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# ZnuABC and ZosA zinc transporters are differently involved in competence development in *Bacillus subtilis*

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**Disruptants of genes encoding the ZnuABC high-affinity zinc incorporator and *zosA* encoding a P-type ATPase for zinc incorporation were identified to show low transformability. The low transformability of the *znuB* cells was rescued by excess zinc addition and epistatic analysis of the mutation revealed no effect on the expression of *comK*, which encodes a master regulator for late *com* operons. We further examined the expression of each late *com* operon in the *znuA* mutant and found that the *znuA* mutation specifically inhibited the expression of *comF*, but not the other late *com* operons. The addition of zinc also rescued the low transformability of the *zosA* cells. In *zosA* cells, transcription of *comK* was severely repressed. Using a strain carrying *comK* driven by a xylose-inducible promoter, we showed that the *zosA* mutation inhibited the post-transcriptional control of *comK*. The addition of zinc also rescued the defect of xylose-inducible *comK* expression in *zosA* cells, suggesting that post-transcriptional control of *comK* requires zinc incorporation. Taken together, we propose that the both ZnuABC- and ZosA-mediated zinc incorporation is involved in competence development, although the two zinc transporters are differently implicated in this developmental process.**

*Keywords*: ABC transporter/genetic competence/P-type ATPase/zinc homoeostasis.

*Abbreviations*: IPTG, isopropyl β-D-thiogalactopylanoside; MC, modified competence; ORF, open reading frame; PCR, polymerase chain reaction; SD, Shine–Dalgarno.

Zinc is an essential metal, but excess zinc is toxic for the cell (*1*). Zinc serves as a co-factor and/or structural component for various enzymes and regulatory proteins (*2*, *3*). Thus, the intracellular zinc concentration is tightly regulated by zinc transporters and zinc sensors. In the Gram-positive bacterium *Bacillus subtilis*, zinc importers, ZnuABC and ZosA are known, and the genes encoding the components for ZnuABC are negatively regulated by the zinc-sensor Zur functioning as a DNA-binding protein (*4*), (Supplementary Fig. S1). This regulatory network constitutes zinc homoeostasis. ZnuA (Formerly YcdH), ZnuB (Formerly YceA) and ZnuC (Formerly YcdI) are a zinc-binding lipoprotein, a membrane permease and an ATP-binding protein, respectively, and are all components of an ABC transporter involved in zinc incorporation (*5*, *6*). The ZnuABC transporter serves as a high-affinity zinc transporter and thus, plays a major role in zinc incorporation at an environmental zinc level <1 μM. ZosA is a P-type ATPase and the *zosA* gene is negatively regulated by PerR-sensing hydrogen peroxide (*7*). Thus, ZosA, a peroxide-induced zinc transporter, has been thought to play a role under certain conditions, e.g. a condition of >1 μM of zinc, where the genes for ZnuABC are repressed, but induced under oxidative stress by hydrogen peroxide treatment (*7*). In the latter condition, activated expression of *zosA* leads to an enhancement of ZosA-mediated zinc transport, which plays an important role in the protection of the cells from oxidative stress (*7*). In addition, the probable membrane metal chaperon YciC may serve as the third zinc importer for low affinity to zinc, although there is no direct evidence (*8*). The *yciC* gene is directly regulated by Zur (*8*, *9*) and YciC might function in ZnuABC-deficient cells grown under a zinc-limited condition.

*Bacillus subtilis* exhibits genetic competence as an adaptive response to stationary-phase stress. The regulatory cascade leading to competence development in *B. subtilis* has been well elucidated (*10*, *11*). The pheromone ComX is a cell density signal, and triggers autophosphorylation of ComP, the sensor kinase in the ComP–ComA two-component system, resulting in activation of ComA, and thus expression of the *srfA* operon. The *srfA* operon encodes the biosynthetic genes for the biosurfactant surfactin (*12*). Activation of ComK requires ComS, which is encoded by another Open reading frame (ORF) in *srfA*. ComS mediates the release of ComK from the MecA-ClpCP-ComK complex, namely proteolysis by ClpCP, leading to the autoactivation of ComK (*13*, *14*). ComK is degraded by ClpCP in the presence of MecA *in vitro*, and the degradation is inhibited by ComS. ComK autoactivation is important for bistability in both competent and non-competent cells (*15*, *16*). Additional activators, such as DegU and repressors, including CodY, AbrB and Rok, are also involved in ComK activation. ComK is a transcriptional regulatory protein that activates the expression of many genes, including late competence operons encoding the protein components needed for the uptake and processing of foreign DNA, *comC*, *comE*, *comF* and *comG* (*17*–*20*).

In this article, we identified gene disruptants for ZnuABC transporter and a *zosA* mutant with low transformability and found that the addition of excess

zinc restored the low transformable phenotype of the both mutants. Furthermore, we confirmed the former report that the lack of ZnuABC perturbs zinc homoeostasis (*5*). It was observed that the *znuA* mutant exhibited a lowered expression of *comF*, one of the late *com* operons, without affecting *comK* expression. On the other hand, we showed that in the *zosA* mutant grown under our moderately zinc-replete conditions used, perturbation of zinc homoeostasis did not occur, based on the monitoring of Zur-regulated *ytiA* gene expression (*21*). We also observed that the *zosA* mutation inhibited the post-transcriptional control of *comK*. Therefore, it is suggested that ZnuABC and ZosA play different roles in regulation of competence development.

## Experimental Procedures

### *Media and materials*

In all of the experiments for competence development, liquid modified competence (MC) medium was used (*22*). Antibiotic III (Difco. Co) was used for the pre-culture under standard conditions. Colonies were counted on Luria–Bertani agar plates (Difco. Co). The bacterial strains and plasmids used in this study are shown in Table I.

### *Transformation and β-galactosidase assay*

Transformation assay was carried out as follows. Overnight culture in Antibiotic medium III was inoculated to MC medium for transformation (5%, v/v). Total DNA containing the appropriate antibiotic resistance gene was added to the 0.1 ml of cell cultures at 2 h after entry into the stationary phase (T2) followed by addition of 2 ml of Antibiotic medium III after 30 min and the cells were further incubated for 2 h. After transformation, the cells were subjected to serial 10-fold dilutions. Each diluted fraction was then plated onto two LB agar plates containing the appropriate antibiotics, and colonies were then counted. The chromosomal antibiotic markers used for the experiments were a Cm resistance marker with an *amyE*::*degU-lacZ* fusion (*23*) and a Tc marker with the *amyE*::*ytiA-lacZ* fusion (*21*). Viable cell numbers were counted by plating the culture onto two LB agar plates containing the appropriate antibiotics after a $10^{-5}$ dilution. The typical experiments among those conducted at least two times are shown in the Tables (Tables III–V). The β-galactosidase activities of *lacZ* fusions were measured as described previously (*24*).

**Table I. *Bacillus subtilis* strains used in this study.**

| Strain | Genotype | Reference or source |
|---|---|---|
| 168 | *trpC2* | Laboratory stock |
| YKVWd | *trpC2 zosA*::Em$^r$ (*zosA-lacZ*) | BSORF[a] (Watabe, K.) |
| YCDHd | *trpC2 znuA*::Em$^r$ (*znuA-lacZ*) | BSORF[a] (Yamane, K.) |
| YCICd | *trpC2 yciC*::Em$^r$ (*yciC-lacZ*) | BSORF[a] (Yamane, K.) |
| OAM584 | *trpC2 yciC*::Em$^r$ (*yciC-lacZ*:: Tc$^r$) | This study |
| OAM551 | *trpC2 znuA*::Em$^r$ (*znuA-lacZ*:: Tc$^r$) | This study |
| OAM552 | *trpC2 znuA*:Pm$^r$ | This study |
| OAM298 | *trpC2 znuB*::Pm$^r$ | This study |
| OAM299 | *trpC2 znuC*::Pm$^r$ | This study |
| OAM553 | *trpC2 zosA*::Em$^r$ (*zosA-lacZ*) Pspac-*zosA* | This study |
| OAM554 | *trpC2 zosA*::Pm$^r$ | This study |
| OAM555 | *trpC2 zosA*::Pm$^r$ *znuA*::Em$^r$ (*znuA-lacZ*:: Tc$^r$) | This study |
| OAM585 | *trpC2 znuA*::Em$^r$-Tc$^r$ *yciC*::Em$^r$ | This study |
| OAM586 | *trpC2 zosA*::Pm$^r$ *yciC*::Em$^r$ (*yciC-lacZ*) | This study |
| RIK796 | *trpC2 amyE::ytiA-lacZ* (Tc$^r$) | (*21*) |
| OAM558 | *trpC2 amyE::ytiA-lacZ* (Tc$^r$) *znuA*::Pm$^r$ | This study |
| OAM559 | *trpC2 amyE::ytiA-lacZ* (Tc$^r$) *zosA*::Pm$^r$ | This study |
| 8G33 | *trpC2 comK-lacZ* (Km$^r$) | (*31*) |
| OAM560 | *trpC2 comK-lacZ* (Km$^r$) *zosA*::Pm$^r$ | This study |
| 13G32 | *trpC2 comG-gfp* (Cm$^r$) | Kuipers, O.P. |
| OAM581 | *trpC2 comG-gfp* (Cm$^r$) *zosA::*Pm$^r$ | This study |
| OAM236 | *trpC2 comG-lacZ* (Cm$^r$) | (*29*) |
| OAM577 | *trpC2 comG-lacZ* (Cm$^r$) *znuA*::Em$^r$-Tc$^r$ | This study |
| BD2524 | *hisA1 leu9 metB5 amyE::*Pxyl-*comK* (Sp$^r$) *comK* (Cm$^r$) *comG-lacZ* (Km$^r$) | (*30*) |
| OAM566 | *trpC2 amyE::*Pxyl-*comK* (Sp$^r$) *comK* (Cm$^r$) *comG-lacZ* (Km$^r$) | This study |
| OAM567 | *trpC2 amyE::*Pxyl-*comK* (Sp$^r$) *comK* (Cm$^r$) *comG-lacZ* (Km$^r$) *zosA*::Pm$^r$ | This study |
| OAM340 | *trpC2 comF-lacZ* (Cm$^r$::Tc$^r$) | (*29*) |
| OAM576 | *trpC2 comF-lacZ* (Cm$^r$::Tc$^r$) *znuA*::Em$^r$-Tc$^r$ | This study |
| OAM344 | *trpC2 comEA-lacZ* (Em$^r$) | (*29*) |
| OAM578 | *trpC2 comEA-lacZ* (Em$^r$) *znuA*::Em$^r$-Tc$^r$ | This study |
| OAM348 | *trpC2 comC-lacZ* (Em$^r$) | (*29*) |
| OAM579 | *trpC2 comC-lacZ* (Em$^r$) *znuA*::Em$^r$-Tc$^r$ | This study |
| **Plasmid** | **Description** | **Reference or source** |
| pPhl2 | Insertion vector, phreomycin resistance | (*24*) |
| pPhl-zosA | pPhl2 carrying a part of *zosA* | This study |
| pPhl-znuA | pPhl2 carrying a part of *znuA* | This study |
| pPhl-znuB | pPhl2 carrying a part of *znuB* | This study |
| pPhl-znuC | pPhl2 carrying a part of *znuC* | This study |
| pMutinIII | Insertion vecter, ampicillin and erythromycin resistance, *lacZI* | (*25*) |
| pMut-zosA | pMutinIII carrying a part of *zosA* | This study |
| pDG148 | Multicopy vector carrying kanamycin resistance, Pspac promoter, and Ampicillin resistance | (*26*) |
| pDG148-znuA | pDG148 carrying the *znuA*ORF | This study |
| pDG148-znuB | pDG148 carrying the *znuB*ORF | This study |
| pDG148-znuC | pDG148 carrying the *znuC*ORF | This study |

[a]http://bacillus.genome.jp/

### Plasmid construction

Synthetic oligonucleotides were commercially prepared by the Tsukuba Oligo Service (Ibaraki, Japan). The plasmids and oligonucleotides used in this study are listed in Tables I and II, respectively. To construct pPhl-zosA and pPhl-znuA, the polymerase chain reaction (PCR) products produced by the oligonucleotide pairs, zosA-E and zosA-Sa and ycdH-Sa and ycdH-Sc, were digested by simultaneous restriction enzyme digestion, using EcoRI and SalII, and SalI and SacI respectively, and cloned into pPhl-2 digested with the same restriction enzyme pairs as used for the PCR product (*24*). To construct pPhl-znuB and pPhl-znuC, PCR products produced by the oligonucleotide pairs, yceA-Sa and yceA-H and ycdI-E and ycdI-Sa, were digested by the combinations of restriction enzymes, SalII and HindIII, and EcoRI and SalI, respectively, and cloned into pPhl-2 digested with the same restriction enzyme pairs as used for the PCR product. To construct pMut-zosA, a PCR product produced by an oligonucleotide pair, pMut-zosA-H and pMut-zosA-B, was digested by HindIII and BamHI, and cloned into pMutIn-III with the same restriction enzymes (*25*). To construct pDG148-znuA, pDG148-znuB and pDG148-znuC, PCR products produced by the oligonucleotide pairs, pDG148-ycdH-H and pDG148-ycdH-Sa, pDG148-yceA-H and pDG148-yceA-Sa and pDG148-ycdI-H and pDG148-ycdI-Sa, were digested by HindIII and SalI, and cloned into pDG148 with the same restriction enzymes (*26*). The sequences of all the cloned PCR products were confirmed.

### Fluorescence microscopy

Cells were grown in MC medium and 500 µl of the culture were centrifuged, and 400 µl of the supernatant were aspirated off. The cells were then resuspended in the remaining 100 µl. Portions (2 µl) of each sample were mounted on glass slides treated with 0.1% (w/v) poly-L-lysine (Sigma). Microscopy was performed with an Olympus BX51 phase-contrast and fluorescence microscope with a 100× UplanApo objective. Images were captured using a coolSNAP-hq charge-coupled device camera (Nippon Roper) and Metavue 4.6r8 software (Universal Imaging). Green fluorescent protein (GFP) was visualized using a WIB filter set (Olympus). Image processing was performed with Adobe Photoshop.

## Results

### Disruption of the genes encoding the ZnuABC transporter for zinc incorporation caused low transformability

In our screening for disruptants with low transformability (*27–29*), the disruptants of *znuA*, *znuB* and *znuC* displayed reduced transformation frequencies (data not shown). To confirm this, three gene-disruption cassettes were back-crossed and the resultant strains exhibited low transformation frequency (Table III). Next, we carried out a complementation test using a multicopy system, i.e. pDG148-znuA, pDG148-znuB and pDG148-znuC, in which an IPTG-inducible promoter is located upstream of each Shine-Dalgarno (SD) sequence for the genes. In all three cases, the introduction of each plasmid into the *znu* disruptant resulted in evident recovery of transformation frequency without the addition of IPTG (Table III). This is due to amplification of the leaky expression of Pspac-driven genes. When IPTG was added to the culture of the *znu* strains with the cognate pDG148-znu plasmid, complementation of the disruption by artificial induction of the corresponding gene was observed, although in the case of *znuC*, only partial complementation was observed due to an unknown reason. Based on these results, it is concluded that each gene disruption of the *znuABC* operon causes a low transformation frequency phenotype.

### Zinc rescue test

We examined whether the addition of excess zinc to the medium restored low transformability in the *znu* gene disruptants, because the Znu ABC transporter incorporates zinc from the environment when cells are grown in low levels of zinc concentration <1 µM. The culture for transformation should contain some concentration of zinc, because Antibiotic medium III is composed of natural products, such as yeast extract containing zinc. Thus, we needed to know the surrounding milieu of the cells with respect to zinc in the culture system used in our laboratory. To do so, we adopted a *lacZ* fusion with *ytiA* encoding L31-like ribosomal protein as a sensor of the zinc concentration, since *ytiA* is regulated by the metalloregulator Zur (*21*). When the cells were grown in zinc-depleted medium, the expression of *ytiA* was derepressed from Zur-dependent control, and when grown in zinc-replete medium, *ytiA* expression was repressed. First, we cultured the *ytiA-lacZ* strain in MC medium using the cells grown on an LB agar plate as the pre-culture, i.e. without the natural products derived from the ingredients of the medium. In this case, the expression of *ytiA-lacZ* was very

**Table II. Oligonucleotides used for this study.**

| Name | Sequence | Product |
|---|---|---|
| zosA-E | 5′-ATGGAATTCAAGTTATCGTTCAACGCGAC-3′ | pPhl-zosA |
| zosA-Sa | 5′-ATGGTCGACGTATGTTTCTAAAGCTCCGC-3′ | pPhl-zosA |
| ycdH-Sa | 5′-ATGGTCGACGCCTTTGCTTGCATTAACGA-3′ | pPhl-znuA |
| ycdH-Sc | 5′-ATGGAGCTCTTTGTTTGTGATTGCAGCGT-3′ | pPhl-znuA |
| yceA-Sa | 5′-ATGGTCGACTTGGAATTCATGCGACG-3′ | pPhl-znuB |
| yceA-H | 5′-ATGAAGCTTATCGATGCTCATATTGGCTG-3′ | pPhl-znuB |
| ycdI-E | 5′-ATGGAATTCTCTCGTCTCATTGAAAGATA-3′ | pPhl-znuC |
| ycdI-Sa | 5′-ATGGTCGACAACGTTTAAACCATTTCCC-3′ | pPhl-znuC |
| pMut-zosA-H | 5′-ATCAAGCTTCGTTCTCAATTAGAGAGGAG-3′ | pMut-zosA |
| pMut-zosA-B | 5′-ATCGGATCCGAACCAATGGCAGCGAAAAT-3′ | pMut-zosA |
| pDG148-ycdH-H | 5′-ATCAAGCTTCTGAAAAGAGGGGATATACGAT-3′ | pDG148-znuA |
| pDG148-ycdH-Sa | 5′-ATCGTCGACTTATGATTTAACCAATAGTGAA-3′ | pDG148-znuA |
| pDG148-yceA-H | 5′-ATCAAGCTTAGAGAGAGGAGAAAAAGGCG-3′ | pDG148-znuB |
| pDG148-yceA-Sa | 5′-ATCGTCGACTTATCGGCTTCTTTTTTTGCGC-3′ | pDG148-znuB |
| pDG148-ycdI-H | 5′-ATCAAGCTTGTGAGAAAGGAAGATTAAC-3′ | pDG148-znuC |
| pDG148-ycdI-Sa | 5′-ATCGTCGACTAGAAAAGCTCGTCGCAT-3′ | pDG148-znuC |

**Table III. Complementation of low transformability in the mutants by IPTG-driven expression of the corresponding gene.**

| Experiment type/Strain | Relevant genotype | IPTG (1 mM) | Viable cells/ml ($\times 10^6$) | Transformed cells/ml ($\times 10^2$) | Frequency ($\times 10^{-4}$) | Relative frequency (%) |
|---|---|---|---|---|---|---|
| Experiment 1 | | | | | | |
| 168 | Wild-type | − | 131 | 64 | 0.49 | 100 |
| YCDHd | *znuA* pDG148 | − | 116 | 3 | 0.026 | 4 |
| | *znuA* pDG-znuA | − | 99 | 14 | 0.14 | 22 |
| | | + | 81 | 86 | 1.06 | 163 |
| Experiment 2 | | | | | | |
| 168 | Wild-type | − | 216 | 492 | 2.27 | 100 |
| OAM298 | *znuB* pDG148 | − | 64 | 4 | 0.063 | 2.8 |
| | *znuB* pDG-znuB | − | 52 | 71 | 1.37 | 60 |
| | | + | 86 | 162 | 1.89 | 83 |
| OAM299 | *znuC* pDG148 | − | 54 | 17 | 0.31 | 14 |
| | *znuC* pDG-znuC | − | 89 | 125 | 1.4 | 62 |
| | | + | 85 | 100 | 1.18 | 52 |
| Experiment3 | | | | | | |
| 168 | Wild-type | − | 271 | 274 | 1.01 | 100 |
| YKVWd | *zosA* | − | 270 | 10 | 0.037 | 3.7 |
| | | + | 190 | 9 | 0.047 | 4.7 |
| OAM553 | *zosA* Pspac-*zosA* | − | 109 | 27 | 0.25 | 24 |
| | | + | 206 | 175 | 0.85 | 84 |

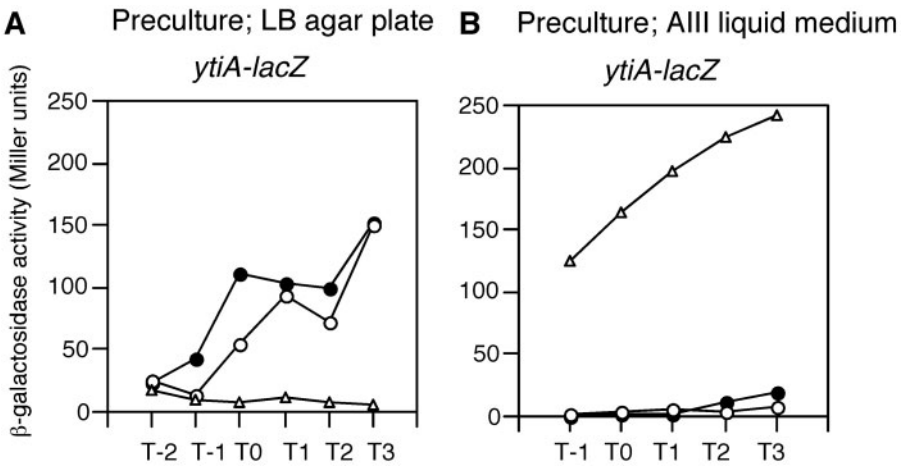


**Fig. 1 Expression of *ytiA* in zinc-replete and -depleted conditions.** Cells were grown in MC medium and sampled hourly. The X-axis represents the growth time in hours relative to the end of vegetative growth (T0). (A) The RIK796 cells grown on LB-agar plate were used as pre-culture. $ZnCl_2$ was added with the inoculation of pre-culture. Zinc concentrations; closed circles, 0 μM; open circles, 0.1 μM; triangles, 1 μM. (B) The cells grown in Antibiotic medium III were used as pre-culture. Closed circles, wild-type; open circles, *zosA*; triangles, *znuA*.

high (Fig. 1A). Then, the addition of zinc to the medium decreased *ytiA-lacZ* expression, and finally 1 μM of zinc completely repressed the expression of *ytiA-lacZ*. This is consistent with the former report (*21*). Next, we examined the expression of *ytiA-lacZ* in our standard conditions using Antibiotic medium III as the medium for pre-culture. We observed the repressed expression of the fusion in wild-type cells (Fig. 1B). Based on the results, it was concluded that the cells grow in milieu containing ~1 μM of zinc in our standard conditions. The addition of 10 μM zinc resulted in a partial recovery of low transformability in the *znuB* cells (Table IV). In the mutants for *znu* genes, severe perturbation of zinc homoeostasis is reported to occur (*5*), which leads to enhancement of the expression of *yciC* due to derepression of the genes from Zur. Thus, it is possible that external zinc could be incorporated by YciC in the *znuB* cells, leading to the recovery of transformation efficiency. To confirm this, we constructed the *znuAyciC* double mutant. The *yciC* single mutant did not display significant defect in transformability (Table V). The double mutant exhibited a synergistic decrease in transformability compared with that in the *znuA* single mutant (Table V). When excess zinc was added to the culture, recovery of low transformation frequencies in the double mutant was scarcely observed (Table V). We note that similar result was obtained in a *znuByciC* mutant (data not shown). The result indicated that the recovery of low transformability in the *znuB* cells mediated by overproduced YciC. Taken together, it was shown that perturbation of zinc homoeostasis impaired the full induction of transformation.

**Table IV. Transformation frequencies in the various mutants under the condition of addition of zinc ion.**

| Experiment type/Strain | Relevant genotype | $ZnCl_2$ (μM) | Viable cells/ml ($\times 10^6$) | Transformed cells/ml ($\times 10^2$) | Frequency ($\times 10^{-4}$) | Relative frequency (%) | Fold (zinc addition) |
|---|---|---|---|---|---|---|---|
| Experiment 1 | | | | | | | |
| 168 | Wild-type | 0 | 105 | 235 | 2.24 | 100 | |
| | | 10 | 110 | 113 | 1.03 | 46 | ×0.46 |
| OAM298 | *znuB* | 0 | 185 | 19 | 0.11 | 4.7 | |
| | | 10 | 128 | 100 | 0.78 | 35 | ×7.4 |
| Experiment 2 | | | | | | | |
| 168 | Wild-type | 10 | 251 | 138 | 0.54 | 100 | |
| OAM552 | *zosA* | 0 | 128 | 0.6 | 0.0046 | 0.9 | |
| | | 10 | 193 | 51 | 0.26 | 49 | ×54 |
| OAM555 | *zosA znuA* | 0 | 55 | <0.01 | <0.0002 | <0.04 | |
| | | 10 | 175 | 15 | 0.086 | 16 | >×400 |

**Table V. Effect of the *yciC* mutation on the rescue by zinc addition in the *znuA* and *zosA* cells.**

| Experiment type/Strain | Relevant genotype | $ZnCl_2$ (μM) | Viable cells/ml ($\times 10^6$) | Transformed cells/ml ($\times 10^2$) | Frequency ($\times 10^{-4}$) | Relative frequency (%) | Fold (zinc addition) |
|---|---|---|---|---|---|---|---|
| Experiment 1 | | | | | | | |
| 168 | Wild-type | 0 | 119 | 367 | 3.08 | 100 | |
| YCICd | *yciC* | 0 | 161 | 197 | 1.16 | 38 | |
| Experiment 2 | | | | | | | |
| 168 | Wild-type | 0 | 163 | 680 | 4.17 | 100 | |
| OAM585 | *znuA yciC* | 0 | 126 | 0.8 | 0.006 | 0.15 | |
| | | 10 | 143 | 3.5 | 0.014 | 0.35 | × 2.3 |
| Experiment 3 | | | | | | | |
| 168 | Wild-type | 0 | 203 | 484 | 4.17 | 100 | |
| OAM586 | *zosA yciC* | 0 | 33 | 1.4 | 0.042 | 1.8 | |
| | | 10 | 259 | 24 | 0.092 | 3.9 | × 2.2 |

### zosA *disruption resulted in low transformability*

In the screening, we also identified YKVWd with a low transformation frequency (data not shown). Similar to the case of the *znu* genes, transformation frequency of the back-crossed *ykvW* strain was assessed and the strain exhibited ~4% of the transformation efficiency of the control parental strain (Table III). The *ykvW* gene has been renamed *zosA*, encoding a zinc-transporting P-type ATPase (*7*). Next, we examined the possibility that the mutation might be polar to the downstream gene, leading to the low transformability. If so, the addition of IPTG should rescue the low transformability in YKVWd, because in the strain, an IPTG-inducible promoter, Pspac, is located upstream of the *ykvY* ORF (Fig. 2). The addition of IPTG did not change the low transformability in YKVWd (Table III). Thus, the mutation is not polar to the downstream gene. Furthermore, we carried out a complementation test of the *zosA* disruption by artificial expression of *zosA* in the strain OAM553. In the strain OAM553, the addition of IPTG leads to an artificial expression of the entire *zosA* ORF (Fig. 2). When OAM553 was grown in competence medium without IPTG, the strain exhibited relatively high transformability. This partial complementation is likely due to leaky transcription from the Pspac-driven *zosA* gene. Complete complementation of the disruption of *zosA* by Pspac-*zosA* was observed with the addition of 1 mM isopropyl-beta-D-thiogalactopylanoside (IPTG) (Table III). It is concluded that the *zosA* disruption caused the low transformability.

### *No perturbation of zinc homoeostasis in* zosA *cells*

The major zinc incorporation transporter is thought to be ZnuABC under conditions where the zinc concentration is on a submicromolar order (*5*). In fact, the disruptant of *znuA* encoding a solute-binding protein for zinc in the ZnuABC transporter exhibited highly derepressed expression of *ytiA-lacZ* (Fig. 1B). This indicates that perturbation of zinc homoeostasis continues in *znuA* cells due to the lack of the ZnuABC transporter. In contrast, in the *zosA* mutant, the expression of *ytiA-lacZ* did not increase compared with that observed in the wild-type cells, although some decrease was observed due to an unknown reason (Fig. 1B). Thus, it is concluded that there is no perturbation of cellular zinc homoeostasis in the *zosA* cells.

### *External zinc reversed low transformability in* zosA *cells*

To examine whether external zinc reverses low transformability in *zosA* cells, we added zinc to the medium and performed a transformation test. The addition of 10 μM of zinc increased transformation frequency in the *zosA* cells from 0.9% to approximately half of

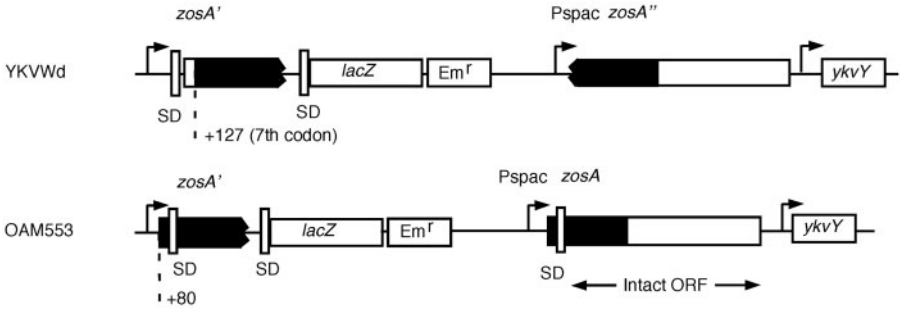


**Fig. 2 Chromosomal structures around *zosA* in YKVWd and OAM553.** Both strains were constructed by Campbell-type single cross-over event of the corresponding plasmids. Bent arrow, promoter; Rectangle, complete or partial ORF; Small rectangle with SD, Shine Dalgarno sequence; Closed box, cloned chromosomal region into the plasmids to construct the strain; Numbers, nucleotide positions relative to the transcription start site.

that observed in the wild-type cells (Table IV). This rescue might be mediated by the incorporation of zinc through the residual zinc transporter, ZnuABC and/or the putative low-affinity zinc transporter, YciC. To determine which of the two contributes to this rescue, we made two types of double-mutants, *znuAzosA* and *yciCzosA* and tested the transformation frequency in the presence or absence of excess zinc. In the *znuAzosA* strain, a very low transformation frequency was observed (Table IV). This decrease would be caused by a synergistic effect of the two mutations. The observed synergy suggests that the two mutations act on different nodes in the regulatory cascade of competence development. The addition of excess zinc rescued the low transformation frequency in the *znuAzosA* strain, suggesting that the putative zinc transporter, YciC, which could mediate this rescue of both mutations simultaneously. In the *yciCzosA* cells, the transformation frequency was not distinguishable from that observed in the *zosA* single mutant (Table V). When excess zinc was added, transformation frequencies in the double mutant did not evidently change (Table V), strongly suggesting that zinc incorporation is mediated by YciC in the *zosA* cells and that the ZnuABC-mediated zinc incorporation was not able to rescue low transformability in this double mutant. Based on these results, it is concluded that ZosA-mediated zinc incorporation is required for full induction of competence development. It should be noted that the putative low-affinity zinc transporter YciC would replace the ZosA function with respect to zinc requirement of transformability, taking into account of the *zosAyciC* cells. This suggested that only low levels of zinc would be required for transformability if zinc homoeostasis is maintained.

### ZosA-*mediated zinc transport is required for* comK *expression*

Next, we aimed to identify the target molecular event regulated by the zinc transport mediated by ZosA and ZnuABC. To do this, we performed an epistatic analysis of the mutations using *srfA-lacZ* and *comK-lacZ*. The *zosA* and *znuA* mutations had essentially no effect on the expression of *srfA-lacZ* (data not shown). Next, we tested the effect of the mutations on the expression of *comK-lacZ*. The expression of *comK-lacZ* severely decreased in the *zosA* strain but not in the *znuA* strain (Figs. 3A and 4A). The results suggested that both mutations might affect different points in the competence development regulation, as well as, the rescue of low transformability by zinc addition, the low level expression of *comK-lacZ* in the *zosA* cells is almost completely rescued by the addition of 10 μM of zinc (Fig. 3A). In addition, the severe decrease in *comK* expression in the *zosA* cells was also observed in a zinc-limited condition in which the expression of the genes for ZnuABC are derepressed, i.e. a condition in which the assay was carried out in a culture using cells grown on an LB agar plate as the pre-culture (data not shown). This again suggested that ZosA defect was not rescued by ZnuABC overproduction. A microscopic analysis using *comG-gfp* (green fluorescent protein) fusion also confirmed a smaller fraction of competent cells among the *zosA* cells, due to a decrease in ComK activity, compared to that observed in wild-type cells (Fig. 5). In the competent cells carrying the *zosA* mutation, weak intensities of ComG-GFP fluorescence were frequently observed, which reflects the relatively low activity of ComK in the competent cells (Fig. 5). This would also contribute low transformability in the *zosA* cells, due to the low level of the competence machinery compared to the wild-type, competent cells.

### *Zinc is required for post-transcriptional control of* comK

The regulation of ComK is achieved in two phases (Fig. 6). One is the post-transcriptional control involving the ComK release from the MecA-ClpCP proteolysis system by ComS, and the other is the transcriptional control affected by many transcription factors. To distinguish which of the phases is the target of ZosA-mediated zinc incorporation, we used the xylose-dependent *comK* transcription system constructed by Hahn *et al.* (*30*). In the strain carrying *comG-lacZ* as an indicator of ComK activity, *comK* is transcribed by the xylose-dependent promoter alone, independent of *comK* expression from the transcription factors, including ComK autoactivation. If the effect of the *zosA* mutation were on the one of the transcription factors, the decrease of *comK-lacZ* expression would be bypassed in this system. As shown in Fig. 3B, the decreasing effect of the *zosA*

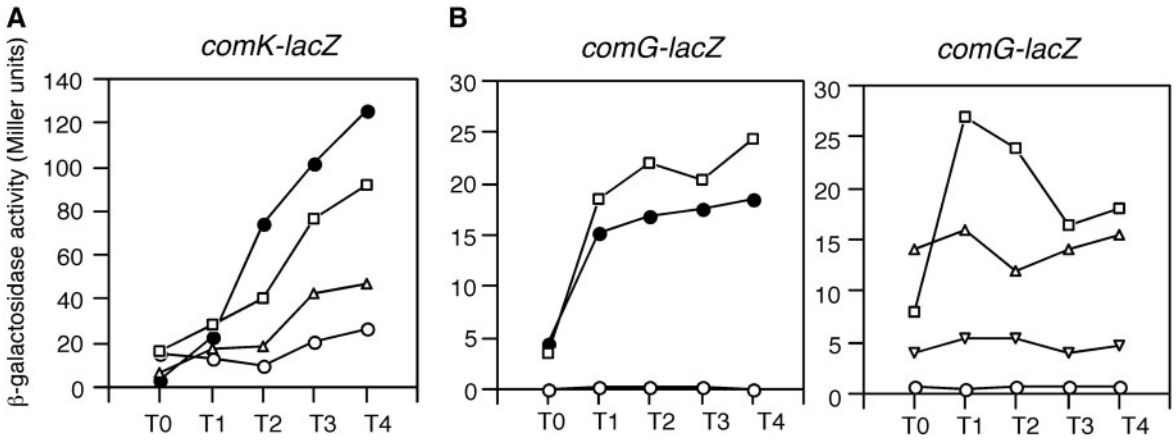


**Fig. 3 Epistatic analysis of *comK* and *comG* by *zosA* mutation.** Cells were grown in MC medium and sampled hourly. The X-axis represents the growth time in hours relative to the end of vegetative growth (T0). The cells grown in antibiotic medium III were used as pre-culture. $ZnCl_2$ was added with the inoculation of pre-culture. The expression of the indicated fusion above the graph was examined in each mutant. (A) Closed circles, wild-type without zinc; open symbols, *zosA*. Zinc concentrations: circles, 0 μM; squares, 10 μM; triangles, 30 μM. (B) The media contained 2.5% xylose to induce *comK*. Left panel. Closed circles, wild-type (*comG-lacZ comK amyE*-Pxyl-*comK*) without zinc; open squares, wild-type with 10 μM of zinc; open circles, *zosA* (OAM567). Right panel. *zosA* (OAM567). Zinc concentrations; Circles, 0 μM; inverted triangles, 3 μM; squares, 10 μM; triangles, 30 μM.

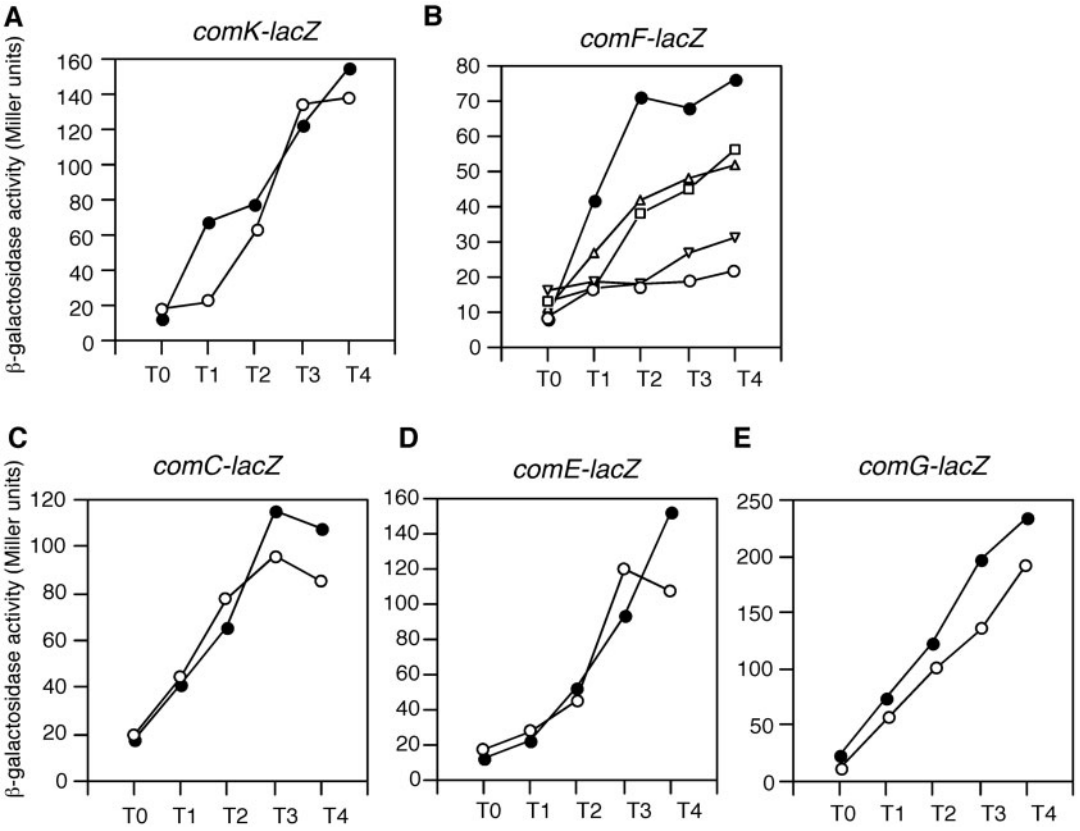


**Fig. 4 Gene expressions of *comK* and late *com* in *znuA* cells.** Cells were grown in MC medium and sampled hourly. The cells grown in Antibiotic medium III were used as pre-culture. The *X*-axis represents the growth time in hours relative to the end of vegetative growth (T0). Closed circles, wild-type without zinc; open circles, *znuA*. The expression of the indicated fusion above the graph was examined in each mutant. (A) *comK-lacZ*. (B) *comF-lacZ*. Reverse triangles, *znuA* with 3 μM of ZnCl2; triangles, *znuA* with 10 μM of $ZnCl_2$; squares, *znuA* with 30 μM of $ZnCl_2$. (C) *comC-lacZ*. (D) *comE-lacZ*. (E) *comG-lacZ*.

mutation was not bypassed. The addition of zinc resulted in a slight increase in *comK-lacZ* expression in this system (Fig. 3B, left). Contrary to this, in the *zosA* mutation background, zinc addition resulted in complete rescue of the decrease in the fusion expression. We note that the extent of the rescue reached a plateau at 10 μM of zinc (Fig. 3B, right). These observations indicate that the effect of the *zosA* mutation on *comK* acts on the post-transcriptional control of *comK*. It should be noted that in the *zosA* cells, ComK

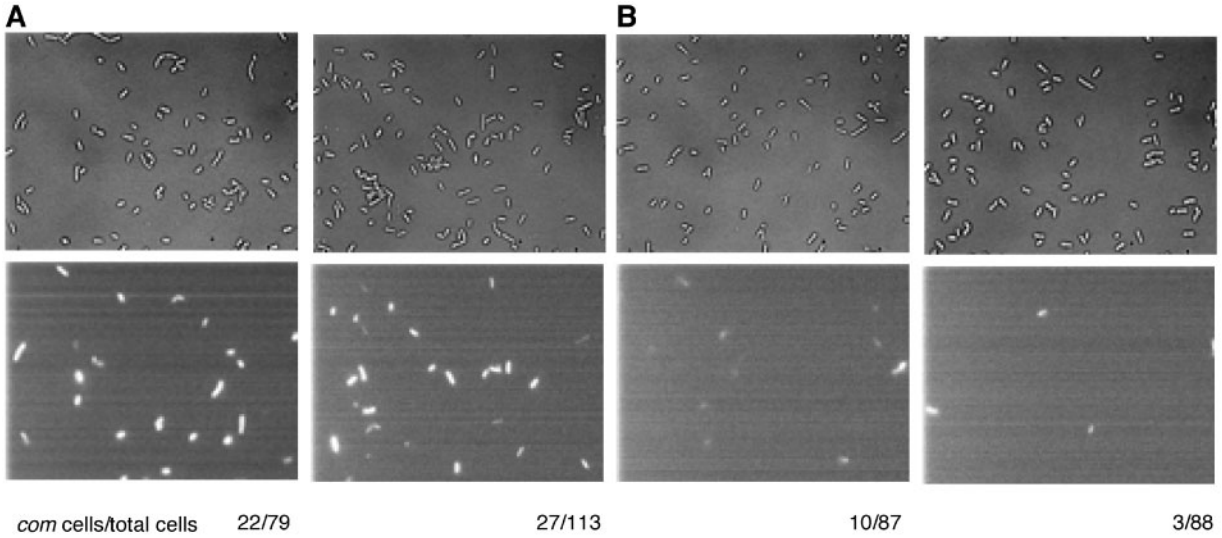


**Fig. 5 Fluorescence microscopic analysis of *zosA* cells using ComG-GFP.** Cells were grown in MC medium. The cells grown in Antibiotic medium III were used as pre-culture. Samples were harvested at T2. (A) wild-type. (B) *zosA*. Upper panels, phase contrast micrographs. Lower panels, GFP fluorescence micrographs. The phase contrast and GFP fluorescence images are captured in the same field of vision.

function is inhibited post-transcriptionally, leading to the repression of *comK* transcription, since *comK* is within the autoregulatory loop. The notion of post-transcriptional control of *comK* is reinforced by the observation that an introduction of the *mecA* mutation, which allows *comK* expression independently from the ClpCP protease and releasing factor ComS, leads to an enhancement of *comK* transcription and late *com* gene expression, bypassing the decreasing effect of the *zosA* mutation (data not shown). On the other hand, the introduction of the *mecA* mutation did not bypass a deficiency due to a lack of positive transcription factors required for *comK* expression, including DegU and ComK (*31*, *32*).

### znuA *mutation specifically decreased* comF *expression*

In *znuA* cells, the expression of *comK* is normal, which may indicate that the expression of the late *com* genes naturally occurs, as in the wild-type cells. Recently, however, it was reported that there is one check-point control by YutB and ComN on *comE* expression, although the expression of *comK* is not impaired in either disruptant of *yutB* or *comN* (*29*). Thus, it was worth examining each late *com* gene transcription event in the *znuA* cells. We introduced the *znuA* mutation into the strain carrying the *lacZ* fusion with each late *com* operon and examined the β-galactosidase activities. As shown in Fig. 4, the expression of *comF-lacZ* was greatly decreased in the *znuA* cells, whereas, the expression of the rest of the late *com* operon fusions, *comC*, *comE* and *comG*, did not change. Next, we tested whether the addition of zinc would rescue the decreased expression of *comF*. The addition of 10 μM of zinc to the culture of the *znuA* cells recovered the expression of *comF*, which is consistent with the results of the transformation assay. These results clearly indicate that ZnuA-mediated zinc incorporation is specifically required for *comF* expression.

## Discussion

We report that perturbation of zinc homoeostasis caused by the lack of the Znu ABC transporter for zinc incorporation resulted in low transformability (Fig. 6). This perturbation causes a decrease in *comF* transcription without affecting *comK* transcription. The ZnuABC high-affinity zinc incorporation system is found in many bacteria and thus, there are several reports showing that the disruption of *znu* genes results in drastic changes in certain phenotypes. In *Streptococcus pneumoniae*, disruption of the genes encoding the ABC transporter for zinc incorporation, Adc, leads to a competence-deficient phenotype, although the molecule targeted by zinc remains unknown (*33*). Furthermore, in pathogenic *Salmonella enterica*, disruption of the ZnuABC system results in reduced virulence (*34*, *35*). These patterns of reduced virulence are likely caused by the impaired growth of the Znu system-deficient bacteria in the host cells. In addition, in *Streptococcus gordonii*, disruption of the repressor of the *adc* operon, leading to an overproduction of the Adc ABC transporter, resulted in a deficiency in both biofilm-formation and genetic competence (*36*). This indicates an involvement of this transporter in these two biological processes.

It was also determined that ZosA-mediated zinc incorporation is required for the post-transcriptional control of *comK* (Fig.6). The question of which molecules and processes are required for interaction with zinc remains a subject for future investigation. ClpX has a zinc-binding domain, thus zinc binding influences the activity of the protein (*37*). It has been reported that ClpX is required for post-transcriptional control of *comK* (*38*). Thus, it is possible that ClpX is a target of ZosA-mediated zinc incorporation. This hypothesis,

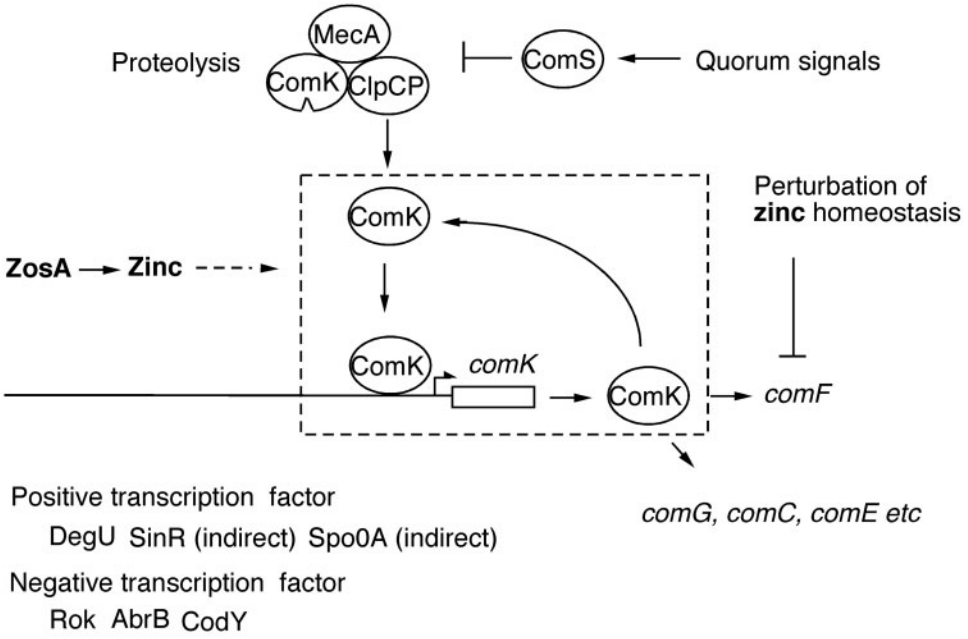


**Fig. 6 Regulation of ComK by zinc transportation: *comK* proteolytic complex and transcription factors are shown.** Dotted box indicates *comK* autoregulatory loop. Oval, protein; Notched oval, degraded protein; Rectangle, *comK* ORF; Bent arrow, promoter; T-bar, ComS action resulting in release of ComK from proteolytic complex and inhibitory effect of perturbation of zinc homoeostasis in the cells lacking the complete ZnuABC transporter; Arrow, gene activation; Dotted arrow, indirect gene activation.

however, has a shortcoming, i.e. the unimpaired expression of *srfA*, which is positively regulated by the proteolytic activity of ClpXP for Spx, was observed in the *zosA* cells (*39*). This means that the *zosA* mutation may not affect the ClpXP activity. Otherwise, the zinc-dependent activities of ClpX required for activation of *srfA* and *comK* might be different. In fact, ClpX has a role in FtsZ assembly independently of ClpP (*40*) and the regulation of ComK by ClpX may not be dependent on ClpXP-dependent proteolysis (*38*). It has been known that P-type ATPase for ion uptake functions with secondary ion export due to its structure and mode of action (*41*). In many cases, what secondary ion is associated with uptake of ion remains unknown. Thus, we could not completely rule out the possibility that the effect of ZosA is dependent on, not zinc transport, but unknown secondary ion transport.

It is a very important point that perturbation of zinc homoeostasis did not occur in the *zosA* mutant, which is based on the observation that the expression of *ytiA-lacZ* is still repressed. In other words, in *zosA* cells, the need of proteins requiring zinc to be active would be fulfilled. This situation is consistent with the former observation that the total cellular zinc content in the *zosA* mutant is similar to that in the wild-type cells, when the cells were grown in the presence of 20 nM of zinc and 20 μM hydrogen peroxide in order to induce *zosA* expression (*7*). The nature of the ZosA transporter, which works as a major zinc transporter at a high environmental zinc level, where the expression of the genes coding for ZnuABC and YciC are completely repressed (*5*), is also compatible with this situation. Since there are many protein ligands for zinc in the cell that results in the cellular free zinc pool being scarce, it is thought to be reasonable that Zur reacts to femtomolar levels of zinc concentration (*42*). Thus, fluctuation of the intracellular free zinc concentration caused by the *zosA* mutation might be transient and subtle, leading to ComK inactivation. Since the intracellular zinc pool is scant, subtle and transient increases in intracellular zinc might transmit specific signal to the target.

It is very intriguing that the expression of the *zosA* gene is upshifted by partial derepression from the PerR sensor for hydrogen peroxide in the transition from the growing phase to stationary phase, probably because of the increased generation of hydrogen peroxide due to aerobiosis (*7*, *43*). In other words, the entry into the stationary phase is transmitted to the regulatory cascade for ComK as an increase in cellular hydrogen peroxide, leading to the up-regulation of *zosA*.

ZosA-mediated zinc incorporation is apparently required for post-transcriptional control of *comK*, while ZnuABC-mediated zinc incorporation specifically activates *comF* transcription. The differential roles of zinc transporter are common phenomena in eukaryotes (*44*). For example, 14 zinc importer genes have been identified in human cells, and tissue-specific expression and specific cellular localization are reported (*45*). Thus, each disruption of the genes encoding zinc transporters shows different phenotypes, such as abnormal morphogenesis of embryo and depletion of thymic pre-T cell in zinc-limiting condition and finally lethality (*44*). This suggested that in *B. subtilis*, two zinc transporters might regulate competence development at different timing in the regulatory cascade, because *comF* activation follows post-transcriptional control of ComK. Taken together, to the best of our

knowledge, this is the first report of the differential roles of zinc transporters in the cellular differential process of prokaryotes.

## Supplementary Data

Supplementary Data are available at *JB* online.

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**Conflict of interest**
None declared.

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