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# Zinc homeostasis mechanism and its role in bacterial virulence capacity FREE

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# Zinc Homeostasis Mechanism and Its Role in Bacterial Virulence Capacity

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**Abstract.** Zinc ion ($Zn^{2+}$) is an essential cofactor required by numerous metalloenzymes and is important for structural and regulatory systems in bacterial cells. $Zn^{2+}$ is a trace element: bacterial cells require only very small quantities. High concentrations of $Zn^{2+}$ are toxic to microorganisms. In the environment, bacteria may be subject to conditions where $Zn^{2+}$ is either very limited or s at a toxic level. Due to the limitation of $Zn^{2+}$ or the toxic effect of high levels of free $Zn^{2+}$ ions, the bacteria need to carefully control the intracellular level of $Zn^{2+}$. This paper aims to determine the mechanisms bacteria use to maintain the intracellular $Zn^{2+}$ concentration and the adaptation mechanisms used by bacteria to grow in a $Zn^{2+}$-limited environment. The role of $Zn^{2+}$ in bacterial pathogenesis and virulence capacity will also be discussed. It is known that in bacteria, $Zn^{2+}$ homeostasis is maintained by $Zn^{2+}$-uptake/import and $Zn^{2+}$-efflux/export systems. These two systems provide a balance between the requirement for the metal and its toxicity and therefore is essential for bacterial growth and survival. Metal ions, including $Zn^{2+}$ have been demonstrated to be involved in various bacterial pathogeneses. Moreover, there is increasing evidence for the importance of $Zn^{2+}$ in the virulence of various bacteria. $Zn^{2+}$ is shown to be involved in biofilm formation, bacterial motility, antibiotic resistance, and survival against oxidative stress. Therefore, the ability of bacterial cells to maintain a homeostasis of $Zn^{2+}$ is crucial for their growth, survival and virulence capacity.

**Keywords**: adaptation mechanism, bacteria cells, microorganism, zinc concentration.



## INTRODUCTION

Zinc ($Zn^{2+}$) has been shown to play a significant role in bacterial cells. In the cell, $Zn^{2+}$ acts as a major structural protein (which stabilizes diverse "Zn-finger" proteins) and as a catalytic cofactor.[1,2,3] However, besides the crucial role of $Zn^{2+}$ for many bacterial cellular processes, this metal ion is also toxic to the cell if present in high concentrations.[4] In the environment, bacteria may be exposed to various ranges of $Zn^{2+}$ concentrations, e.g., a $Zn^{2+}$ limited environment or a toxic level of $Zn^{2+}$. Therefore, to be able to survive in these conditions, many bacteria have several mechanisms to maintain a sub-toxic $Zn^{2+}$ concentration. To maintain the homeostasis of the intracellular $Zn^{2+}$ level, bacterial cells use a $Zn^{2+}$ regulator which precisely regulates the expression of $Zn^{2+}$-transporters. This strategy is used by bacterial cells in response to either $Zn^{2+}$ starvation or $Zn^{2+}$ toxicity. Such a transcriptional regulation by global regulator proteins will regulate $Zn^{2+}$-efflux and $Zn^{2+}$-acquisition to import or export $Zn^{2+}$ across cell membranes.[5]

Recently, there has been increasing evidence for the importance of $Zn^{2+}$ in the pathogenesis and virulence of various bacteria, including *E. coli*,[6] *S. pneumonia,*[7] *S. aureus,*[8] *L. monocytogenes,*[9] *P. mirabilis,*[10] and *Enterococcus faecalis*.[11] $Zn^{2+}$ has been shown to play a role in various bacterial pathogeneses, including the formation of bacterial biofilms, bacterial intracellular growth, bacterial survival during the infection process, antibiotic sensitivity, and bacterial ability for colonization.

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This paper will present a review of the mechanisms used by bacterial cells to control the intracellular level of $Zn^{2+}$. The role of $Zn^{2+}$ in bacterial pathogenesis and virulence will also be discussed.

## ZINC HOMEOSTASIS MECHANISMS IN BACTERIA

There are several strategies used by bacteria to maintain intracellular $Zn^{2+}$ in a biological level. This review paper will cover the strategies used by bacterial cells in response to $Zn^{2+}$ starvation or toxicity to maintain a homeostasis of $Zn^{2+}$. The mechanisms used by bacteria to maintain the homeostasis of $Zn^{2+}$ are via transcriptional regulation by metal-sensing metalloregulatory proteins, zinc uptake regulator (Zur), and $Zn^{2+}$ efflux and acquisition across cell membranes.[5] The $Zn^{2+}$ sensor, Zur, is a member of the Fur family, which in the presence of normal levels of $Zn^{2+}$ represses the expression of the $Zn^{2+}$-uptake ABC transporter, *znuABC*.[12,13]. However, when the level of $Zn^{2+}$ in the environment is limited, this regulator will activate the $Zn^{2+}$-uptake ABC transporter *znuABC* to scavenge this metal ion from the environment. In addition, there is another regulator, namely ZntR, which is a MerR regulator. This regulator activates the transcription of a gene encoding a P-type ATPase that exports $Zn^{2+}$ from the cytoplasm to the periplasm of the cell.[5] In most bacteria, Zur act as the global regulator that regulates the expression of a number of genes required to adapt to conditions of $Zn^{2+}$ deprivation. In their microenvironments, organisms are continually exposed either the persistence of a deleterious level of metal ions, the lack of essential metal ions, or the persistence of heavy metal pollutans; the metalloregulatory proteins regulate the expression of the genes that enable the microorganism to survive in these harmful microenvironments.[14]

### Zinc uptake systems in bacteria

To control the homeostasis of $Zn^{2+}$; bacteria have ability to import or to export $Zn^{2+}$ from their cells via a $Zn^{2+}$-uptake system. Many bacterial species have two types of $Zn^{2+}$-uptake systems: the high affinity $Zn^{2+}$-uptake systems and the low affinity uptake systems. These two types of systems are used under different conditions.[15] During conditions of $Zn^{2+}$-starvation, the high affinity $Zn^{2+}$-uptake systems are up-regulated, while during conditions of toxic levels of $Zn^{2+}$, the efflux system is up-regulated. In moderate conditions, the low affinity uptake transporters are used to maintain the biological level of $Zn^{2+}$in the cell.[15] The high-affinity $Zn^{2+}$-uptake systems belong to the ABC transporter family. These transporters consist of three proteins, ZnuA, ZnuB and ZnuC, encoded by the *znuABC* genes.[16] It has been reported that the deletion of *znuA*, *znuC*, *znuB* or *znuABC* in various bacteria decreases the $Zn^{2+}$-uptake.[17] The $Zn^{2+}$-uptake system ZnuABC is a P-type ATPase transporter. The energy required to transport the metal ions of this system comes from the hydrolysis of ATP, which is undertaken by ZnuC; whereas ZnuA is the soluble $Zn^{2+}$-binding periplasmic protein that interacts with the inner membrane permease, ZnuB.[18] In addition to the high-affinity $Zn^{2+}$-uptake system, there is a low-affinity $Zn^{2+}$-uptake system, but little is known about this system. In *E. coli* and *S. enterica* Serovar Typhimurium,[19,20] ZupT, a low affinity uptake protein has been shown to be involved in bacteria grown in a $Zn^{2+}$ scarcity environment. It has also been suggested that the expression of ZupT is not regulated by Zur,[20] However, it is reported that ZupT does not import $Zn^{2+}$ specifically, but also imports a broad range of other metal ions, such as $Cd^{2+}$, $Fe^{2+}$, cobalt ($Co^{2+}$), $Cu^{2+}$ and $Mn^{2+}$.[21]

### Zinc efflux systems in bacteria

In conditions where $Zn^{2+}$ is at a toxic level, bacterial cells will express efflux systems and export $Zn^{2+}$ out of the cytoplasm to prevent an overabundance of this ion in the cell cytoplasm.[22] Three families of protein exporters have been identified in bacteria, namely the Cation Diffusion Facilitator (CDF), Resistance Nodulation Division (RND) efflux pumps, and P-type ATPases families.[23] The CDF family is a ubiquitous family of proteins found in most living organisms.[23] The exporters in the CDF families are the ZitB and YiiP proteins in *E. coli* that use the free energy derived from hydrogen influx to export heavy metal ions, including $Co^{2+}$, $Cd^{2+}$, $Zn^{2+}$ and $Ni^{2+}$, from the cytoplasm to the periplasm by paasing it through the inner membrane.[17] Another CDF transporter, CzcD, has also been identified. It functions as a $Zn^{2+}$ exporter.[24] A CDF family transporter, ZntA, which is involved in the resistance to $Zn^{2+}$ and $Co^{2+}$, has been identified in *S. aureus*: it is regulated by ZntR.[25] It should be noted that the ZntA and ZntR proteins in *E. coli* are different from ZntA and ZntR in *S. aureus*. In *E. coli*, ZntA and ZntR are designated as a transporter and a regulator belonging to the P-type ATPase family of transporters, while in *S. aureus,* ZntA is an export transporter belonging to the CDF family.

The next family of exporters, the P-type ATPase family, is found in both the eukaryotes and bacteria. These transport proteins are controlled by either MerR/ZntR regulators or by members of the ArsR/SmtB superfamily of repressors. These two types of families are also found in connection with other metal transporters.[13] The P-type ATPases perform active ion transport across the membrane using the free energy of ATP hydrolysis.[26] An example of a P-type ATPase exporter is ZntA, which has been identified in *E. coli*.[27] ZntA is regulated by the MerR-like ZntR regulator via the binding of the apo-ZntR dimer to the promoter of *zntA*. It represses the transcription of zntA.[17] Another example is ZitB, which also found in *E. coli*. It has been suggested that ZitB plays a role in the maintenance of $Zn^{2+}$ homeostasis at low $Zn^{2+}$ concentrations, whereas in toxic concentrations of $Zn^{2+}$, ZntA is more active.[28] *zntA* is regulated by ZntR, an MerR-family.[13] The ZntR repress *zntA* expression when intracellular $Zn^{2+}$ levels exceed sub-toxic levels.[29]

The other family, RND, is widespread, especially among Gram-negative bacteria, and catalyzes the active efflux of many antibiotics and chemotherapeutic agents. These efflux systems have very large periplasmic domains, and form tripartite complexes with the outer membrane channels and periplasmic adaptor proteins.[30] These families consist of three complex proteins: the cytoplasmic membrane-associated protein, a periplasmic membrane fusion protein, and an outer-membrane channel protein.[31] The RND efflux systems have been identified in *Caulobacter crescentus,* namely *czrCBA* and *nczCBA*.[31] It is reported that CzrCBA functions to export $Cd^{2+}$ and $Zn^{2+}$ from the bacterial cytoplasm whereas NczCBA exports $Ni^{2+}$ and $Co^{2+}$ as well as having a secondary role, exporting $Cd^{2+}$ and $Zn^{2+}$.[31] A study of *Ralstonia metallidurans* also reported that there is a CzcABC system that functions export $Zn^{2+}$, $Co^{2+}$ and $Cd^{2+}$ and the regulation of this system appears to involve the CzcS/CzcR two-component regulatory system.[24,13] A diagrammatic representation of the $Zn^{2+}$-uptake and $Zn^{2+}$-efflux systems in Gram-negative and Gram-positive bacteria are shown in Figures 1 and 2, respectively.

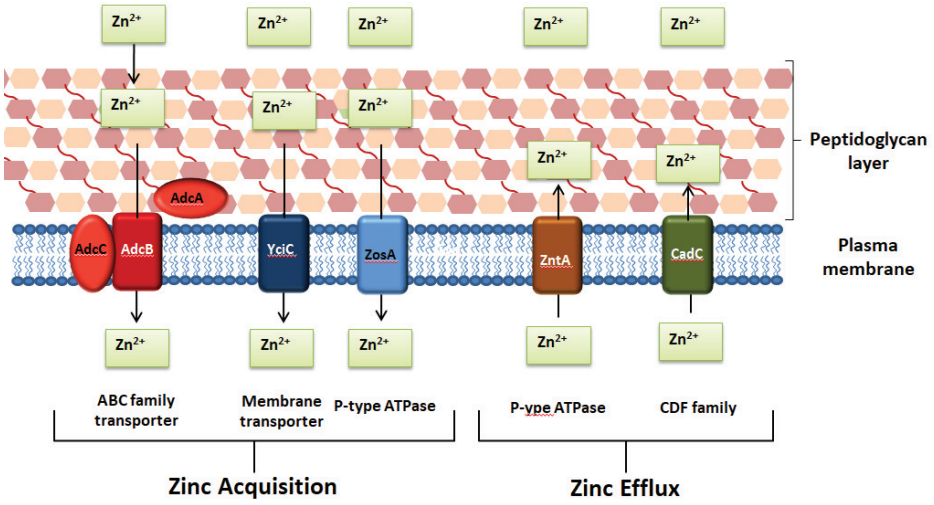


**FIGURE 1.** The mechanisms of Zinc acquisition and efflux in Gram-positive bacteria. The $Zn^{2+}$-acquisition and efflux system in Gram-positive bacteria are shown diagrammatically. The light green box represent $Zn^{2+}$ ions ($Zn^{2+}$). $Zn^{2+}$ is imported via a number of mechanisms: (i) ZnuABC (red), an ABC transporter, (ii) a low affinity $Zn^{2+}$ importer YciC (dark blue; found in *B. subtilis*) and (iii) ZosA (clight blue; found in *B. subtilis*), a P-type ATPase family protein. $Zn^{2+}$ can be exported from the bacterial cytoplasm by; (i) ZntA (brown), a P-type ATPase and (ii) the CadC (green) CDF-family protein. Adopted from Suryawati, B.[32] (unpublish).

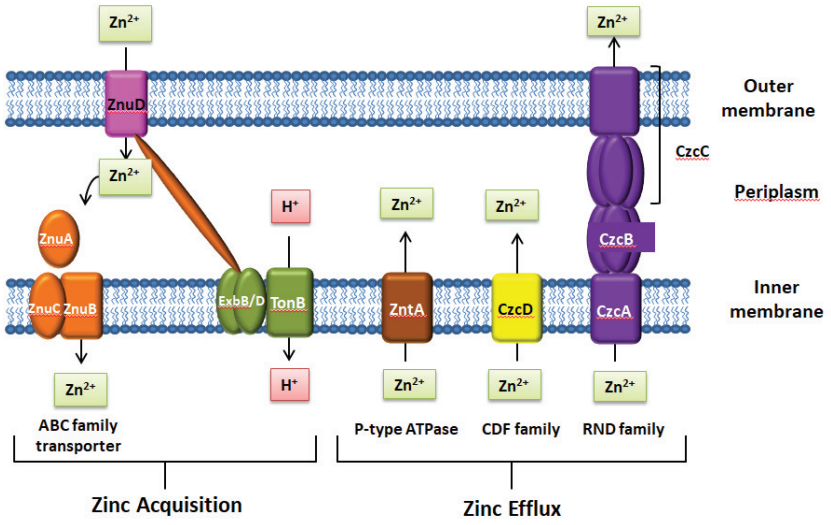


**FIGURE 2.** The mechanisms of Zinc acquisition and efflux in Gram-negative bacteria. The $Zn^{2+}$-acquisition and efflux systems in Gram-negative bacteria are shown diagramatically. The light green boxes represent $Zn^{2+}$ ions ($Zn^{2+}$). One mechanism of $Zn^{2+}$ import happens via the $Zn^{2+}$-regulated TonB-dependent outer membrane receptor, ZnuD (magenta) which is energised by a $Zn^{2+}$-regulated TonB-ExbB-ExbD (green) system. This system requires the proton motive force (represented by hydrogen ions, $H^+$, pink boxes). Zn transport across the inner membrane is mediated by the ZnuABC transporter (orange), an ABC transporter family member. $Zn^{2+}$-efflux systems are represented by; the P-type ATPase ZntA (brown), the CDF-family CzcD (yellow), and a tripartate RND family transport system CzcABC (purple). Adopted from Suryawati, B.[32] (unpublish).



## THE ROLE OF ZINC IN BACTERIAL PATHOGENESIS

There is increasing evidence for the importance of $Zn^{2+}$ in the pathogenesis and virulence of various bacteria, including *E. coli*,[6] *S. pneumonia*,[7] *S. aureus*,[8] *L. monocytogenes*,[9] *P. mirabilis*,[10] and *Enterococcus faecalis*.[11] It has been shown that disruption of the $Zn^{2+}$-uptake systems, ZnuABC importers, affects bacterial virulence and pathogenesis. For example, it has been demonstrated that ZnuC plays a role in a bacteria's ability to cause infection. The deletion of *S. enterica* Serotype Typhimurium *znuC* leads to reducing bacterial virulence in the *znuC* mutant strain compared to the WT strain in BALB/c mice. This indicates that this $Zn^{2+}$ -uptake system plays a critical role during mouse infection.[33] In addition, a study of ZnuC in *P. mirabilis* showed that the $Zn^{2+}$-binding ATP-binding protein, ZnuC, enables the bacteria to grow better in a condition of $Zn^{2+}$ limitation. In that study, ZnuC was also shown to play a required role in *P. mirabilis* motility.[10] It has also been shown that this metal ion is important in bacterial colonization, such as in *S. pneumoniae*: a reduction of $Zn^{2+}$ uptake via deletion of both the $Zn^{2+}$-uptake gene *adcA* and a $Zn^{2+}$-binding lipoprotein, *adcAII*, prevented *S. pneumoniae* from colonizing the nasopharynx of mouse models or causing pneumonia and sepsis in mouse models.[34]

On the other hand, $Zn^{2+}$ also plays an essential role in the host cell self-defense mechanism.[35] A study showed that $Zn^{2+}$ is used by host cells to eliminate bacteria from the site of infection.[36] For example, it metal chelation from the wound is an effective means for inhibiting microbial growth inside abscessed tissue caused by *S. aureus* infections.[8]

## THE ROLE OF ZINC IN BACTERIAL VIRULENCE

### The effect of zinc limitation in bacterial biofilm formation

Biofilm formation is important for the survival of bacteria in hostile environments, since bacteria within a biofilm are usually more resistant to antibiotics and disinfectants than when in a planktonic form.[37] $Zn^{2+}$ has been shown to play a role in the ability of bacteria to produce a biofilm. It was found that $Zn^{2+}$ depletion via metal chelation specifically prevented biofilm formation in *Staphylococcus epidermidis* and in methicillin-resistant *S. aureus* (MRSA).[38,39] In this biofilm inhibition in these bacteria, $Zn^{2+}$ affected the bacterial surface protein Aap

(accumulation-associated protein) which contains a G5 domain (a self-association repeat sequence element). This G5 domain is $Zn^{2+}$-dependent; the limitation of the $Zn^{2+}$ effect on the activity of the G5 domain therefore disturbs the biofilm formation stages in *Staphylococcus* spp.[38] It has similarly been shown that $Zn^{2+}$ is required for SasG (a surface protein) mediated biofilm formation in *Staphylococcus* spp.[39]

$Zn^{2+}$ can also affect the bacterial pilus assembly and the production of curli, which is important in bacterial biofilm formation.[40] Labrie *et al.* showed that low concentrations of $Zn^{2+}$ in the medium inhibited biofilm production in *Actinobacillus pleuropneumoniae*.[41] In addition, the mutation of genes encoding L31 ribosomal protein YkgM and ZitA in *E. coli* greatly affected biofilm formation under fluidic conditions; but no inhibition was observed in static conditions.[40] Both YkgM and ZitA are Zur regulated genes. Other studies have shown that $Zn^{2+}$ chelation especially blocks the formation of biofilm of *S. epidermidis* and methicillin resistant *S. aureus*.[38] In contrast, some studies have found that the addition of $Zn^{2+}$ to growth media inhibits the formation of biofilms.[42] It has been discussed above that excessive amounts of $Zn^{2+}$ have a deleterious effect on the cell. In the case of biofilm production, it has been shown that the addition of small concentrations of $Zn^{2+}$ significantly inhibit biofilm formation in a dose-dependent manner in various bacteria,[42] which may indicate that there is an inhibition of bacterial growth due to a toxic level of $Zn^{2+}$. The role of $Zn^{2+}$ in biofilm production was also observed in *Salmonella enterica sv* Typhymurium grown in $Zn^{2+}$-limited conditions.[43] In these growth conditions, there was an increased production of the quorum sensing signal AI-2, indicating that there was a reduction of biofilm formation ability.[43]

## The effect of zinc limitation on bacterial motility

$Zn^{2+}$ has also been shown to play a role in the motility of various bacteria.[6,10,44] Sub-optimal levels of $Zn^{2+}$ can affect the motility of a bacterial cell. The ability of *E. coli* to migrate is affected by an inactivation of the $Zn^{2+}$ transport systems.[6] A study of *P. mirabilis* also showed that swimming/swarming motility requires an effective $Zn^{2+}$ acquisition system.[10] The presence of a functional $Zn^{2+}$-binding ATP-binding protein, ZnuC, allows *P. mirabilis* to grow better under $Zn^{2+}$ limitation and ZnuC has been shown to be required for *P. mirabilis* motility.[10] In *S. pneumoniae*, a reduction of $Zn^{2+}$ uptake via deletion of both the $Zn^{2+}$-uptake gene *adcA* and a $Zn^{2+}$-binding lipoprotein, *adcAII*, prevented *S. pneumoniae* from establishing an infection in mouse models.[34] $Zn^{2+}$ has been shown to affect the expression of FliC, the structural subunit of *Salmonella* phase 1 flagella, in a *znuABC Salmonella enterica* sv. Typhimurium mutant strain grown in $Zn^{2+}$ starvation culture, which caused a reduction in motility.[43] It is suggested that $Zn^{2+}$ is involved in the initiation of the transcriptional regulatory of flagella assembly.[43] The down-regulation of all the genes involved in the biosynthesis of flagella indicates that this metal ion is an essential cofactor of the protein in this pathway.[43]

## The effect of zinc limitation on bacterial antibiotic sensitivity

$Zn^{2+}$ acts as a cofactor for various enzymes, including enzymes which are involved in resistance to antibiotics. β-lactamase is one of the enzymes which is produced by most Gram negative bacteria. This enzyme contributes to bacterial antibiotic resistance by inactivating β-lactam antibiotics via catalyzing hydrolysis of the four-membered β-lactam ring.[45] There are two group of β-lactamases: serine β-lactamases and metallo-β-lactamases (MBLs). The MBLs require one or two $Zn^{2+}$ ions in their active sites to catalyze the hydrolysis of all classes of β-lactam antibiotics, including penicillins, cephalosporins, and carbapenems.[45] The role of $Zn^{2+}$ in β-lactamase activity is well known, especially for MBLs, where the MBL enzymes are inactive in the absence of $Zn^{2+}$. A previous study showed that adding $Zn^{2+}$ reverses the antibiotic resistance of a carbapenem-resistant strain of *A. baumannii*.[46]

## The effect of zinc in bacterial survival against oxidative stress

Reactive oxygen species (ROS), such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals, are generated by the partial reduction of oxygen.[47] Redox cycling reactions of active metal ions such as $Fe^{2+}$, $Cu^{2+}$, chromium ($Cr^{2+}$), $Co^{2+}$ and $Zn^{2+}$ produce reactive radicals. These metal ions can also function as signal mediators in signalling pathways in eliminating the deleterious effects on the cells of redox reactions. Therefore, perturbing the intracellular homeostasis of the metal ions may also lead to the cells undergoing an oxidative stress and may also increase the formation of reactive oxygen radicals.[48] Specifically, the role of metal $Zn^{2+}$ in cell protection is carried out via its antioxidant function. $Zn^{2+}$ is a redox inert metal; even though this ion is not involved in oxidation-reduction reactions, this metal ion is involved in the protein sulphhydryl groups protecting against free

radical attack.[48] $Zn^{2+}$ may also inhibit the formation of free radicals by competition with redox-active metals such as $Fe^{2+}$.[49,50] It has also been suggested that the $Zn^{2+}$-uptake protein, ZosA, protects thiols from oxidation.[51]

## SUMMARY

$Zn^{2+}$ is a trace element which is crucial for a range of metabolic processes within bacterial cells. Maintaining a sub-toxic level of intracellular $Zn^{2+}$ is crucial for bacterial growth and survival. Bacteria use a global regulator Zur to control the amount of $Zn^{2+}$ entering the cells or chelating $Zn^{2+}$ from the cytoplasm. Zur regulates the $Zn^{2+}$ -import and $Zn^{2+}$-export system. There are two types of $Zn^{2+}$-import systems: the high-affinity $Zn^{2+}$-uptake systems, which belong to the ABC transporter family, and the low-affinity import systems. The $Zn^{2+}$ protein export system families which have been identified include the CDF, RND, and P-type ATPases families. $Zn^{2+}$ has a significant role in bacterial pathogenesis and virulence. The ability of bacteria to maintain a homeostasis of $Zn^{2+}$ is essential to their survival in hostile environmenst and to their success as pathogens.

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