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Mini review

Environmental effects on enzyme efficiency involved in bacterial defence systems

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Summary. Studies on bacterial defence systems are crucial for understanding their mechanism of action and thus, for development of more efficient anibiotics. The enzymes included in these processes are often metalloenzymes, with a metal ion cofactor in their active center. The function of these enzymes is naturally affected by the availability of metal ions. Nevertheless, those enzymes suggested to be metal ion independent, may also be influenced by metal ions encountered in the biological environment. Two types of bacterial defence systems are discussed in this review. (1) TEM-1 β-lactamase, protecting bacteria from β-lactam antibiotics, is not a metalloenzyme but it offers potential sites for metal ion binding. (2) NColE7 is the nuclease domain of the colicin E7 bacterial toxin, being a metallohydrolase purified together with Zn(II). It is suggested by the collected results that non-native metal ions may modify the catalytic mechanism, providing a chance to design more efficient antibiotic compounds.

Keywords: colicin E7 nuclease domain, bacterial defence, metal ions, TEM-1 β-lactamase.

METAL IONS AFFECT THE BIOACTIVITY OF COORDINATING MOLECULES

Metal ions in biological systems play diversified roles. By coordination to electron pair donor groups of various ligands, such as drug molecules, small molecular components of sera and cells, as well as macromolecular components (*e.g.*, proteins, nucleic acids), metal ions may influence their overall structure or conformation, activate or inhibit their reactions. Metal ions may directly participate in hydrolytic or redox reactions depending on their properties and complexation modes. While it is quite general to neglect the possibility of the interaction of organic drug molecules or metal ion independent enzymes with metal ions, such conjunctions may alter/determine the mechanism of action.

A nice example of the importance of the drug – metal ion interaction is provided, for example, by anticancer chemotherapeutics targeting multidrug resistant cells. These cells overexpress the P-glycoprotein pump, which provides resistance against the drug molecules by promoting their efflux from the cell. However, the efflux of those drug molecules, that are able to chelate *e.g.*, Fe(III) ions, causes iron deprivation and, thereby, inhibition of cell proliferation (Pape et al. 2021). This emphasises the importance of the maintenance of the metal ion homeostasis in each type of cells (Fnu and Weber 2021). Such chelator drugs may also act through binding to iron-containing histone demethylase active center (Rai et al. 2012). On the other hand, complexation with oher metal ions may also play a role in their anticancer function (Richardson DR. 2002; Heffeter et al. 2019). The systematic study of the metal ion complex formation of drug molecules contributes to better understanding of their multiple pathways of action (Kovács et al. 2024).

Similarly, the interaction of metal ions with enzymes, in

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spite of the hypotheses that they carry out their functions independently of metal ions, may also lead to changes in catalytic efficiency (Sun et al. 2024; Faixová and Faix 2002). Metal ions are directly involved in approximately half of enzymatic reactions. Metal ion exchange in metalloenzymes may lead to severe changes in the function of the enzyme (Mordasini et al. 2003; Sas et al. 2006; Shabani et al. 2011; Prejanò et al. 2020), while the new metal ion may still allow for catalytic activity, if the replacement metal ion has similar properties to the native one (Bauer et al. 1997; Paul-Soto et al. 1999; Hemmingsen et al. 2001). In most of the experiments with non-native metal ions, inhibition of metalloenzymes was observed in the presence of foreign metal ions, but in few cases the enzyme showed enhanced activity (Robertson and Villafranca 1993; Medyantseva et al. 1998; Mahmoudi et al. 2003; Han et al. 2008; Deb et al. 2013; Srivastava and Anand 2015). The quantitative characterization of these effects is made difficult by the multiple interactions of metal ions with the enzyme either at the active center, substrate binding pocket or at an allosteric metal ion binding site, causing structural changes (Wright et al. 2007; Valasatava et al. 2018), as well as with the substrate, intermediate or product of the catalytic reaction or with, other molecules in the reaction mixture possessing metal ion complexation ability, such as buffering molecules (Nagaj et al. 2013; Zawisza et al. 2013; Leite et al. 2000; Forero et al. 2023). These effects also depend on the concentration of the components, pH, and temperature of the reaction mixture (Medyantseva et al. 1998; Kiss et al. 2012; Ferreira et al. 2015). Furthermore, the dynamics of the active site of a metalloprotein can be modified by the foreign metal ion (Czyrko et al. 2018; Balogh et al. 2020).

Applications of (metallo)enzyme targeting are widespread, ranging from medicinal to environmental, agriculture or industrial fields (van Assche and Clijsters 1990; Łukowski and Dec 2018; Guo et al. 2019; Ivošević DeNardis et al. 2019; Golub et al. 2022; Moianos et al. 2023) On this line, recently, we have studied the effect of non-endogeneous/non native metal ions on the catalytic activity of two enzymes participating in the bacterial defence system (Nafaee et al. 2023a, 2023b, 2023c). TEM-1 β-lactamase and the nuclease domain of the colicin E7 (NColE7) are both hydrolytic enzymes cleaving β-lactam antibiotics and DNA, respectively, to promote bacterial survival under stress conditions.

INTERACTIONS OF TEM-1 B-LACTAMASE WITH Ni(II), Cd(II), AND Hg(II)

Similarly to many other proteins, that are supposed to function independently of metal ions, TEM-1 β-lactamase offers a large number of donor atoms for potential metal ion binding. Fig. 1 highlights the amino acids possessing coordinating side-chains. In addition, the amide oxygens and nitrogens may also offer complexing sites. As a plasmid mediated enzyme, TEM-1 β-lactamase is found in various bacterial species, easily acquires mutations and developes into extended-spetrum β-lactamases (Bradford 2001; Weinreich et al. 2006; Jacquier et al. 2013). Therefore, TEM-1 β-lactamase is a frequent target of antibacterial studies. Furthermore, the mutations may lead to new metal ion binding sites. In spite of this, no experiments with TEM-1 β-lactamase considering the possible effects of metal ions on its enzymatic activity are available in the literature, apart form a recent publication on Ni(II), Cd(II), and Hg(II) complexation with the enzyme (Nafaee et al. 2023a).

Interaction of TEM-1 β-lactamase histidines with metal ions was already proven by immobilized metal ion (Zn(II) and Ni(II)) affinity chromatography (Lawung et al. 2001; Yang et al. 2020; Nafaee et al. 2023c). Mass spectrometry revealed that one Ni(II) is bound strongly, but up to three metal ions can bind at tenfold metal ion excess (Nafaee et al. 2023c). The soft sulphur-containing methionine residues may coordinate the soft Cd(II) and Hg(II) ions. The slight change observed in the circular dichroism spectra upon interaction with metal ions suggested negligible change of the protein secondary structure, either because of the metal ion binding to surface histidines (Ni(II) and eventually Cd(II)), or to weak binding to sulphur donor groups (Hg(II) or eventually Cd(II)). Indeed, mass spectrometry and ^{119m}Hg perturbed angular correlation spectroscopy detected weak binding of Hg(II) (Nafaee et al. 2023a).

Catalytic experiments were carried out with ampicillin as a substrate. Slow, non-catalytic conversion of the primary product of the hydrolytic reaction (ampicilloic acid) to a decarboxylated secondary product (ampilloic acid) further complicated the evaluation (Fig. 2). Ni(II) and Cd(II) clearly promoted the catalytic activity of the enzyme, while Hg(II) had an inhibitory effect in a concentration dependent manner (Nafaee et al. 2023a). Fig. 3 shows this phenomenon for the Ni(II)-containing systems. It was suggested that Ni(II) and Cd(II) ions, being close to the substrate binding site, could activate ampicillin for hydrolysis due to their Lewis acidity (Gensmantel et al. 1980; Deshpande et al. 2004), in a manner similar to many hydrolytic metalloenzymes. On the other hand, Hg(II) bound to the soft sulfur donor groups (Deshpande et al. 2004) close to the active center of the enzyme, could hinder the catalytic action (Nafaee et al. 2023a).

Metal ion interaction with ampicillin was supported by *E. coli* growth assays in minimal media. While Cd(II) affected the bacteria only in the mM concentration range and small starting number of cells in the culture, Hg(II) and Ni(II) inhibited the growth of the cells even at μM concentrations. Hg(II) proved to be the most toxic among the

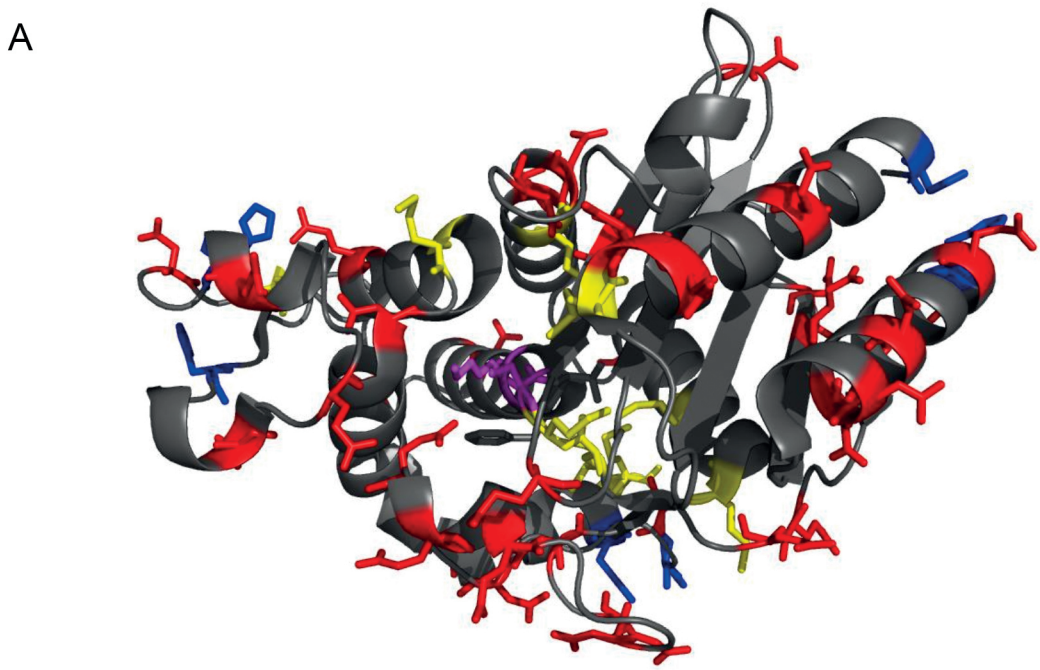


Fig. 1. A, Crystal structure of mature TEM-1 β-lactamase (PDB ID: 1ZG4). The active site amino acids Ser70, Lys73 are magenta; the O-donor providing carboxylate-containing Asp and Glu amino acids are red; the His amino acids with N-donor atoms are blue and the S-donor atom containing Cys and Met amino acids are yellow, all highlighted by stick representation. **B**, The sequence of the investigated enzyme with the active site residues and the electron pair donor containing amino acids highlighted by the same colors as in panel **A**.

applied metal ions (Nafaee et al. 2023a). In parallel experiments, including ampicillin, the antibiotic could surprisingly neutralize this toxic effect, most probably by complexing the metal ions and forming less toxic agents – with Hg(II) at the highest efficiency. It shall be noted that the metal ion concentration in the *in vitro* catalytic experiments described above, was three orders of magnitude less than in the cell culture. Therefore, their interaction with the substrate was less pronounced in the *in vitro* catalytic experiments.

INTERACTIONS OF NColE7 WITH Cu(II), Cd(II), AND Ni(II)

NColE7 is the nuclease domain of the colicin E7 bacterial toxin. Contrary to TEM-1 β-lactamase, NColE7 is a metalloenzyme purified together with Zn(II) (Czene et al. 2014). The sequence of the enzyme is shown in Fig. 4 together with its structure. The protein was purified in its inactive complex with immunity protein (Im7) in most of the studies (Ko et al. 1999; Doudeva et al. 2006; Huang and Yuan 2007). This resulted in a loss of the metal ion upon acidification step for the separation of the two proteins. However, when it was both purified and crystallized together with Im7, Zn(II) was also found in the active center (Ko et al. 1999). Still, there is a debate about the metal ion cofactor, as for the closely related NColE9 enzyme, with highly similar active site, Ni(II) or Mg(II) ions were proposed to be necessary for the catalytic activity (Pommer et al. 1998, 1999; Maté and Kleanthous 2004).

Surprisingly, the purified apo-form was found to be more active than the Zn(II)-bound form (Nafaee et al. 2023b). This form was established by admixture of ethylenediaminetetraacetate (EDTA – a strong chelating agent to remove the metal cofactor) excess in previous studies, while in theory one equivalent of EDTA should result in complete removal of the metal ion, due to the very high stability of its Zn(II) complex compared to those formed with proteins. The negatively charged EDTA excess, however, interacts with the

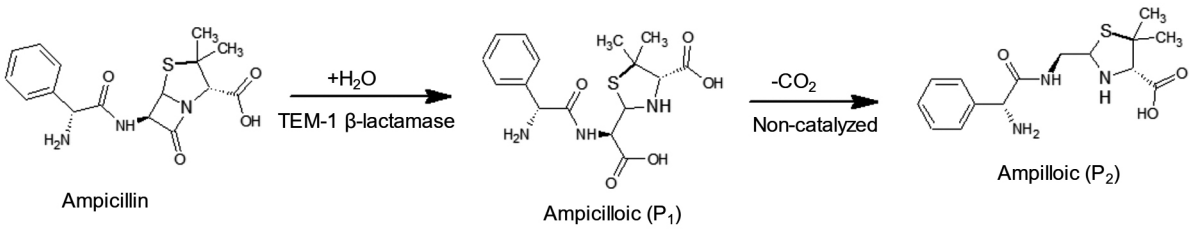


Fig. 2. The schematic structures of the substrate, and the primary (P_1 = ampicilloic acid) and secondary (P_2 = ampilloic acid) products of the reaction taking place in the catalytic assay.

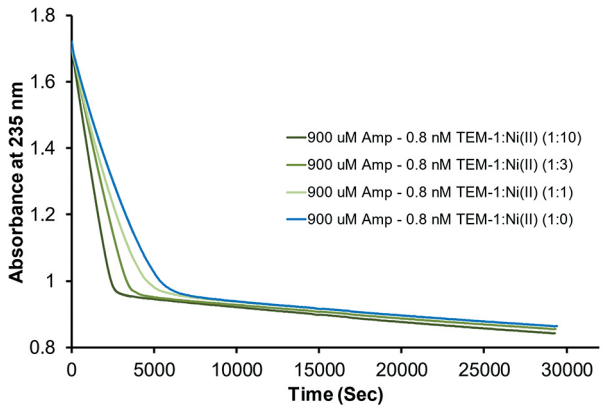


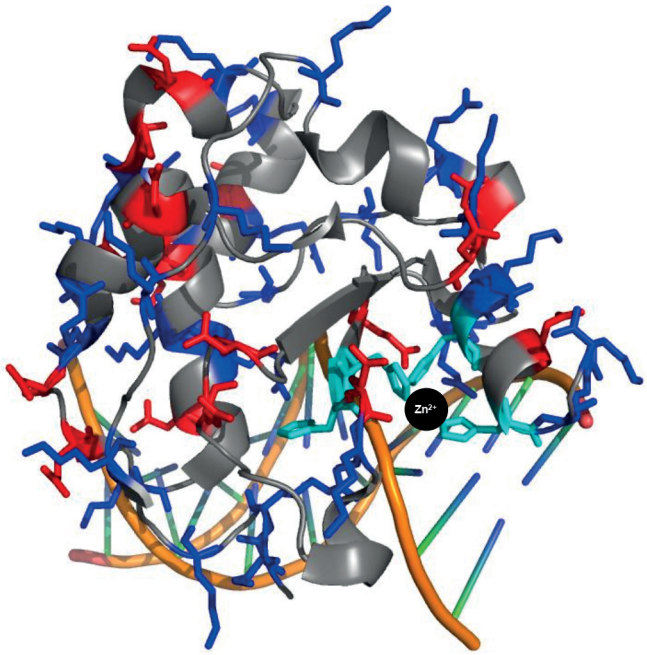
Fig. 3. The concentration dependent effect of Ni(II) ions on the hydrolytic process of ampicillin catalyzed by TEM-1 β-lactamase in 50 mM phosphate buffer (pH 7.0) at 30 °C. In the parallel experiments 900 μM ampicillin was hydrolysed by ~0.8 nM enzyme in the presence of 0, 1, 3 and 10 equivalents of Ni(II) referred to the enzyme concentration.

positively charged side-chains of the enzyme, which in fact are enriched in the substrate (DNA) binding site (Nafaee et al. 2023b). Similar binding mode was observed earlier with phosphate ions bound to an NColE7 mutant in its crystal structure (Czene et al. 2014). Therefore, EDTA excess could also inhibit the enzyme by preventing the substrate binding through competition. Although the enzyme is less active in its Zn(II)-bound form, it is still very active and can kill the bacterial cells. It is worth mentioning that the enzyme is inhibited by an excess of metal ion, most probably due to weak coordination to the fourth histidine, preventing the production of the nucleophylic OH^- ions.

Mainly due to the ambiguity of the identity of the metal ion cofactor, the effect of non-native metal ions on the structure and catalytic activity of the enzyme was checked. Both the native NColE7 enzyme and its R447G mutant showed the same behavior in the experiments. R447 is an important residue at the N-terminus of the enzyme – the opposite end of the sequence relative to the active site at the C-terminus, that comes close to the active center and activates the substrate in the catalytic process. Experiments were carried out with both the native NColE7 enzyme and its R447G mutant but the evaluation of the changes in the catalytic efficiency was rather straightforward for the mutant due to its decreased activity (Nafaee et al. 2023b). Small but significant changes in the circular dichroism spectra were observed upon addition of Zn(II) to both the native and mutant apoenzymes (Németh et al. 2014). Fig 5. shows that Cu(II) and Cd(II) ions caused similar changes to those observed with Zn(II), but the effect of Mg(II) is negligible, due to the hard character of this metal ion preventing strong binding to the histidines in the active center. This behavior made it impossible to follow competition reactions between Zn(II) and other metal ions by this method (Nafaee et al. 2023b). Mass spectrometry could unequivocally prove the binding of a Zn(II) to the active center, which could not be replaced by Cu(II), Ni(II) or Cd(II), although Cu(II) forms complexes of three orders of magnitude higher stability (log*K* ~ 8: Kállay et al. 2006, 2009; Jakab et al. 2008; Valensin et al. 2009; Fragoso et al. 2013; Székely et al. 2024) with flexible peptides containing three histidines than Zn(II) (log*K* ~ 5: Jakab et al. 2008; Kállay et al. 2009; Miller et al. 2018). This supports that the metal ion binding site of NColE7 is by nature optimized for Zn(II) binding (Németh et al. 2015). On the other hand, Cu(II), Ni(II) or Cd(II) could occupy the catalytic site upon prior removal of Zn(II) ions (Hannan et al. 1999; Kleanthous et al. 1999; Doudeva et al. 2006; Nafaee et al. 2023b).

In the catalytic activity experiments, addition of 1 eq of Cu(II) ions to the apo KGNK mutant enzyme had similar effect to Zn(II), while 1 eq of Cd(II) ions did not decrease the hydrolytic activity to the same extent due to the lower affinity towards the catalytic site than Zn(II), as supported by mass spectrometry (Nafaee et al. 2023b). The enzyme became extremely active in the presence of 1 eq of Ni(II) ion, and could not be significantly inhibited by the excess of Ni(II) ions, similar to the WT NColE7 (Fig. 6). This suggested that the exchange of the metal ion altered the mechanism of action of the enzyme. While in the Zn(II)-containing enzyme the role of the metal ion is suggested to bind and activate

A



B

GPLGSPEFKRNKPGKATGKGKPVNNKWLNNAGKDLGSPVPDRIANKLRDKEFKSFDDFRKKFWEE

VSKDPELSKQFSRNNNDRMKVGKAPKTRTQDVSGKRTSFELHHEKPISQNGGVYDMDNISVVTPKR

HIDIHRGK

Fig. 4. A, Crystal structure of NColE7 (PDB ID: 3FBD). The active site histidines are light blue (three of them are coordinated to Zn(II) and the fourth His residue promotes water deprotonation for nucleophylic attack); the negatively charged O-donor providing carboxylate-containing Asp and Glu amino acids are red; the positively charged N-donor atom containing Lys and Arg amino acids are dark blue (the positively charged amino acids rarely participate in metal ion coordination, in spite of their N-donor atom content), all highlighted by stick representation. B, The sequence of the investigated enzyme using the same color code for highlighting the amino acids as in panel A.

the substrate molecule and the nucleophilic hydroxyde is generated by a fourth non-coordinating histidine side-chain close to the active center (Huang and Yuan 2007), in Ni(II)-containing enzyme the water molecule is supposed to be acitvated directly by the metal ion neighbouring the scissile phosphodiester group (Doudeva et al. 2006).

CONCLUSIONS

The above findings suggest that the non-native metal ions in their environment may have a significant effect on both metal ion dependent and independent enzymes. Surprisingly, both types of enzymes are often activated by metal ions, while most of the literature studies reported inhibiton effect of the foreign metal ions. This study draws the attention to the importance of the properties of metal ions and donor groups, as well as concentrations ranges of the interacting agents applied in the reaction mixtures. Understanding of the environmental effects on the enzymatic function and eventual modification of the catalytic mechanism by the foreign metal ion may provide a chance to design more efficient antibiotic compounds in the future. Although the available information on the synergic effect of metal ions and antibiotic drugs is limited yet, the research in this field will contribute to the wise application of food supplements containing essential trace elements together with antibiotic ligands and vitamins. Such novel ‚antibiotic cocktails' may be efficient against antibiotic resistant bacterial strains that express, for example, extended-spectrum β-lactamases and evolve mainly in the clinics, causing serious health threat. In parallel, the tuning of the nuclease effect and development of novel metallonucleases may find widespread biotechnological applications in the future by themselves in the fight against bacteria, viruses or cancer cells, or as controlled

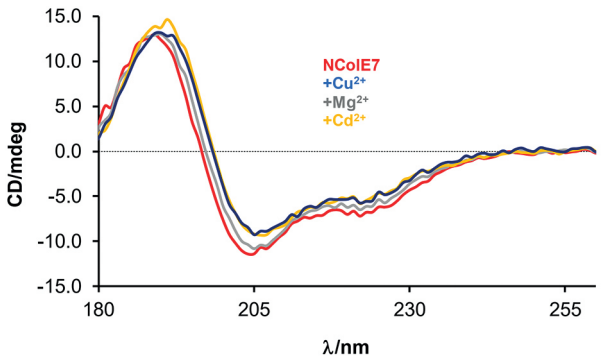


Fig. 5. Circular dichroism spectra of NColE7 and its complexes with selected metal ions. The spectra were recorded at AU-CD beamline of the ASTRID2 synchrotron at the ISA, Aarhus University, Denmark (Miles et al. 2007, 2008) using camphor-sulfonic acid for calibration of the instrument. All spectra were recorded with 1 nm steps and a dwell time of 2 s per step, using a 0.2 mm pathlength quartz cell (SUPRASIL, Hellma GmbH, Germany) in the wavelength range of 180–260 nm. The raw spectra were baseline-corrected with a water, or an appropriate buffer, spectrum. The protein concetration was kept constant at 32 μM and the metal ion-to-protein ratio was 3:1.

domains of artificial nucleases (Chandrasegaran and Carroll 2016; Németh et al. 2018; Dixit et al. 2024; Pacesa et al. 2024), with possible applications in gene engineering.

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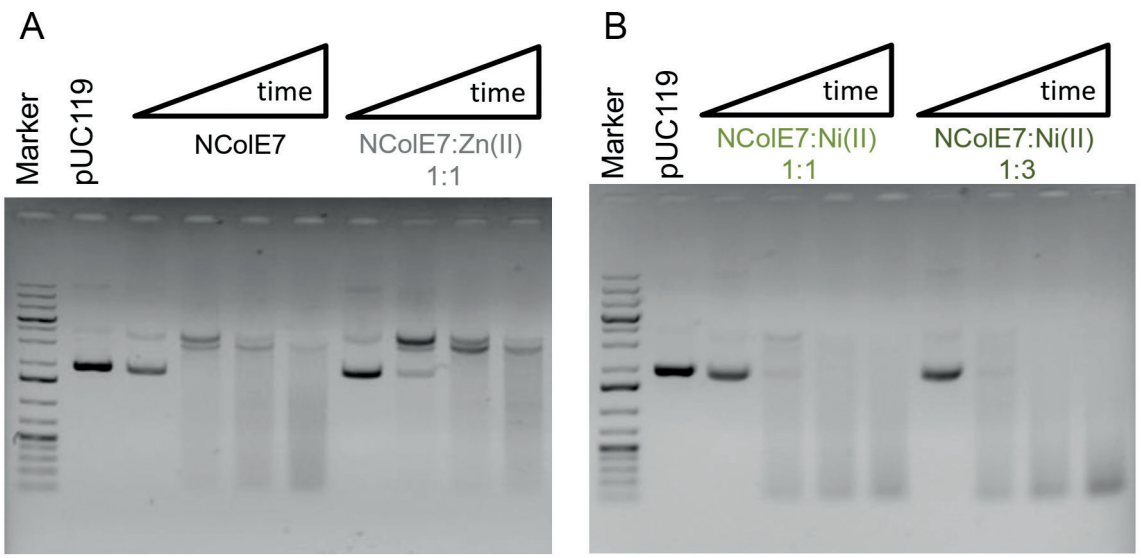

Fig. 6. Detection of the pUC119 plasmid DNA (c = 74 μM for base pairs (bp); 3162 bp) cleavage by 0.28 μM NColE7 enzyme using 1% (w/v) agarose gel electrophoresis in the absence and presence of various metal ions. A, DNA cleavage with the apo-NColE7 enzyme only and in the presence of Zn(II) ions at 1:1 molar ratio. B, DNA cleavage with NColE7 enzyme in the presence of Ni(II) ions at 1:1 and 1:3 enzyme-to-metal ion molar ratios. Each well of the agarose gel in a cleavage experiment represents the catalytic activity after 0, 15, 60, or 120 min, from left to right. The untreated pUC119 plasmid was loaded on each gel as a negative control.

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