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ZINC UNDER CONTROL: A REVIEW OF PROTEINS RESPONSIBLE FOR Zn(II) HOMEOSTASIS IN BACTERIA AND FUNGI

The rapid emergence of antimicrobial resistance poses a significant challenge and represents a global threat to modern society. In order to create effective, and selective medicines, it is essential to understand the difference in human and pathogen metabolism. One of them is the mechanism of Zn(II) uptake. Pathogens have evolved mechanisms to acquire this essential metal ion from their host's environment, including the production of zincophores and zinc transporters.

Arms race: pathogens *vs* antibiotics

Bacteria were first identified in 1670 by Antoni van Leeuwenhoek **[1]**. At that time, they were not suspected of being pathogenic, and only in the 19th century did Joseph Lister put forward the theory that they caused diseases. In 1928, Alexander Fleming discovered the first antibiotic, penicillin **[2]** and marked the first milestones in the history of antibiotic research. Bacteria began to be isolated, cultivated and identified as potential disease agents or producers of bioactive metabolites. Thanks to new antibiotics, in the middle of the 20th century, many diseases that had reached epidemic proportions at that time were combat. However, it quickly turned out that bacteria are able to develop, acquire and spread numerous resistance mechanisms **[3]**. Consequently, the introduction of subsequent antibiotics, sooner or later, turned out to be ineffective and the 'golden era' of antibiotics ended very fast.

Secret pathways: Zn(II) transport in bacteria and fungi

A major challenge in developing new, effective, and pathogen-specific treatments that minimize serious side effects in patients is that bacteria and fungi share key metabolic pathways with humans.

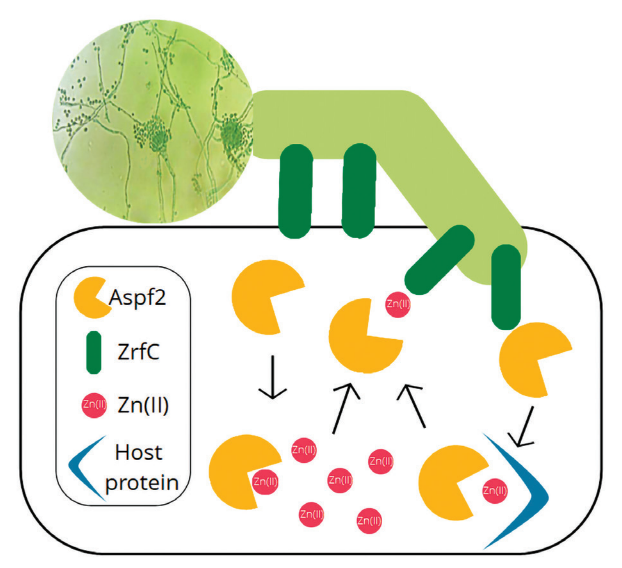

Fig. 1 - Schematic model of *A. fumigatus* Zn(II) scavenging from host cells. After invasion of the host cell, Aspf2 is expressed and secreted. It binds Zn(II), either in the form of free Zn(II) or from Zn(II)-binding proteins of the host. Reassociation with the *Aspergillus fumigatus* cell surface and Zn(II) transportation into the cell occurs via a Aspf2-ZrfC interaction

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Especially in the case of fungi, which are also eukaryotes. Thus, to develop highly specific antifungal and antibacterial drugs, it is important to understand and target differences in human and pathogen metabolism. Although such pathogen-selective targets are rare, there is at least one significant difference between bacterial, fungal and mammalian cells - the transport of transition metal ions, such as Zn(II). In my work, I focused on fragments of: (i) Aspf2 zincophore, and (ii) ZrfC transporter from *Aspergillus fumigatus*, (iii) ZnuA and (iv) AztC from *Paracoccus denitrificans* and (v) AdcA from *Streptococcus mutans*.

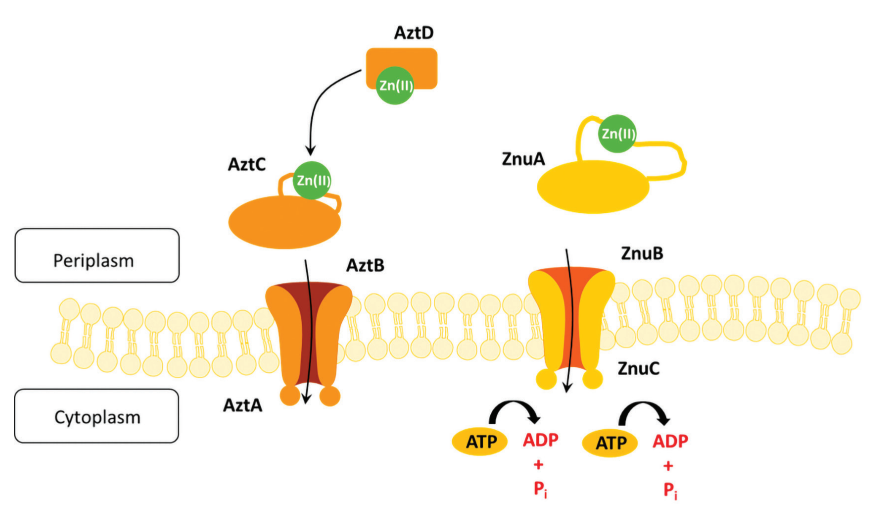

Fig. 2 - A simplified model for zinc acquisition through ABC transporters in *P. denitrificans*

Detective on the trail: Zn(II) binding and acquisition strategies by Aspf2

A well-adapted saprophytic and an opportunistic fungal pathogen, *Aspergillus fumigatus*, produces many small airborne spores, proliferates in the lungs of immunocompromises patients. To acquire Zn(II) during its starvation, *A. fumigatus* uses 'zincophore' system. It includes the Aspf2 zincophore **[4]** and ZrfC transporter (Fig. 1).

To understand the transport of Zn(II) in *A. fumigatus*, I focus on Zn(II) and Ni(II) complexes of four Aspf2 zincophore regions: (i) Ac-ARHAKAH-NH_2, (ii) Ac-MHRLYHVP-NH_2, (iii) Ac-MHRLYHVPAVGQG-WVDHFAD-NH_2, and (iv) Ac-PNCHTHEGGQLHCT.

I pointed out that above pH 6, the Ac-PNCHTHEGGQLHCT *C*-terminal Aspf2 fragment becomes the primary zinc-binding site. At around physiological pH (pH 7.4), Zn(II) is bound to two histidine imidazoles and two cysteine thiolates. Moreover, at pH 7.4, the same *C*-terminal part of the zincophore binds Ni(II) via analogous coordination mode $\{2N_{im}, 2S^-\}$, but with a smaller affinity than Zn(II). Thus, the Aspf2 zincophore strongly prefers to bind Zn(II) over Ni(II) **[5]**.

Guardian of the balance: ZrfC as the unsung hero in *Aspergillus fumigatus* pathogenesis

To fully understand the transport of Zn(II) in *A. fumigatus*, I also focus on Zn(II) and Ni(II) complexes of two ZrfC regions: (i) Ac-TGCHSHGS-NH_2 and (ii) Ac-MNCHFHAGVEHCIGAGESESGSSQ-NH_2. The results clearly indicate that Ac-MNCHFHAGVEHCIGAGESESGSSQ-NH_2 has a much higher affinity for Zn(II) and it suggests that most probably this region is involved in Zn(II) transport, because this fragment has a much higher affinity for Zn(II) than Ac-PNCHTHEGGQLHCT fragment. The analyzed ZrfC transporter fragments also bind Ni(II) and the Ac-MNCHFHAGVEHCIGAGESESGSSQ-NH_2 fragment has the highest affinity toward Ni(II). Moreover, almost in the whole pH range, it prefers to bind Ni(II) rather than Zn(II). However, the free Ni(II) concentration is most likely several orders of magnitude lower than that of free Zn(II) and also the binding of Ni(II) to the ZrfC transporter is not equal to its transmembrane transfer **[6]**. Moreover, in a theoretical situation in which equimolar amounts of Zn(II), Ni(II) and Ac-PNCHTHEGGQLHCT would be available, more than 85% of the zincophore would bind Zn(II), what additionally guarantees the Zn(II) specificity. Moreover, in experimental conditions, Ni(II) does not displace Zn(II) from this binding site because this Zn(II) binding mode (typical for zinc fingers **[7]**) is thermodynamically very stable **[8]**.

First contact agents: SBPs from *Paracoccus denitrificans* as molecular scouts in Zn(II)-uptake

Paracoccus denitrificans is an aerobic and mesophilic Gram-negative bacterium, which encodes two Zn(II) ABC transporter systems (Fig. 2): (i) AztABCD and (ii) ZnuABC **[9]**. The ATP binding cassette contains three components: (i) the ZnuA periplasmic Zn(II) binding protein (SBP), (ii) the ZnuB membrane protein, (iii) the ZnuC ATPase **[10]**. Both SBP differ in the length but also the composition of a His-rich, flexible loop **[11]**.

In order to gain insight into Zn(II) transport in *P. denitrificans*, I focus on Zn(II) and Ni(II) complexes of two ZnuA regions (Ac-HEHEHEHEHEHEHEHEH-NH_2 and Ac-GGEHEHEHEHEHEHEHEHEHDGHGHAEEQAHHDHDHSG-NH_2) and one AztC region (Ac-GGGHYHYIDGKAVFHAG-NH_2). I indicated that at acidic pH, the strongest ligand is the longest ZnuA fragment. At physiological pH, the metal is bound to four imidazole nitrogens of histidine residues belonging to different sets of imidazole rings, due to the polymorphic character of this binding sequence. On the other hand, the preferences towards Ni(II) of the tested fragments are more pronounced than those of Zn(II). In the entire pH range, Ni(II) binds with the highest affinity to longer fragment of ZnuA with a $\{6N_{im}\}$ binding mode. The Ac-GGGHYHYIDGKAVFHAG-NH_2 ligand has the lowest affinity towards Ni(II) among the three tested fragments **[12]**.

Silent weapon: AdcA in the art of Zn(II) acquisition in *Streptococcus mutans*

Streptococcus mutans is a Gram-positive, key pathogen of dental caries **[13]**. Different from other pathogenic bacteria that encode multiple Zn(II) import systems, *S. mutans* encodes a single, highly conserved and high affinity zinc ABC-type importer known as AdcABC **[14]** (Fig. 3), which consists of the (i) AdcA, zinc-binding lipoprotein **[15]**, (ii) AdcB - a membrane

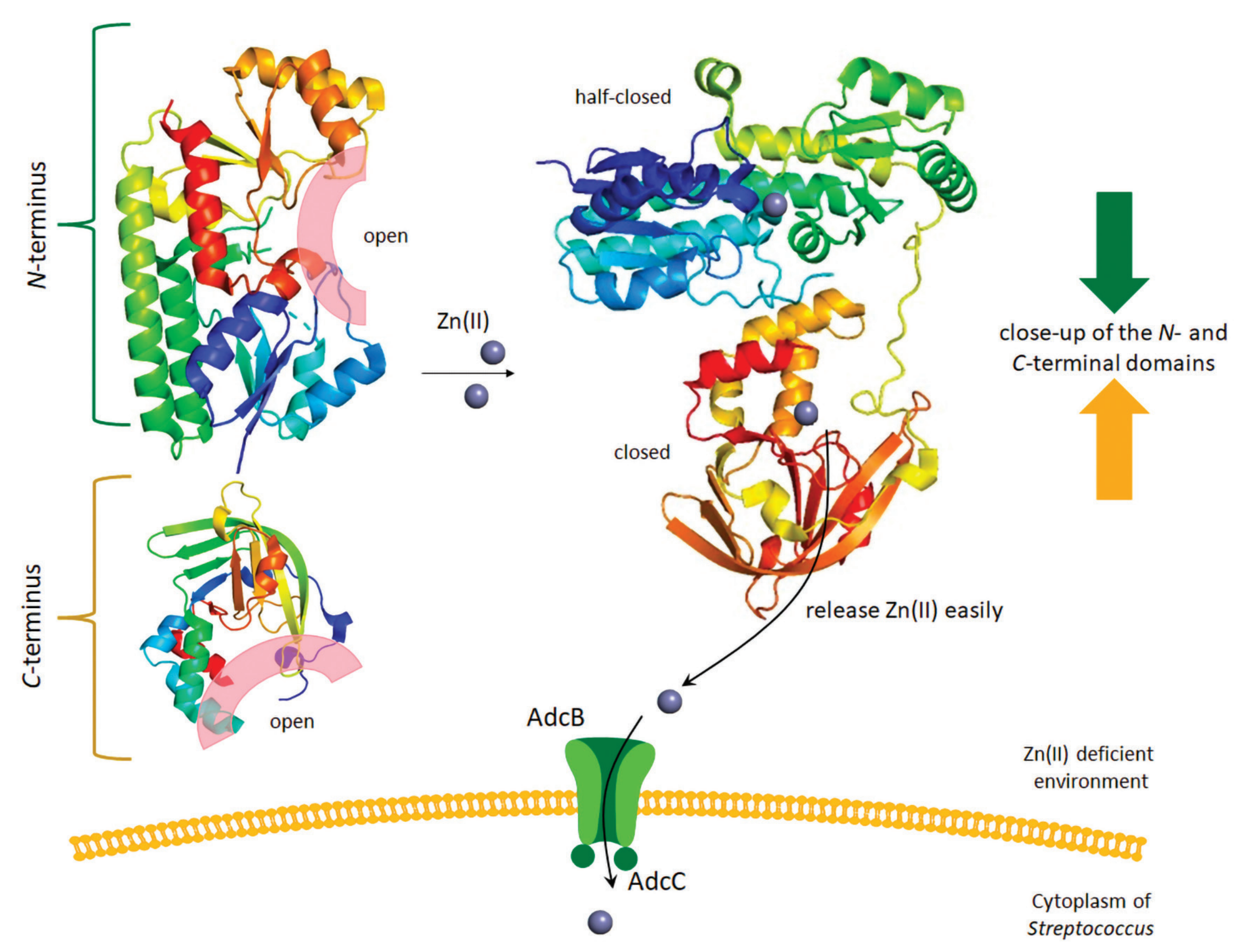

Fig. 3 - The model of Zn(II) uptake in *Streptococcus* via AdcA in a Zn(II)-deficient environment

permease and (iii) AdcC - a cytoplasmic ATPase **[16]**. To investigate the transport of Zn(II) in *S. mutans*, I focus on Zn(II) and Ni(II) complexes of two AdcA regions: (i) Ac-EGHGHKGHHHA-NH$_2$ and (ii) Ac-HGIKSQKAEHFH-NH$_2$. In almost the entire pH range, the Ac-EGHGHKGHHHA-NH$_2$ ligand is preferred over the Ac-HGIKSQKAEHFH-NH$_2$ one. Ni(II) also chooses this fragment, but the situation changes dramatically in alkaline environment. It shows that the poly-His complexes are more tempting metal binding sites at lower pH values. For both ligands, Zn(II) and Ni(II) binding preferences are concerned and these two metal ions bind with almost identical affinity. Of course, this much higher than expected affinity of Ni(II) towards both regions that should be specific for Zn(II) is corrected by physiological concentrations of Zn(II) and Ni(II) in the saliva, teeth, and dental plaque. The significantly lower concentration of Ni(II) guarantees efficient Zn(II) transport using the AdcA protein **[17]**.

Conclusions

This work determined the relationship between the coordination mode, structure and thermodynamic stability of Zn(II) and Ni(II) complexes with unstructured fragments of (i) Aspf2 zincophore **[5]**, (ii) ZrfC transporter **[8]**, (iii) ZnuA solute binding protein **[12]**, (iv) AztC solute binding protein **[12]**, and (v) AdcA lipoprotein **[18]**. The analysis unequivocally shows that: (i) the number of amino acids in the sequence and their position strongly influence the properties of Zn(II) complexes, (ii) the most effective coordination modes for Zn(II) complexes are the $\{2N_{im}, 2S^-\}$ and $\{4N_{im}\}$ coordination modes, the second one with a polymorphic motif, and (iii) for Ni(II), the most effective binding modes turned out to be the $\{6N_{im}\}$ and the $\{2N_{im}, 2S^-\}$ type of coordination. The knowledge collected in this work should be used when designing new, highly specific drugs based on STAMP technology, where the specifically targeted antimicrobial peptides are connected with targeting domains with a short, flexible linker **[18]**.

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