) interaction with ClpC1 is currently unclear.

function(s) of this machine (including its physiological substrates) have been difficult to explore [14,15].

In a first step, to better understand the role of ClpC1 within *Mtb* and identify possible interacting partners (substrates and/or adaptors) of ClpC1, Ziemski *et al.* [16] performed a bacterial adenylate cyclase two-hybrid (BACTH) screen in *E. coli*. The screen identified 196 unique interaction partners, approximately half of which were 'conserved hypothetical' proteins (Fig. 1A). Of the remaining interaction partners, the second largest group (25 ORFs) belonged to the 'virulence, detoxification and adaptation' class of proteins, 24 of which were components of Type II TA systems. Given the abundance of TA systems in the *Mtb* genome (79 in total) and their link to stress response pathways, virulence and persistence [17], this group of interacting proteins was further characterized. Surprisingly, both antitoxins and toxins were identified as potential *Mtb* ClpC1-interacting proteins, although no members of the ribosome-dependent ribonuclease TA systems (i.e. RelBE and HigAB) were identified. To validate the identification of TA systems in their screen, Ziemski *et al.* examined the turnover of two TA systems. Consistent with the identification of VapB20 as a ClpC1-interacting protein, the antitoxin was degraded by ClpC1P1P2 *in vitro*, while both the cognate toxin and TA complex remained stable. Importantly, the turnover of VapB20 (and RelB—not identified in the screen) only occurred in the presence of ClpC1 (and not ClpX), indicating that ClpC1 likely plays a crucial role in activation of many Type II TA systems in *Mtb*. However, given RelB was not identified as a ClpC1-interacting protein, the ClpCP protease likely regulates the turnover of additional antitoxins (and possibly other ORFs). In contrast to the validated identification of antitoxins as ClpCP substrates, the significance of the 16 ClpC1-interacting toxins (i.e. VapC11) identified in the screen requires further examination. Are these toxins degraded or do they somehow act as regulated adaptors for the delivery of their cognate antitoxin?

Finally, Ziemski *et al.* employed the genetic screen to examine the interaction of ClpC1 (using the NTD)

with the putative adaptor protein *Mtb* ClpS (which was missing from *Mtb* ORF library). Consistent with the docking mechanism of *E. coli* ClpS to its cognate unfoldase ClpA [7,18], an interaction between the two proteins was observed. To validate this interaction, the team performed additional *in vitro* experiments using model substrates, confirming that *Mtb* ClpS was indeed responsible for the recognition of a model N-degron substrate [19] and showed for the first time that *Mtb* ClpS is essential for the turnover of a model N-degron substrate (FR-li-GFP) by ClpC1P1P2. Additionally, they showed that, similar to *E. coli* ClpS [6], *Mtb* ClpS also inhibited the *in vitro* turnover of a model C-degron substrate, GFP-ssrA and the auto-degradation of its cognate unfoldase.

Overall, the study identified ~ 200 ClpC1-interacting proteins, and although many of the interacting proteins remain to be validated, either as substrates or adaptor proteins and the physiological significance of these interacting proteins remains unknown, the study has laid the foundations to define the interactome of this essential protease [16]. Future studies, however, are necessary to fully characterize the remaining candidate proteins and their physiological significance, further defining the *Mtb* ClpC1 interactome to identify which, if any, of these interacting proteins are adaptor-regulated substrates such as N-degron substrates and which are novel ClpC1 adaptor or otherwise interacting proteins. Defining the adaptor and substrate protein network of this essential AAA+ protease is crucial for our understanding of this promising antibiotic target and control of this important human pathogen.

Acknowledgements

The work in the Lab of KT is supported by the Max Planck Society and the Deutsche Forschungsgemeinschaft.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

DAD, RA and KT wrote the commentary.

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