

YcdY Protein of *Escherichia coli*, an Atypical Member of the TorD Chaperone Family[∇]

David Redelberger,¹ Farida Seduk,¹ Olivier Genest,¹ Vincent Méjean,¹
Silke Leimkühler,² and Chantal Iobbi-Nivol^{1*}

Laboratoire de Chimie Bactérienne, IFR88-CNRS, Aix-Marseille University, 13402 Marseille Cedex 20, France,¹ and Institute of Biochemistry and Biology, University of Potsdam, 14476 Potsdam, Germany²

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The TorD family of specific chaperones is divided into four subfamilies dedicated to molybdoenzyme biogenesis and a fifth one, exemplified by YcdY of *Escherichia coli*, for which no defined partner has been identified so far. We propose that YcdY is the chaperone of YcdX, a zinc protein involved in the swarming motility process of *E. coli*, since YcdY interacts with YcdX and increases its activity *in vitro*.

Prokaryotic molybdoenzymes require specific chaperones for both protection and maturation (3). Using the TorD chaperone of *Escherichia coli* as a model, we found that TorD protects the apofom of its partner TorA, a trimethylamine oxide (TMAO) reductase and maintains it in a competent conformation that allows the insertion of the molybdenum cofactor into the catalytic site (4–6, 8). It was also proposed that these chaperones prevent premature targeting of their partner by a quality control activity (11, 19). These accessory proteins belong to the large TorD family that can be divided into five subfamilies (3, 9, 18). The TorD, DmsD, and NarJ subfamilies are genetically linked to the TMAO, dimethyl sulfoxide (DMSO), and nitrate reductases, respectively, while FdhX is the chaperone of certain formate dehydrogenases (3; our unpublished results). The fifth subfamily including the YcdY homologs does not seem to be genetically connected to molybdoenzymes. In *E. coli*, no molybdoenzyme coding gene was found in proximity to *ycdY*. Two genes are present upstream of *ycdY*, namely, *ycdX* and *ycdW*. *ycdX* codes for a 27-kDa protein, and its structure has been solved (17). YcdX contains a trinuclear zinc catalytic site, and it belongs to the polymerase histidinol phosphatase (PHP) protein superfamily, but its physiological role is yet unknown. In contrast, YcdW was characterized as a NADPH-linked glyoxalate reductase in *E. coli* (15). Surprisingly, it was proposed that YcdY is required for swarming motility (10). In this study, we show that YcdX is also involved in swarming and that YcdY acts as a chaperone for YcdX.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains used in this work are BL21(DE3) [*F*[−] *ompT hsdS_B(r_B[−] m_B[−]) dcm gal(DE3)*] (Novagen), BTH101 [*F*[−] *cya-99 araD139 galE15 galK16 rpsL1 (Str^r) hsdR2 mcrA1 mcrB1*] (12), and from the Keio collection (1), the wild-type strain (BW25113) and derivatives JW1018 (*ycdY*), JW1017 (*ycdX*), and JW5146 (*ycdW*). The mutant strains were checked by PCR. The strains were grown in Luria broth (LB)

medium or M9 medium as specified in the text. When necessary, ampicillin (50 μg · ml^{−1}) was added to maintain plasmid selection.

Swarming experiments. Semisolid medium was made of tryptone (10 g/liter), glucose (5 g/liter), NaCl (10 g/liter), and agar (0.4%) (10). The plates were dried for 15 min at 43°C. Cell samples (2 μl) from an exponentially growing culture were inoculated, and the plates were incubated at 24°C for 48 h.

Plasmid constructions. After PCR amplification of their coding sequence, the *ycdY* and *ycdX* genes were cloned into the KpnI-Sall cloning sites of pET-52b(+) vector (Novagen), resulting in pTycdY and pTycdX plasmids, respectively. These constructions led to the fusion of a streptavidin (Strep) tag StrepTagII (IBA) to the N-terminal extremity of the proteins, allowing the production of Strep-tagged YcdY and YcdX proteins. Transformations into strain BL21(DE3) were carried out by the method of Chung and Miller (2).

Two-hybrid assays. The PCR products of the *ycdX* and *ycdY* genes were fused to the sequences encoding T18 and T25, the two catalytic domains of the adenylate cyclase, using plasmids pEB355 and pEB354, respectively (12). After cotransformation with the two plasmids expressing the fusions, the BTH101 strain (*cya*) was grown overnight at 30°C in LB medium supplemented with ampicillin (125 μg · ml^{−1}), kanamycin (40 μg · ml^{−1}), and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The efficiency of the interaction was quantified by measuring β-galactosidase activity by the method of Miller (13).

Chemical cross-linking experiments. Interaction of YcdY with YcdX was tested using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as a cross-linker. Proteins (1 mg/ml) and EDC (5 mM) in a final volume of 20 μl were incubated for either 10 or 30 min at room temperature. The interactions were analyzed by SDS-PAGE and revealed by Coomassie blue staining.

Purification of recombinant proteins. Recombinant YcdY and YcdX were purified from the soluble extract of strain BL21(DE3) containing either pTycdY or pTycdX grown aerobically at 37°C and induced for 4 h with IPTG (1 mM). The StrepTagII fused to the N-terminal extremity of the proteins allows a one-step purification by affinity chromatography using streptavidin-coupled columns as described by the manufacturer (IBA). Proteins were eluted in buffer containing Tris-HCl (100 mM; pH 8), NaCl (150 mM), and desthiobiotin (2.5 mM).

Analytical procedures. Alkaline phosphatase activity was measured by incubating purified YcdX (1 μM) with *p*-nitrophenyl phosphate (pNPP) (0.1 M) in a Tris-HCl buffer (50 mM; pH 8) for 20 min at 37°C (16). The activity was measured spectrophotometrically at 410 nm. Phosphatase activity was expressed in nanomoles of hydrolyzed pNPP per minute and per milligram of protein. The commercial alkaline phosphatase used as a control was from Sigma.

Proteins were revealed after 15% SDS-PAGE by Coomassie blue staining. Protein concentration was measured using the Bio-Rad protein assay.

Trypsin digestion and MALDI-TOF MS. Polyacrylamide gel slices were put into a 96-well microplate (Greiner) for sample digestion. A robotic workstation (Freedom EVO 100; TECAN) was used to perform automated sample preparation including multiple steps: washes, reduction and alkylation, digestion by trypsin (proteomics grade; Sigma), and extraction and drying of mixed peptides (7). Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) protein identification was performed as previously described (5).

* Corresponding author. Mailing address: Laboratoire de Chimie Bactérienne, IFR88-CNRS, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France. Phone: 33491164427. Fax: 33491718914. E-mail: iobbi@ifr88.cnrs-mrs.fr.

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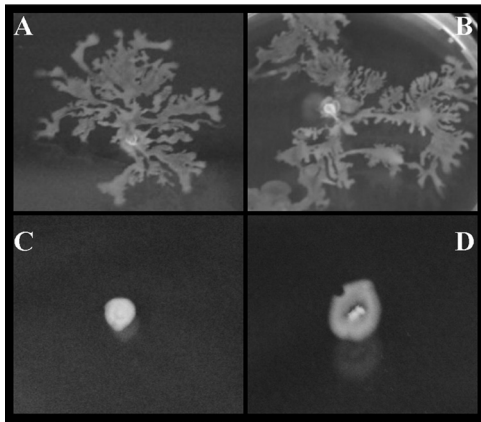


FIG. 1. Swarming motility phenotypes. *E. coli* bacteria (2 μ l) were inoculated onto semisolid medium and incubated for 48 h at 24°C. (A) Wild-type control (BW25113), (B) Δ *ycdW* mutant (JW5146), (C) Δ *ycdX* mutant (JW1017), (D) Δ *ycdY* mutant (JW1018).

Urea denaturation. To partially denature YcdX, the protein (31 μ M) was incubated with urea (5 M) at 70°C. After 1 h, the sample was dialyzed against buffer containing Tris-HCl (100 mM; pH 8) and NaCl (150 mM).

ICP experiments. Proteins (20 μ M) were incubated with a zinc solution (0.1 mM) for 2 h at 37°C. The excess zinc was then removed by dialysis against Tris-HCl buffer (50 mM; pH 8). Metal analysis was performed using a Perkin-Elmer Optima 2100DV inductively coupled plasma optical emission spectrometer (ICP-OES). The samples were wet ashed with 32.5% nitric acid (Suprapur; Merck) for 12 h at 100°C (14). Samples were filled to a 10-fold volume with water prior to ICP-OES analysis. The multielement standard solution XVI (Merck) was used as a reference.

RESULTS AND DISCUSSION

Genetic link between *ycdY* and *ycdX*. To better understand the function of YcdY and to check whether it is functionally related to the TorD chaperone family, we first examined its genetic environment. The *ycdY* gene most probably belongs to a three-gene operon. Indeed, *ycdY* is preceded by two other genes transcribed in the same orientation, *ycdW* and *ycdX* (<http://genolist.pasteur.fr/Colibri/>). The distances between the *ycdW* and *ycdX* genes and the *ycdX* and *ycdY* genes are 53 and 23 nucleotides, respectively. A promoter upstream from *ycdW* has been reported (15), but no obvious promoter upstream of either *ycdX* or *ycdY* has been found. This could indicate that these three genes are organized as a single transcriptional unit. The operon organization of *ycdW*, *ycdX*, and *ycdY* is also corroborated by a survey of bioinformatics prediction tools (<http://www.genome.sk.ritsumei.ac.jp/odb/> and www.operondb.cbc.umd.edu). Interestingly, from a search of a data bank (<http://www.genome.jp/kegg/ssdb/>), it is clear that when *ycdY* homologs are present, they are found close to *ycdX* homologs in 100% of the genomes, while *ycdW* can be missing. We thus concluded that *ycdY* is genetically and probably physiologically connected to *ycdX*.

Since the knockout of *ycdY* leads to a swarm defect (10), we checked the swarming phenotype of *ycdW* and *ycdX* gene knockout mutants. The *ycdY* (JW1018), *ycdX* (JW1017), and *ycdW* (JW5146) deletion mutants from the Keio collection (1) and the wild-type strain (BW25113) were inoculated on semi-solid agar medium. Figure 1 shows that the wild-type strain (BW25113) and the *ycdW* mutant present a normal swarming

phenotype, whereas *ycdY* and *ycdX* mutants show no swarming ability. The wild-type swarming phenotype of the *ycdW* mutant clearly indicates that there is no polar effect due to the drug resistance marker. Thus, *ycdY* and *ycdX* products are clearly involved in this swarming process. These observations support the idea of a relationship between the two proteins. Since YcdY is a member of the TorD family of specific chaperones, we investigated whether YcdY is involved in YcdX maturation.

Interaction between YcdY and YcdX. To test whether YcdY and YcdX were partners, the bacterial two-hybrid system based on functional reconstitution of adenylate cyclase activity was employed. *ycdY* and *ycdX* were cloned into the bait and prey vectors pT18 and pT25, respectively, and complementation experiments were performed. The results in Fig. 2 show that there is a signal of interaction between the two proteins. However, compared to the signal of the Zip interaction, the signal is weak, although it is at least three times higher than the background activities (Fig. 2A). To confirm the YcdX-YcdY interaction, we incubated each protein and a mixture of both proteins with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), a chemical cross-linker for 30 min. Results of the binding experiment were revealed by submitting the samples to 15% SDS-PAGE, followed by Coomassie blue staining. Figure 2B shows that an additional band appears in the lane containing the mixed proteins and that this band corresponds to the sum of the molecular masses (about 53 kDa) of the two Strep-tagged proteins. This additional band was present even after an incubation of 10 min (data not shown). The YcdX-YcdY complex was further analyzed by mass spectrometry. The global mass obtained by MALDI-TOF MS indicates that the mass of the complex ($53,374 \pm 100$ Da) matches the sum of the masses observed for the Strep-tagged YcdY and YcdX proteins, $23,715 \pm 50$ and $29,591 \pm 60$ Da, respectively. Moreover, trypsin digestion of excised gel slices followed by MALDI-TOF mass spectrometry indicated unambiguously that YcdY and YcdX were present in the additional band. Indeed, 9 and 7 different peptides covering 40% of the sequences of YcdX and YcdY, respectively, were identified (Table 1). These results confirm the presence of the two proteins after cross-linking and thus the interaction between them. In conclusion, results from both *in vitro* and *in vivo* experiments indicate that YcdY interacts with YcdX.

Role of YcdY in YcdX maturation. First, we tried to ascertain the enzymatic activity for YcdX. Since the trinuclear catalytic site of YcdX is similar to those of enzymes able to hydrolyze phosphoester bonds (17), we hypothesized that YcdX might have a similar activity. Thus, purified YcdX was tested in an assay for alkaline phosphatase activity, and the activity of YcdX was compared to that of alkaline phosphatase. As shown in Fig. 3A, YcdX is able to hydrolyze pNPP (135 nmol of hydrolyzed pNPP per min per mg of protein), and under our experimental conditions, its enzymatic activity corresponded to half of that of the commercial alkaline phosphatase (Fig. 3A). This indicates that phosphatase activity could be the physiological enzymatic activity of YcdX. In contrast, under these experimental conditions, YcdY showed no alkaline phosphatase activity (Fig. 3A).

To test whether YcdY influences the activity of YcdX, YcdY was included in the enzymatic assay. Thus, YcdY was incubated for 5 min with YcdX at 37°C prior to the addition of the

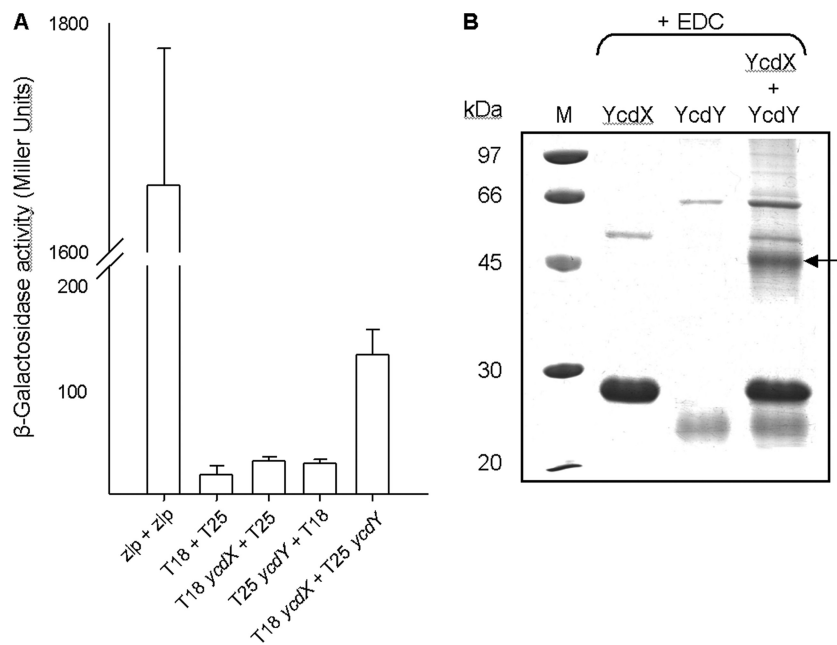


FIG. 2. YcdY interacts with YcdX. (A) Interaction between YcdX and YcdY analyzed by a bacterial two-hybrid approach. β -Galactosidase activities are expressed in Miller units. Error bars indicate standard errors (SE) ($n = 3$). (B) Purified proteins (1 mg/ml) were incubated with EDC (5 mM), a chemical cross-linker, for 30 min alone or together. Samples were submitted to 15% SDS-PAGE. Proteins were visualized by Coomassie blue staining. The arrow points to the cross-linked complex of YcdY and YcdX. The positions of molecular mass markers (M) (in kilodaltons) are shown to the left of the gel.

substrate. Figure 3A reveals that the presence of YcdY does not affect the level of YcdX enzymatic activity. To partially unfold YcdX, the enzyme was frozen and thawed twice before alkaline phosphatase activity was measured. Under these conditions, the activity showed no significant alteration (126 nmol of hydrolyzed pNPP per min per mg of protein). Interestingly, the presence of YcdY in the assay significantly increased YcdX activity (55%). A similar result was obtained when YcdX was partially denatured by using urea. Indeed, after dialysis, the urea-denatured YcdX possessed a decreased activity of about

50% (62 nmol of hydrolyzed pNPP per min per mg of protein [Fig. 3B]), and its zinc content analyzed by ICP-OES represented about 40% of that of native YcdX. Incubation of the denatured enzyme with either zinc (10 μ M) or YcdY did not modify the level of the enzymatic activity, while when both zinc and YcdY were added, the activity level reached 70% of that of the native protein (Fig. 3B). We thus propose that YcdY plays a role in the proper folding of YcdX or in zinc insertion.

YcdY does not bind zinc. To better understand the role of YcdY as a chaperone for YcdX, the YcdX apoenzyme was produced in strain BL21(DE3)/pTycdX grown in minimal medium in order to reduce zinc incorporation into YcdX. The enzyme was purified and exhibited residual activity (37 nmol of hydrolyzed pNPP per min per mg of protein) probably due to a remaining trace of zinc during growth (Fig. 4). Incubation of YcdX with a solution of Zn (10 μ M) did not modify the activity (30 nmol/min/mg). In contrast, when YcdX was incubated with YcdY, the activity increased twofold and even more when zinc was also present (53 and 71 nmol/min/mg, respectively), indicating that YcdY allows a better YcdX activity. Thus, YcdY might play a direct role during metal insertion into the YcdX catalytic site. We therefore checked whether YcdY was able to bind the metal to deliver it to YcdX. YcdY and YcdX, used as a control, were incubated with a zinc solution. Dialyzed samples were then submitted to ICP-OES analysis as described in Materials and Methods. Figure 5 clearly shows that the zinc content of YcdX was increased in the presence of YcdY, while YcdY binds only small amounts of zinc by itself and this binding is probably not significant. These data rule out the possibility of YcdY transporting or inserting the metal into the catalytic site of YcdX. However, YcdY facilitates YcdX mat-

TABLE 1. Peptide identification after trypsin digestion of the YcdY-YcdX cross-linked complex

| Protein | Positions ^a | Peptide sequence |
|---------|------------------------|---------------------------|
| YcdX | 62–70 | VVDGVGILR |
| | 71–77 | GIEANIK |
| | 111–136 | ATNTQAMATIASGNVHIISHPGNPK |
| | 137–142 | YEIDVK |
| | 151–166 | HQVALEINSSFLHSR |
| | 175–181 | EVAAAVR |
| | 208–218 | ILDAVDFPPER |
| | 219–225 | ILNVSPR |
| | 227–234 | LLNFLESR |
| YcdY | 10–17 | VLGSLYYR |
| | 18–32 | QPQDPLLVPLFTLIR |
| | 36–50 | LAANWPLEQDELLTR |
| | 80–92 | SAWVEDATEAEVR |
| | 93–98 | AFLSER |
| | 151–161 | VEAHATTPFWR |
| | 162–168 | TMAPLTR |

^a The start and end positions of the peptides on the protein sequence are shown.

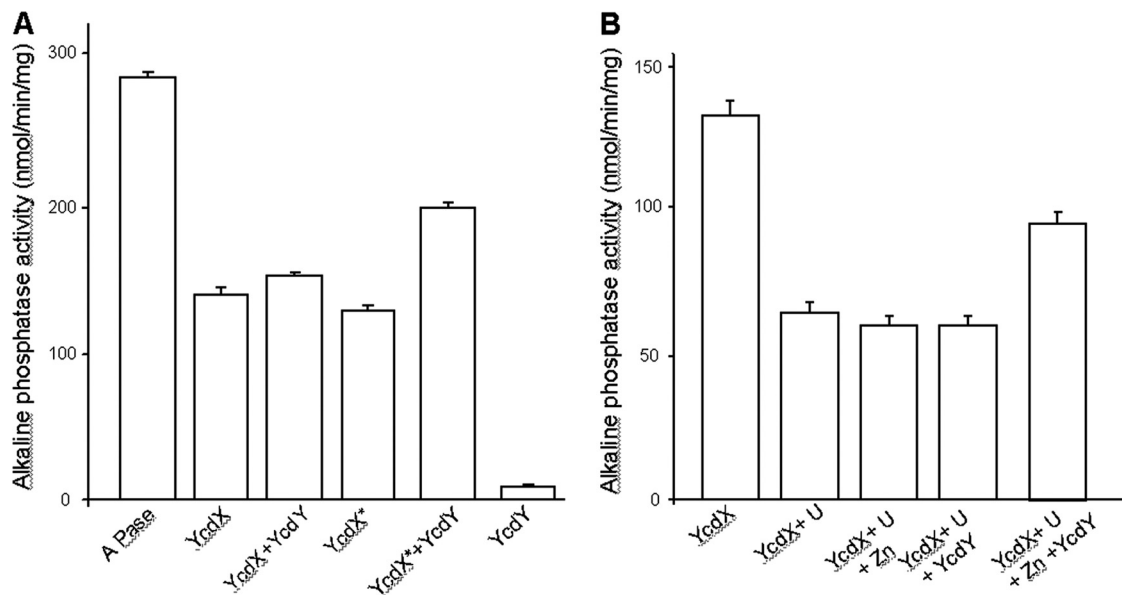


FIG. 3. Effect of YcdY on alkaline phosphatase activity of YcdX. (A) Alkaline phosphatase activity was measured in samples containing purified YcdX (0.125 nmol) or YcdX frozen and thawed twice (YcdX*) (0.125 nmol) in the presence or absence of YcdY (0.125 nmol). Alkaline phosphatase activities of commercial alkaline phosphatase (A Pasa) (0.125 nmol) and YcdY (0.125 nmol) were measured under the same conditions. Error bars indicate SE ($n = 5$). (B) Alkaline phosphatase activity was measured in samples containing purified YcdX (0.125 nmol), dialyzed YcdX treated with urea (U), and dialyzed YcdX treated with urea and incubated with either YcdY (0.125 nmol) or zinc (10 μ M) or both. Error bars indicate SE ($n = 3$).

uration, since the amount of incorporated zinc increased about 35% in the presence of YcdY. This point agrees with YcdY inducing an YcdX-competent form for metal binding.

In conclusion, this study showed that in contrast to the other members of the TorD chaperone family that are specific chaperones for molybdoenzymes, YcdY is genetically and physiologically linked to a zinc protein. YcdY cannot bind the metal, but it significantly increases YcdX phosphatase activity probably by inducing a specific active conformation of YcdX. We propose a model in which by binding YcdX, YcdY causes a fold in YcdX that facilitates the correct insertion of the zinc

into the catalytic site. Moreover, the presence of YcdY seems necessary for the physiological activity of YcdX, since deletion of one of the two genes leads to the same phenotype. We thus propose that YcdY is a specific chaperone like the members of the TorD family but that it is devoted to a zinc protein instead

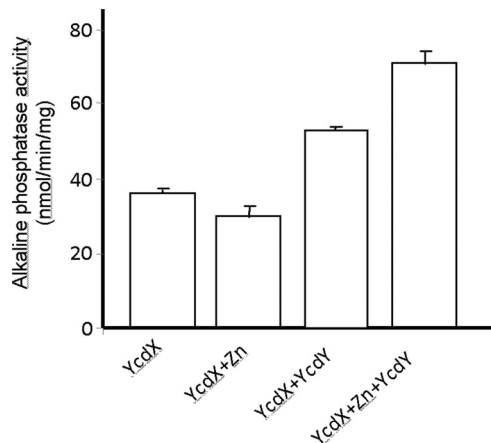


FIG. 4. Effect of YcdY on alkaline phosphatase activity of YcdX produced from minimal medium. YcdX (0.125 nmol) was purified from cells grown on minimal medium. Its activity was measured in the presence of either zinc (10 μ M) or YcdY (0.125 nmol) or both. Error bars indicate SE ($n = 4$).

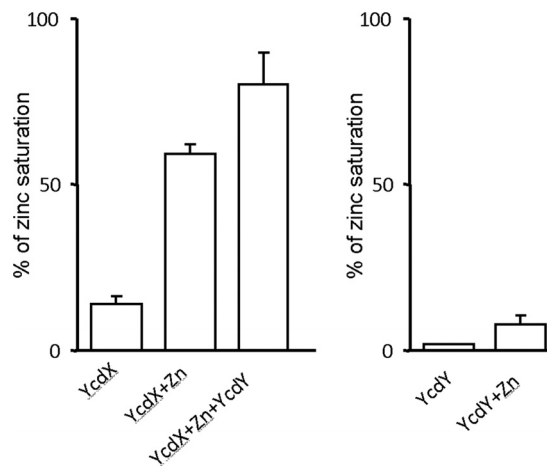


FIG. 5. YcdY increases zinc binding of YcdX. (A) ICP analysis was performed on samples containing purified YcdX (20 μ M) and purified YcdX (20 μ M) incubated with a zinc solution (0.1 mM) in the presence or absence of YcdY (20 μ M). The percentage of zinc incorporation corresponds to the ratio between the amount of zinc measured and the full zinc saturation of YcdX (3 Zn molecules per protein [17]). (B) ICP analysis was performed on samples containing purified YcdY (20 μ M) and YcdY incubated with a zinc solution (0.1 mM). The percentage of zinc incorporation corresponds to the ratio between the amount of zinc measured and the full zinc saturation of YcdY (1 Zn molecule per protein). Error bars indicate SE ($n = 3$).

of a molybdoprotein and does not bind the metal itself. This study thus demonstrates that the members of the TorD family are able to facilitate the insertion of different metals and metal-containing cofactors.

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