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Bacterial Transition Metal P_{1B}-ATPases: Transport Mechanism and Roles in Virulence

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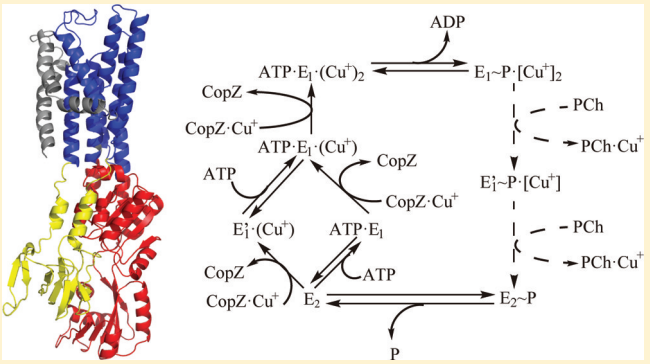
ABSTRACT: P_{1B}-type ATPases are polytopic membrane proteins that couple the hydrolysis of ATP to the efflux of cytoplasmic transition metals. This paper reviews recent progress in our understanding of the structure and function of these proteins in bacteria. These are members of the P-type superfamily of transport ATPases. Cu^+-ATPases are the most frequently observed and best-characterized members of this group of transporters. However, bacterial genomes show diverse arrays of P_{1B}-type ATPases with a range of substrates (Cu^+, Zn^{2+}, Co^{2+}). Furthermore, because of the structural similarities among transitions metals, these proteins can also transport nonphysiological substrates (Cd^{2+}, Pb^{2+}, Au^+, Ag^+). P_{1B}-type ATPases have six or eight transmembrane segments (TM) with metal coordinating amino acids in three core TMs flanking the cytoplasmic domain responsible for ATP binding and hydrolysis. In addition, regulatory cytoplasmic metal binding domains are present in most P_{1B}-type ATPases. Central to the transport mechanism is the binding of the uncomplexed metal to these proteins when cytoplasmic substrates are bound to chaperone and chelating molecules. Metal binding to regulatory sites is through a reversible metal exchange among chaperones and cytoplasmic metal binding domains. In contrast, the chaperone-mediated metal delivery to transport sites appears as a largely irreversible event. P_{1B}-ATPases have two overarching physiological functions: to maintain cytoplasmic metal levels and to provide metals for the periplasmic assembly of metalloproteins. Recent studies have shown that both roles are critical for bacterial virulence, since P_{1B}-ATPases appear key to overcome high phagosomal metal levels and are required for the assembly of periplasmic and secreted metalloproteins that are essential for survival in extreme oxidant environments.



The homeostasis of transition metals (Cu^+, Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}) is central to bacterial physiology. Bound to proteins, these essential micronutrients participate in biochemical pathways ranging from cellular respiration to gene expression.[1] Consequently, these are key structural and catalytic components of a large fraction of bacterial proteomes.[1−3] Since these processes occur in different compartments (cytoplasm, periplasm, cell surface, and secreted proteins fractions), the subcellular distribution of metals and their targeting to specific proteins acquire particular relevance. Conversely, the capability of metals to participate in Fenton reactions and directly interact with DNA and proteins renders them as potent cytotoxics if present at high levels or free in the cellular milieus.[1,4,5] Accordingly, even relatively simple bacterial organisms have a number of soluble metal chaperones/complexing molecules that in coordination with membrane transporters maintain the homeostasis of the various transition metals. Among membrane metal transporters, some protein families uniquely transport transition metals (ZIP, CTR, etc.) while others are members of larger protein families with many diverse roles (ABC-ATPases, permeases, porins, or P-type ATPases[a]).[6−8]

Transition metal transport P_{1B}-ATPases couple the energy provided by ATP hydrolysis to the efflux of cytoplasmic substrates. As all P-ATPases, they follow the well-described Albers-Post E1/E2 transport cycle,[9,10] although singular mechanistic elements are also present to satisfy the physical−chemical characteristics and biological roles of transition metals.[11,12] Since P_{1B}-ATPases drive cytoplasmic metal efflux, they contribute to maintain cytoplasmic metal levels. However, their participation in the assembly of periplasmic and secreted metalloproteins has been shown.[13−16] Both general functions appear essential for bacterial infection of host organisms.[11,13−17]

Founding members of the P_{1B}-ATPases subgroup were bacterial Cu^+-ATPases, and soon after homologous genes were described in eukaryotes.[18,19] Mammals typically have two genes encoding for P_{1B}-type Cu^+-ATPases (ATP7A and ATP7B in humans),[20] while plants contain numerous P_{1B}-ATPases able to transport Cu^+, Zn^{2+}, Cd^{2+}, and Co^{2+}.[21] Mutations in these lead to deleterious or even lethal imbalances in metal homeostasis. Bacteria, in particular pathogenic and symbiotic species, present a greater diversity of P_{1B}-ATPases.[18,22] On one hand, there is a

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broader spectrum of ATPases with diverse metal specificity (Cu^+, Ag^+, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Co^{2+}, and perhaps others) with the associated distinct structural characteristics. On the other, bacterial genomes frequently contain homologous genes with specific alternative functions. Here, we describe the structural determinants of the substrate diversity, the mechanism of transport, and the alternative functional roles that P_{1B}-ATPases have in bacteria.

STRUCTURE OF P_{1B}-TYPE ATPASES

P_{1B}-type ATPases are polytopic membrane proteins. They contain 6 or 8 transmembrane segments (TM) where metal translocation sites are located and well described cytoplasm facing hydrophilic domains involved in ATP binding and hydrolysis (ATP-BD), energy transduction (A domain), and metal-controlled regulation (N- and C-terminal metal binding domains (MBD))[12,18,19,23] (Figure 1A). Distinct from other subfamilies of P-ATPases, P_{1B}-ATPases additional TMs are located on the amino side of the core structure.[b] This arrangement, along with the presence of conserved amino acids responsible for ion binding located in M4, M5, and M6, is the hallmark of the P_{1B}-subgroup of P-ATPases.[18,19] Early studies described the atomic resolution structures of the various cytoplasmic domains and pointed out their high similarity to those of other P-ATPases (Ca^{2+}-ATPase, H^+-ATPase, Na^+,K^+-ATPase).[9,24,25] The first high resolution (3.2 Å) structure of a P_{1B}-ATPase that includes the transmembrane region was reported by Gourdon et al.[23] (Figure 1B). The structure of a truncated form (lacking the N-MBD) of *Legionella pneumophila* CopA, a typical Cu^+-ATPase, was obtained by locking the enzyme in a Cu^+-free E2 conformation. This major accomplishment confirmed the location of transmembrane metal binding sites (TM-MBS), described the architecture of TMs, and revealed the peculiar arrangement of the first two TMs (MA and a kinked MB) forming a singular platform at the entrance of the metal path.

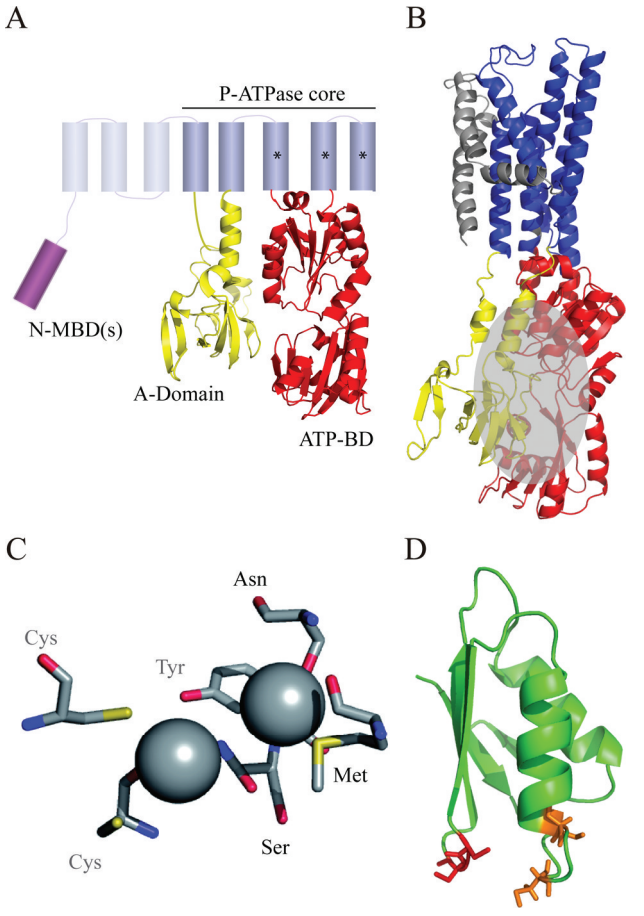

Figure 1. Structural features of P_{1B}-ATPases. (A) Topology of a typical P_{1B}-ATPase. Asterisks indicate the position of amino acids forming TM-MBS(s). (B) Crystal structure of LpCopA (PDB 3RFU). Cytosolic A-domain and ATP-BD are shown in yellow and red, respectively. The gray helices correspond to MA and MB. The gray globe indicates the predicted N-MBD contact with A-domain and ATP-BD.[31] (C) Model of Cu^+-ATPase TM-MBSs. The model was built by using SERCA 1SU4 structure as template. N-terminal Cys had to be manually reoriented to coordinate Cu^+. (D) Model of the N-MBD of *P. aeruginosa* CopA2. Modeling was done using Menkes 4th N-MBD structure 1AW0. In orange are indicated the conserved Cys of the CXXC domain common to most Cu^+-ATPases N-MBDs. In red is the CC motif specific of FixI/CopA2-like ATPases.

Metal translocation across the membrane involves metal coordination by P_{1B}-ATPase TMs. This is accomplished by invariant amino acids located in TMs flanking the large cytoplasmic ATP-BD[12,18] (Figure 1A). TM-MBSs of Cu^+-ATPases and Zn^{2+}-ATPases have been characterized.[26−30] Cu^+-ATPases bind two Cu^+ ions in a trigonal-planar geometry. The first of these sites is constituted by two Cys in M4 and a Tyr in M5, while an Asn in M5 and Met and Ser in M6 form the second site[26,27] (Figure 1C). The structure of LpCopA, although in Cu^+-free form, confirmed the spatial proximity of these residues.[23] However, in this E2 form structure the arrangement of amino acids in the TM-MBSs is not compatible with metal binding. This is in line with the sites placed in a conformation (E2) facilitating outwardly moving substrate release rather than binding. In the case of Zn^{2+}-ATPases, although binding a single ion in the transmembrane region,[28] the coordinating amino acids appear in the same location: two Cys in M4, an Asp in M6, and probably a Lys in M5.[28−30] Considering the binding stoichiometry and the number of coordinating residues, tetragonal coordination geometry appears likely.

A specific feature of P_{1B}-ATPases is the presence of cytosolic MBDs. These have a regulatory role controlling the rate of transport (see below). Low-resolution structures obtained by cryoelectron microscopy[31] and biochemical studies[32−34] show that the N-MBD lean against the ATP-BD and A-domains (Figure 1B). In prokaryotic ATPases these domains are mostly located at the N-terminus (N-MBD), although several C-terminal MBDs have also been reported.[35,36] In Cu^+- and Zn^{2+}-ATPases, typical N-MBDs are 60−70 amino acids long with a $\beta\alpha\beta\beta\alpha\beta$ ferredoxin-like fold. This is also characteristic of soluble Cu^+-chaperones such as human Atox1, yeast Atx1, and bacterial CopZ.[37,38] Eukaryotic Cu^+-ATPases have several of these domains as tandem repeats, while bacterial and archaeal present only 1 or 2. In general, Cu^+-ATPase N-MBDs present the metal binding consensus signatures CXXC, while Zn^{2+}-ATPases coordinate the metal with an additional carboxylic group, DCXXC, $CXXC(X)_{42-44}E$, or CCXXE in eukaryote proteins.[39] This different metal coordination confers higher affinity for Zn^{2+} over Cu^+ to the Zn^{2+}-ATPases regulatory domains and thus higher selectivity.[39] Cu^+-ATPases belonging to the CopA2/FixI subgroup (see below) have an additional CC sequence upstream the classical CXXC motif (Figure 1D). Alternative metal binding domains such as His-rich regions present in all Cu^{2+}-ATPases and in some Cu^+-ATPases

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(LpCopA), and $(XH)_n$ repeats in the N-terminus end of Zn^{2+}-ATPases, have not been structurally characterized.[18]

C-MBDs are less common among bacterial ATPases. While most are homologous to N-MBD and soluble metal chaperones, distinct C-MBD has been characterized in a P_{1B-5}-ATPase from the Gram-positive *Acidothermus cellulolyticus*.[36] This domain presents some degree of homology to the hemerythrin domain from the *Desulfovibrio vulgaris* chemotaxis protein (DcrH-Hr).[40] In vitro structural characterization demonstrated that it houses a di-iron center.[36] Proteins with hemerythrin domains have been proposed to accomplish different functions such as O_2 sensing and transport, iron storage, and NO sensing.[40]

■ TRANSPORT MECHANISM OF P_{1B}-ATPASES

P_{1B}-ATPases follow an ion transport mechanism similar to the classical Albers–Post cycle described for P_2-ATPases; however, they have significant peculiarities required for handling transition metals[11,32] (Figure 2). These have been characterized

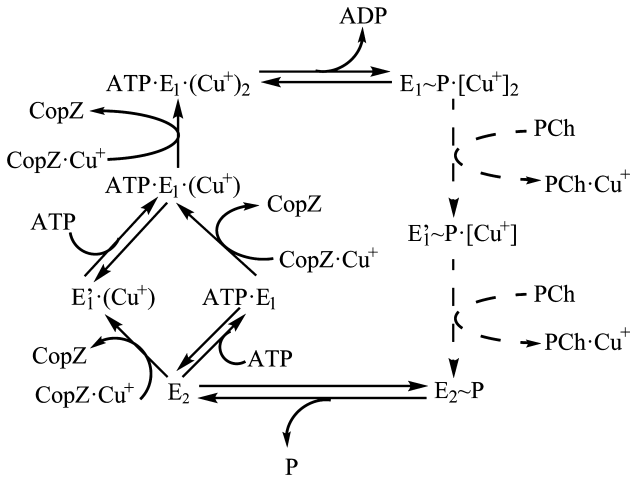

Figure 2. Catalytic and transport cycle of Cu^+-ATPases. Cytoplasmic Cu^+ binding to two transmembrane metal binding sites (TM-MBSs) is coupled to ATP hydrolysis and enzyme phosphorylation ($E1P(Cu^+)_2$). Subsequently, the enzyme undergoes a conformational change (to E2P) leading to the TM-MBSs opening to the extracellular/periplasmic compartment with the consequent metal release. Enzyme dephosphorylation allows the return to the E1 form with TM-MBSs facing the cytoplasm. It is relevant that the E2 → E1 transition is accelerated by ATP (or ADP) acting with low affinity; i.e., a modulatory mode. Note the irreversibility of the Cu^+ transfer from Cu·CopZ to TM-MBS and that binding of ATP is required to full occupancy of the transport site. Discontinued lines indicate proposed steps in the cycle. PCh indicates a hypothetical periplasmic Cu^+-chaperone/acceptor.

by studying archaeal and bacterial archetypical Cu^+-ATPases.[33,41,42] In the Albers–Post mechanism, the enzyme adopts two major conformations E1/E2 in the absence of ATP, E1 being favored upon intracellular metal binding to the TM-MBS. Full occupancy of the TM-MBSs is required for catalytic phosphorylation by ATP of an ubiquitous Asp residue.[26,27] Enzyme phosphorylation drives conformational changes required for metal translocation across the permeability barrier. Upon metal release, the enzyme assumes an E2P form and following dephosphorylation proceeds to E2.[35] Unique aspects of substrate transport by P_{1B}-ATPases originate in the chemistry and consequent physiology of transition metals. In cells, "free" transition metal concentrations are kept at extremely low levels by the formation of adducts with small molecules and metallochaperone proteins.[43] As a consequence, metals do not access transport sites hydrated but bound to complexing molecules, and at some point the metal is transferred from the chaperone to the transporter. Maintenance of low cytoplasmic metal quotas requires the largely irreversible binding of metals to TM-MBSs such that these are not released free into the cytoplasm. A similar phenomenon could be expected at the exit sites where binding of metal to specific periplasmic chaperones, rather than the release of free ion, can be predicted. The absence of significant free metal levels also implies that the transport energetics is not governed by electrochemical gradients but rather by the ligands affinity constants. Probably because of these characteristics, P_{1B}-ATPases transport metals at relatively slow rates compared to P_2-ATPases.[41,44]

The central event of metal transport is the ion binding to TM-MBS (Figure 2). In the case of Cu^+-ATPases it has been shown that Cu^+ access the sites bound to the corresponding chaperone (CopZ) present in most organisms.[45] Gourdon et al. proposed that the amphipathic kinked MB of the Cu^+-ATPase might serve as the docking point for the Cu^+-chaperones during metal delivery to transport sites.[23] This hypothesis is based on the possibility that the largely electronegative surface exposed in the kinked MB might interact with the electropositive areas observed in Cu^+-chaperones[37,38] (Figure 3A). Computer simulated docking of these structures or cross-linking experiments might provide supporting evidence for these ideas. Upon docking of the Cu^+-chaperone, the ion would be transferred to three invariant residues (Met, Glu, and Asp located at the cytoplasmic end of M1, M2, and M3, respectively) (Figure 3B); subsequent to this transient ion binding, Cu^+ would access the TM-MBS. While still speculative, this is a remarkable observation that generates a number of interesting testable hypotheses. For instance, is the kink in MB and the proximity of residues forming the "prebinding" site maintained in other enzyme conformations? How is the ion transferred to the TM-MBS? The possibility that the N-MBD (missing in the structure) might interact with the MB platform delivering Cu^+ to TM-MBS has also been considered. However, the location of N-MBDs leaning against the ATP-BD and A domain has been shown by cryoelectron microscopy[31] and cross-linking experiments[32] (Figure 1B). Then interaction of the N-MBD with the MB platform would require a large movement (180°) of the N-MBD. What would drive this conformational change? A possibility is a conformational change induced by Cu^+ binding; however, Cu^+ binding to the exposed CXXC site does not generate much change in the N-MBD structures.[37]

Once the docking complex is established, Cu^+ subsequently binds one of the two TM-MBSs. The transfer of Cu^+ occurs unidirectionally from the chaperone to the TM-MBS; i.e., there is no metal transfer from the ATPase to the chaperone.[32] This apparent irreversibility originates in the high affinity of the ATPase for the Cu^+·CopZ complex along with the lack of interaction of the apo-chaperone with the TM-MBS access sites. As a complementing element to irreversibility of metal delivery, the high TM-MBS Cu^+ affinities (fM range) prevent backward release rather than determine the metal transfer.[32] The critical role of specific protein–protein interactions in the Cu^+ transfer is confirmed by the observation that fully occupancy of TM-MBSs requires the presence of ATP. That is, ATP is required for a second Cu^+·CopZ binding the enzyme and delivering a second ion (Figure 2). It is tempting to

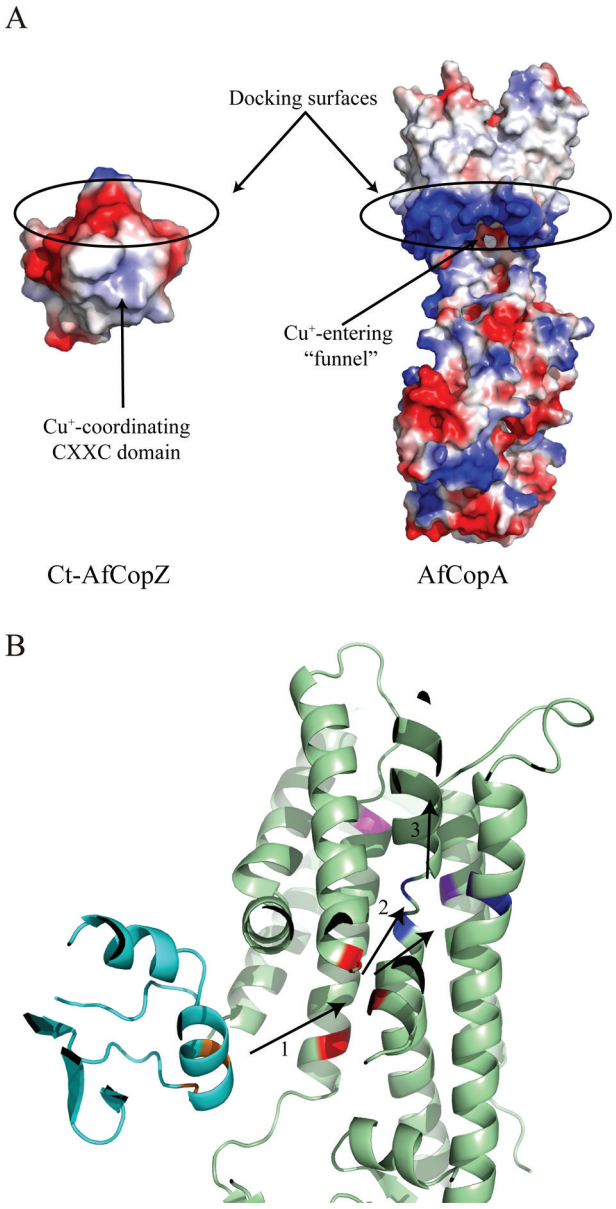

Figure 3. Cu^+ transit from the Cu^+ chaperone to the extracellular space/lumen/periplasm. (A) Electrostatic map of modeled *Archaeoglobus fulgidus* C-terminal domain of the Cu^+ chaperone CopZ (using *E. hirae* CopZ 1CPZ as template) and *A. fulgidus* CopA (using *L. pneumophila* CopA 3RFU as model). Red indicates negative charges and blue positive ones. (B) Metal transport pathway from CopZ to CopA prerelease sites. *A. fulgidus* Ct-CopZ is shown in cyan and *A. fulgidus* CopA in green. Cu^+ is transferred from the CXXC domain (orange) in the chaperone to the predocking amino acids in the ATPase (red) (1). Subsequently, the metal reaches one of the two TM-MBSs (blue and purple) (2) indistinctively. Only when ATP is bound to the ATP-BD, the second TM-MBS is occupied. This triggers ATP hydrolysis and release of Cu^+ which is facilitated by amino acids in the luminal side of the ATPase (pink) (3).

speculate that ATP either maintains MB in a chaperone-interacting conformation or prevents the likely inhibitory interaction of N-MBDs with the ATP-BD[32,34] (see below). Occupancy of TM-MBSs does not appear to be sequential. CopZ-mediated Cu^+ transfer to ATPase mutants separately lacking one or other TM-MBSs has shown a "parallel" arrangement of the TM-MBSs; i.e., CopZ delivers one Cu^+ per ATPase molecule indistinctly to site I or II.[32] Consequently, it could be hypothesized that Cu^+ transfer from the chaperone occurs to the amino acids at the prebinding site, which would subsequently transfer the metal to either TM-MBS (Figure 3B). However, contrary to the TM-MBSs, the Cu^+ affinity of the prebinding site has to be low, since no Cu^+ binding to this hypothetical site is observed in vitro.[26] Then, metal release from the chaperone would be based in lowering the Cu^+ affinity of the chaperone rather than directly competing for the metal.

As discussed earlier, metal release is coupled to enzyme dephosphorylation and is likely the transport rate-limiting step.[41] Barry et al. have shown that the luminal loop linking MA and MB in human ATP7A plays an important role in transient Cu^+ binding and release.[46] The authors postulate that the high number of Met and His in the loop stabilizes Cu^+ in its passage through the membrane explaining the higher dephosphorylation rates shown by ATP7A over ATP7B. Interestingly, the structure of LpCopA shows residues (Glu189, Glu99, Met100, and Met711) close to periplasmic loops that might constitute a "prerelease" metal interacting site (Figure 3B). However, among these, only Glu189 is conserved in all Cu^+-ATPases. The remaining residues (or conservative replacements) are present in only 25−30% of the available Cu^+-ATPase sequences. It has also been hypothesized that P_{1B}-ATPases require specific periplasmic/lumenal "partners" in order to release the metal.[13,46] In bacterial pathogen genomes, the presence of a high number of homologous P_{1B}-ATPases that play different biological functional roles (paralogs) suggests the requirement for different periplasmic partners in order to yield proper delivery of transition metals to secreted protein targets. Putting together these observations, it could be speculated that periplasmic chaperones might accelerate metal release by interacting with extracellular loops affecting the geometry of a "prerelease" site (Figure 2).

N-MBDs appear as regulatory domains. These are not essential for ATP hydrolysis and metal transport by bacterial P_{1B}-ATPases, although they are involved in regulation of the turnover rates with minimal changes in metal $K_{1/2}$ for enzyme activation.[35,42,47,48] This regulation would be mediated by a metal-dependent interaction between N-MBD and the catalytic ATP-BD.[32−34] Cu^+ binding to N-MBD prevents its interaction with ATP-BD affecting rate limiting steps of the catalytic cycle. Studies of the *Thermotoga maritima* Cu^+-ATPase suggest that deletion of N-MBD prevents the enzyme from undergoing conformational changes, leading it to cycle at faster rates.[33] Consistent with a regulatory role, N-MBDs exchange Cu^+ with the corresponding chaperones with $K_{eq} \approx 1$.[49,50]

Available structures of the Ca^{2+}-ATPase in various conformations, together with extensive biochemical studies, have illustrated the conformational coupling of the cytoplasmic substrate binding to TM-MBS with the phosphorylation of the ATP-BD.[9,10] The structural similarities among P-ATPases allow the prediction that P_{1B}-ATPases will undergo similar catalytic and transport steps with the consequent outward transport of cytoplasmic metal ions. However, phenotypes observed upon mutation of *Synechocystis sp.* PCC6803 CtaA and *Enterococcus hirae* CopA among others (see below FixI/CopA2-like Cu^+-ATPases) suggested that they might well be Cu^+ influx ATPases.[13,51,52] This apparent discrepancy was explained by experiments in which the direction of transport of *Pseudomonas aeruginosa* CopA2, *Synechocystis sp.* PCC6803 CtaA, and *E. hirae* CopA Cu^+-ATPase was determined.[11,13] It was observed that these enzymes are indeed only able to drive cytoplasmic Cu^+ efflux, although at a very slow rate compared

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to homologous Cu^+-ATPases. This slow turnover, incompatible with a role in Cu^+-detoxification, appears to be adequate for the function of these proteins in the assembly of copper-containing cytochrome *c* oxidases.[13−16] However, compatible with Cu^+ detoxifier function, *E. hirae* CopA transport rates were higher,[11] and when expressed in *Escherichia coli* ΔCopA strain, it was able to complement the copper-sensitive phenotype (Raimunda and Argüello, unpublished results).

The electrogenicity of transport has significant impact in the function of alkali metals and proton transporting P_2- and P_3-ATPases.[53] Therefore, the putative presence of counterions inwardly transported by P_{1B}-ATPases and the influence of the membrane potential on transport rates might be speculated about. In this direction, the electrogenicity of Cu^+-ATPases has been postulated;[54] however, it might be proposed that the slow rate of transport of P_{1B}-ATPases,[41,44,45] as well as the unlikely presence of substrate electrochemical gradients, would render this putative feature inconsequential for the physiological role of these enzymes. In any case, the slow turnover of P_{1B}-ATPases makes rigorous testing of these hypotheses technically difficult.

■ SUBSTRATE SELECTIVITY OF P_{1B}-ATPASES

As indicated above, P_{1B}-ATPases can transport various metal substrates. Coordination chemistry indicates that the architecture of TM-MBS will determine whether a given metal binds to the transport sites and whether it can activate the ATPase and be translocated.[26,28−30,55] In parallel, as the metals are bound to chaperone or complexing molecules, the interactions of these molecules with the protein for metal delivery would also influence the ATPase specificity. Evidence of the direct and specific interaction among soluble metal chaperones and ATPases is only available for Cu^+ transport (see above). In this context, the analysis is complicated by the diverse TM-MBS signatures, the likely effect of second coordination spheres during binding to TM-MBS, and the lack of information on the speciation of cytoplasmic metals other than Cu^+.

Early studies using bioinformatics approaches provided initial criteria to predict the P_{1B}-ATPases selectivity.[18] These revealed that similar to P_2-ATPases, P_{1B}-ATPases showed potential metal coordinating conserved residues in TMs flanking the ATP-BD (M4, M5, M6) (Figure 1A). This analysis allowed postulating subgroups of P_{1B}-ATPases with different metal transport specificities (Figure 4). Proteins in the P_{1B-1}-subgroup are Cu^+ exporters and constitute the largest and best-characterized group of P_{1B}-ATPases.[11,12,18] Within the P_{1B-1} group, two clusters can be differentiated (Figure 4): the classical Cu^+-ATPases and the FixI/CopA2-like ATPases. The latter one includes a number of Cu^+-ATPases that have a higher affinity for Cu^+, albeit a much lower transport rate.[11,13] Both groups contain identical TM-MBSs that bind the metal with high affinity (fM range) in a trigonal-planar coordination.[23,26,27] Residues forming these metal-binding sites are shown in Figure 1C. However, highlighting the complexity of metal selectivity, the yeast PCA1 P_{1B}-ATPase is an example where the predictability is altered. PCA1 contains the hallmark residues that form the Cu^+ binding sites, but it transports Cd^{2+}.[56] While further biochemical characterization is necessary to confirm the specificity of this pump, mutations of amino acid others than those involved in metal coordination restore Cu^+ transport capability (Lee, L., University of Nebraska, personal communication), suggesting that either the binding geometry or the second sphere of coordination affects the enzyme selectivity.

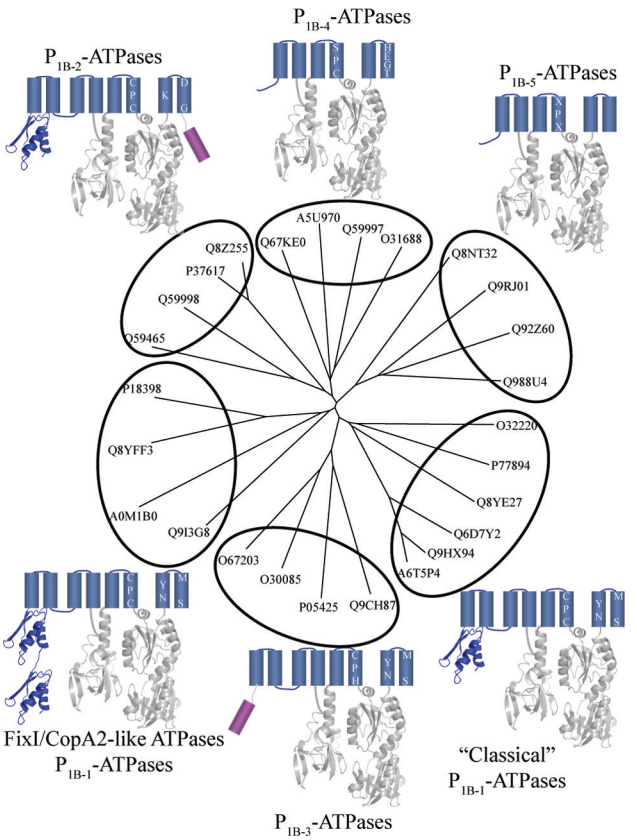

Figure 4. Subfamilies of P_{1B}-ATPases. Sequences used for the tree were: *Symbiobacterium thermophilum* Q67KE0, *M. tuberculosis* A5U970, *Synechocystis* sp. PCC 6803 Q59997, *Bacillus subtilis* O31688, *Corynebacterium glutamicum* Q8NT32, *Streptomyces coelicolor* Q9RJ01, *Sinorhizobium meliloti* Q92Z60, *Mesorhizobium loti* Q988U4, *B. subtilis* O32220, *M. tuberculosis* P77894, *Brucella melitensis* Q8YE27, *Erwinina carotovora* Q6D7Y2, *P. aeruginosa* Q9HX93, *Klebsiella pneumonia* A6T5P4, *Lactococcus lactis* Q9CH87, *E. hirae* P05435, *A. fulgidus* O30085, *Aquifex aeolicus* O67203, *P. aeruginosa* Q9I3G8, *Gramella forsetii* A0M1B0, *B. melitensis* Q8YFF3, *S. meliloti* P18398, *Helicobacter pylori* Q59465, *Synechocystis* sp. PCC 6803 Q59998, *E. coli* P37617, *S. enterica* Q8Z255.

The P_{1B-2}-ATPase subgroup includes Zn^{2+} exporters. The better characterized member, *E. coli* ZntA, has one TM-MBS.[57] It is apparent that at least two Cys in M4 and Asp in M6 are part of the metal site[28−30,55] (Figure 4). The nonphysiological Pb^{2+} and Cd^{2+} are also substrates of ZntA, since they are able of activate the enzyme and catalyze formation of acyl phosphate intermediaries.[57] Interestingly, ZntA TM-MBS binds Co^{2+}, Ni^{2+}, and Cu^{2+} with high association binding constants, although these metals do not activate the enzyme.[58] This observation indicates that selectivity is not determined by binding affinities but by a fitting coordination. Similar phenomena are observed in metal regulatory proteins.[59] In this way, TM-MBS occupancy by transported substrates leads to distinct catalytic conformation/s resulting from the geometry of metal coordinating side chains. This idea is also supported by the capability of ZntA carrying Ser substitutions of both Cys in M4 to bind metals even though the enzyme is inactive.[28]

Subgroup P_{1B-3} includes Cu^{2+}-ATPases. In this case, the cytosolic oriented Cys in M4 is substituted by His, which explains the change in selectivity from Cu^+ to Cu^{2+}.[48] Imidazolium, an intermediate Lewis base, is expected to form a stronger adduct with Cu^{2+}—an intermediate Lewis acid—than

with Cu^{+}—a soft Lewis acid.[1] Further characterization of this subgroup is needed to explain why bacteria carrying this gene require a Cu^{2+} exporter.

Proteins in the P_{1B-4} subgroup lack N- and C-MBDs and present only six TMs. While signature amino acids Cys and Ser in M4 and HEGT in M6 are observed in this group,[18] the residues involved in metal coordination have not been identified. Nevertheless, the experimental evidence suggests that these are likely Co^{2+}-ATPases that might accept other substrates. *In vivo* characterization of *Synechocystis sp.* PCC6803 CoaT has indicated that the protein transports Co^{2+}.[60] On the other hand, *Cupriavidus metallidurans* CH34 CzcP transports also Zn^{2+} and Cd^{2+} at rates even higher than Co^{2+}.[61]

P_{1B-5} groups a number of "rare" and uncharacterized P_{1B}-ATPases, where identification of conserved residues forming putative TM-MBS is not clear. ATPases in this subgroup with a C-MBD sharing some degree of homology with hemerythrin iron-binding domain has been proposed to be involved in iron transport (see above).[36] Interestingly, an unusual number of genes coding for "rare" P_{1B-5}-ATPases have been found in genomes of pathogenic organisms such as *Mycobacterium tuberculosis*.

METALS AND VIRULENCE

The association of metals with the immune system is well-known. Dietary metal deficiencies compromise the animal immune response.[62,63] For instance, iron deficiency limits T-cell proliferation and diminishes the activation of the CD28 receptor,[64] Zn^{2+} deficiency causes an imbalance in the population of Th1 and Th2 cells,[65] and copper is involved in interleukin 1 release.[66] In contrast, in the course of the inflammatory response, local or systemic metal deficiencies are self-provoked.[67] Upon release of the cytokine interleukin 6, the hormone hepcidin is produced inhibiting iron release from the enterocyte and causing a generalized drop of iron levels in the blood.[68] At a local level, the Zn^{2+} and Mn^{2+} chelator calprotectin removes metals from abscesses caused by *Staphylococcus aureus*,[69] leaving the surrounding tissues unaffected. To keep metal levels in body fluids as low as possible, several different metal chelators are used, such as the mentioned calprotectin, but also siderocalins, lactoferrin, and others.[70−72] Perturbations of this nutritional immunity, either by dietary metal supplementation during the infection or by other pathological conditions such as hemochromatosis, can seriously aggravate infection, sometimes with fatal results.[73,74] The purpose underlying nutritional immunity is to deprive invading microbes of essential nutrients in order to slow down their growth and to hamper their protective machinery.

The importance of metals in virulence also lies in the presence of free radicals that the host normally generates to combat infections. For instance, in the phagosome the immune cell will trigger the production of reactive nitrogen and oxygen species to damage the endocyted pathogen.[75] Plants use similar mechanisms to attack invading microbes, albeit reactive species are typically extracellularly produced.[76] Since metals are an essential component of many free-radical detoxifying proteins, such as catalases, superoxide dismutases, or ascorbate peroxidases,[77,78] in several instances they are required for host colonization and virulence.[79−82]

As a result, a competition is established between host and pathogen for metals. This struggle is a turning point for the outcome of the invasion. For instance, loss of the biosynthetic capabilities of bacterial metal chelators or in their uptake results in a loss of virulence both in animal and in plant hosts.[83,84] Similarly, mutation in bacterial transition metal importers would results also in loss of virulence, as is the case for *E. coli* Zn^{2+} transporters ZupT and ZnuABC and *Haemophilus influenzae* ZevAB.[85,86] This struggle for metals is carried out during the entire process of combating the pathogen, from early invasion to the phagosome. In the phagosome, free $Fe^{2+/3+}$ and possibly Mn^{2+} are recovered by NRAMP transporters, while ZIP transporters are involved in Zn^{2+} removal.[7] Their role is essential, and their loss results in higher susceptibility to disease.[87] On the bacterial side, bacterial NRAMP, ZIP, and ABC transporters are employed to counterbalance the phagocytic cell metal transporters.[85,88,89]

Therefore, and in general terms, one of the host strategies against an infection is to minimize access to essential transition metals. However, this is not the case for copper (Figure 5).

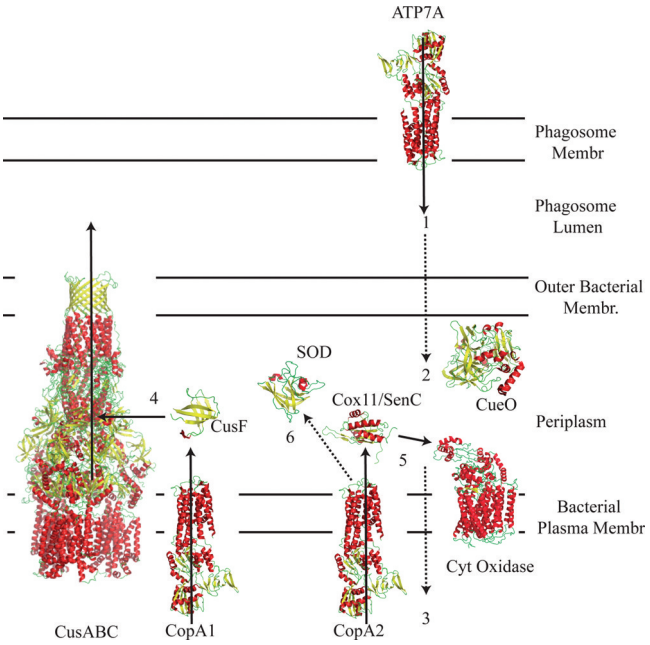

Figure 5. Hypothetical Cu^{+} homeostasis in the phagocytic cell/microbe interface. Cu^{+} is introduced in the phagosome by ATP7A (1), and it crosses the outer membrane reaching the periplasm, where some is oxidized to Cu^{2+} by CueO (2). However, some Cu^{+}still crosses to the bacterial cytoplasm (3). Excess cytosolic Cu^{+} is transported back to the periplasm by CopA1-like ATPases, where it binds to periplasmic Cu^{+}-chaperones such as CusF, which transfers Cu^{+} to CusABC-like transporters to be translocated toward the extracellular space (4). Cu^{+} is also used to synthetize Cu-proteins. CopA2-like transporters are responsible for this, transferring Cu^{+} to other periplasmic Cu^{+} chaperones, such as SenC, which would subsequently donate the metal to cytochrome oxidases (5). Other periplasmic Cu^{+} chaperones must exist to transfer the metal to other periplasmic apoproteins, such as Cu, Zn superoxide dismutases (6).

Wagner et al.[90] observed $Cu^{+/2+}$ accumulation in the phagosome of macrophages previously stimulated with interferon γ or that had phagocytized *Mycobacterium avium*. Moreover, proinflammatory agents in macrophages result in localization of the Cu^{+}-ATPase ATP7A to the endocytic compartment.[91] Loss of function on this Cu^{+} transporter resulted in a higher survival rate of bacteria. Some plants also seem to be using copper as an antibiotic agent. This is the case with rice (*Oryza sativa*) that accumulates relatively high levels of $Cu^{+/2+}$ in their xylem to prevent microbial proliferation.[92]

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As a response, the invading bacteria have to remove Cu^+ from their subcellular compartments (Figure 5). For instance, the Cu^+ introduced by the phagocytic cell, which can produce oxidative damage,[5] could be oxidized to a less harmful Cu^{2+}. This reaction is carried out in the periplasm by multicopper oxidases.[93] Recent studies on systemic virulence of *Salmonella enterica* have shown that the multicopper oxidase CueO is an important player for pathogenesis.[94] Periplasmic Cu^+ is also pumped out of the cell by RND transporters.[94] In *M. tuberculosis*, these transport systems and other copper-detoxifying proteins play an important role in virulence.[95,96] Occasionally, as for certain strains of *Xanthomonas oryzae*, bacteria elicit molecules that promote $Cu^{+/2+}$ removal by the host organism itself.[92]

P-TYPE ATPASES AND VIRULENCE

Since P_{1B}-ATPases translocate metal outside of the cell, it would be expected that Cu^+-ATPases play a role in virulence. In this direction, it has been observed that mutation of Cu^+-ATPases often leads to reduced colonization and virulence.[13,17,91] This role of Cu^+-ATPases in protection from phagocytosis would also explain why these transporters are present in most of the bacteria sequenced to date. Rather than protecting against high concentrations of exogenous copper, they would be involved in detoxifying the Cu^+ produced by predatory amoeba or immune cells.

It is also interesting to note that many pathogenic bacteria have multiple isoforms of Cu^+-ATPases encoded in their genome. Given that bacteria tend to keep their genome size as compact as possible, multiple copies of a gene would indicate functional diversity. Paralogous ATPases have the same structural characteristics as "classical" Cu^+-detoxifying ATPases, albeit they transport at lower turnover rate,[11] which might indicate a coupling of metal translocation with metal transfer to an apoprotein, although the exact mechanism of this process remains to be determined. In one studied example among these FixI-like ATPases play a role in delivering metal to cytochrome *c* oxidases.[13−16] Mutation of *P. aeruginosa* CopA2, a FixI-like protein, results in a higher sensitivity to oxidative stress and reduced virulence, although it has no role in Cu^+ detoxification.[13] It would be possible that different isoforms of cytochrome oxidases would play a role in host colonization, either under low O_2 pressure, such as in symbiotic nitrogen fixation, or when this pressure is higher (leaves, phagosomal oxidative burst), as a way to detoxify oxygen. This is a mechanism that has not been observed in pathogenic bacteria but that some free living-nitrogen fixating bacteria, such as *Azotobacter vinelandii*, use to protect their enzymes from high oxygen pressures.[97]

In other cases, such as *Salmonella typhimurium*, the additional Cu^+-ATPases seem to be only associated with metal detoxification.[98,99] This could be due to a recent genomic change that has had no time to drift or to a specialization to better detoxify Cu^+ and other metals.[98] In the future, we might see more of this type of duplication to counteract the use of Cu^+ as antimicrobial. This is a practice standard in agriculture to prevent plant diseases.[100] In healthcare, the use of copper surfaces is showing promising results in reducing nosocomial infections and in a strategy to fend off multidrug resistance infections, such as methicillin-resistant *S. aureus*.[101,102] However, as antibiotic resistance has become widespread, an increase in copper tolerance could potentially be observed. In this sense, the thorough study of the structure of bacterial Cu^+-ATPases and their differences with eukaryotic ones might provide tools to combat future Cu^+-hypertolerant bacteria.[102]

A closer look at the P-type ATPases of pathogenic and endosymbiotic bacteria reveals the frequent presence of "strange" ATPases belonging to subgroups P_{1B-4} and P_{1B-5}. Very little is known about these ATPases from a biochemical and functional perspective.[12,18] Microarray data suggest that some of them are up-regulated by host invasion, which hints at a role in colonization and perhaps virulence.[103] Although their metal substrate is not clear, it has been hypothesized that they are metals other than Cu^+ or Zn^{2+}. Therefore, it would be expected that they play a role in loading periplasmic and secreted apoproteins with these metals. However, very little is known about these proteins and what role they might be playing in the organism.

FUTURE DIRECTIONS

In the past decade, our understanding of the workings of P_{1B}-ATPases and their physiological role has increased exponentially, culminating with the recent characterization of the first Cu^+-ATPase crystal structure. As has happened with other crystallized P-ATPases, we shall expect to obtain "snapshots" of P_{1B}-ATPases in the other conformations of the catalytical cycle: both when the metal is provided free and during the interaction with metal delivering and removing chaperones. A refinement of these structures will also help us to obtain a closer look at the TM-MBSs of classical Cu^+-ATPases and extrapolate these to homologous subfamilies of metal ATPases. From a mechanistic point of view, description of the metal release process appears as the next challenge. Initial reports on this subject suggest that novel models are in play. Characterization of the release sites will explain how the accepting protein determines the biological role of a P_{1B}-ATPase and how its specificity is established. New roles will likely appear for the already characterized Cu^+, Zn^{2+}, and Co^{2+}-ATPases, in particular functions associated with the synthesis of periplasmic and plasma membrane metalloproteins. Moreover, the narrower distribution of P_{1B-4} and P_{1B-5} ATPases and their connection with pathogens make them interesting study topics. Beyond these, the abundance of metal ATPases in pathogenic bacteria makes them good targets for novel drug developments in treating re-emerging diseases such as tuberculosis.

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■ ABBREVIATIONS

A-domain, actuator domain; ATP-BD, ATP binding domain; MBD, metal binding domain; TM, transmembrane segment; TM-MBS, transmembrane metal binding site.

■ ADDITIONAL NOTES

[a]For simplicity P-type ATPases will be referred as P-ATPases, P_{1B}-ATPases, etc.
[b]The nomenclature of TMs proposed by Gourdon et al.[23] will be used in this review.

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