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# Molecular Analysis of the Copper-Responsive CopRSCD of a Pathogenic *Pseudomonas fluorescens* Strain

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**CopRS/CopABCD is one of the known systems that control copper homeostasis in bacteria. Although CopRS/CopABCD homologues are found to exist in *Pseudomonas fluorescens*, the potential role of this system in *P. fluorescens* has not been investigated. In this study a genetic cluster, consisting of *copR*, *S*, *C*, and *D* but lacking *copAB*, was identified in a pathogenic *P. fluorescens* strain (TSS) isolated from diseased fish. The *copRSCD* cluster was demonstrated to be required for full copper resistance and regulated at the transcription level by Cu. Expression of *copCD* is regulated directly by the two-component response regulator CopR, which also regulates its own expression. Interruption of the regulated expression of *copR* affected bacterial growth, biofilm formation, and tissue dissemination and survival. A mutant CopR, which lacks the N-terminal signal receiver domain and is constitutively active, was found to have an attenuating effect on bacterial virulence when expressed in TSS. To our knowledge, this is the first report that suggests a link between CopR and bacterial pathogenicity in *P. fluorescens*.**

***Keywords*:** CopABCD, copper resistance, CopR, *Pseudomonas fluorescens*, virulence

To most living organisms copper is an essential metal ion that is required at trace amounts for biological processes such as those that involve oxidases, monooxygenases, and dioxygenases, which depend on copper for biological functions. However, copper excess is detrimental to the cells, as copper can interact with the free thiol groups of proteins and may thus impair the activity of these proteins. In addition, copper can also participate in biochemical reactions that lead to the generation of reactive oxygen species. As a result, microorganisms have developed various mechanisms for the control of copper homeostasis. It has been found that *Escherichia coli* employs several systems for the maintenance of copper homeostasis, which include the CueR-regulated Cu(I)-translocating P-type ATPase (CopA; Petersen and Moller, 2000; Rensing *et al.*, 2000; Grass and Rensing, 2001; Outten *et al.*, 2001), the CusR/CusCFBA regulon (Munson *et al.*, 2000; Franke *et al.*, 2003), the CpxR- and YedW-regulated systems (Yamamoto and Ishihama, 2005), and the PcoRS/PcoABCD system (Brown *et al.*, 1995).

CopRS/CopABCD, a copper-responsive system that is related to the *E. coli* PcoRS/PcoABCD, was first identified in *Pseudomonas syringae* (Mellano and Cooksey, 1988; Mills *et al.,* 1993). CopABCD function in the binding of copper while CopR and CopS form a two-component signal transduction system that regulates the expression of *copABCD* (Cha and Cooksey, 1991). A two-component signal transduction system typically consists of a membrane-associated histidine sensor kinase and a cytoplasmic response regulator. Extracellular stimuli are sensed by the sensor kinase, which, in response to the signal, becomes activated by autophosphorylation and subsequently transfers the phosphoryl group to the cognate response regulator, thereby activating the latter (West and Stock, 2001). In the case of CopRS/CopABCD, the sensor protein kinase CopS is activated in the presence of copper; the activated CopS then presumably activates the cognate response regulator CopR by phosphorylation. Structurally, CopR can be divided into the N-terminal receiver domain, which is the target of phosphorylation-mediated activation by the cognate sensor kinase, and the C-terminal effector domain, which, upon activation, can interact with the target promoters. Phosphorylation of the receiver domain induces a conformational change that results in the activation of CopR and the subsequent binding of the activated regulator to the target DNA.

Recently, a copper-transporting P1-type ATPase (CueA) has been identified in *P. fluorescens* and demonstrated to be involved in copper resistance and bacterial fitness (Zhang and Rainey, 2007). CopRS and CopABCD homologues have also been identified in *P. fluorescens* by genome sequencing, but the functions of these proteins have not been investigated. With an aim to illustrate copper homeostatic mechanisms in *P. fluorescens*, we cloned the genetic cluster containing *copRSCD* from a pathogenic *P. fluorescens* strain and analyzed its effect on copper tolerance and bacterial virulence. Our results indicate that the temporal expression of *copR* is not only required for full copper resistance, which also involves CopCD, but also is required for effective bacterial dissemination and survival during infection.

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**Table 1.** Plasmids used in this study

| Plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| p704T | $Tc^R$; suicide plasmid | This study |
| p7TS | $Tc^R$; suicide plasmid | This study |
| pACYC184 | $Tc^R$; cloning vector | New England BioLabs |
| pBR322 | General cloning vector | New England BioLabs |
| pBT | $Ap^R$; cloning vector | Zhang *et al*. (2008) |
| pBT3 | $Ap^R$; cloning vector | Zhang *et al*. (2008) |
| pBTR | $Ap^R$; expressing *copR* | This study |
| pC85 | $Ap^R$; expressing *copR* mutant | This study |
| pC94 | $Ap^R$; expressing *copR* mutant | This study |
| pC104 | $Ap^R$; expressing *C104* | This study |
| pDN18 | Broad host range vector | Marx and Lidstrom (2001) |
| pETCR | $Kn^R$; expressing *copR* | This study |
| pET258 | $Kn^R$; expression vector | Zhang and Sun (2007) |
| pGP704 | Suicide plasmid | Miller and Mekalanos (1988) |
| pJCO | $Tc^R$; pJT expressing *copC* | This study |
| pJC104 | $Tc^R$; pJT expressing *C104* | This study |
| pJRO | $Tc^R$; pJT expressing *copR* | This study |
| pJT | $Tc^R$; pDN18 derivative | This study |
| pJT601 | $Tc^R$; pJT carrying *D601-luc* fusion | This study |
| pJT210 | $Tc^R$; pJT carrying *D210-luc* fusion | This study |
| pS601 | $Kn^R$; carrying *D601-lacZ* fusion | This study |

[a] $Ap^R$, ampicillin resistant; $Kn^R$, kanamycin resistant; $Tc^R$, tetracycline resistant

## Materials and Methods

### Bacterial strains and growth

*E. coli* strains DH5α (TaKaRa, Japan), BL21(DE3) (Tiangen, China), S17-1λpir (Biomedal, Spain), and the *P. fluorescens* strain TSS (isolated from diseased fish, kanamycin-resistant) were cultured in Luria-Bertani lysis broth (LB) medium (Sambrook *et al*., 1989) at 37°C (for *E. coli*) or 28°C (For *P. fluorescens*). Appropriate antibiotics were supplemented at the following concentrations: ampicillin (Ap), 100 μg/ml; kanamycin (Kn), 50 μg/ml; and tetracycline (Tc), 15 μg/ml. The mean generation time (*g*) of bacterial growth was calculated as described by Eagon (1962).

### Plasmid construction

The plasmids and the primers used in this study are listed in Table 1 and Table 2, respectively. pBTR was constructed by inserting the *copR* coding sequence into pBT3 at the *Eco*RV site. The *copR* mutants encoding the 137~221, 128~221, and 128~231 regions of CopR (amplified by PCR with the primer pairs F32/R32, F19/R32, and F19/R9, respectively) were inserted into pBT3 at the *Eco*RV site, resulting in pC85, pC94, and pC104 respectively. pETCR was generated by inserting the coding sequence of *copR* into pET258 between the *Nde*I/*Xho*I sites. pJT was constructed by inserting a linker (5'-AATTCATTTAAATGTTTAAACG-3') into pDN18 between the *Eco*RI/*Bam*HI sites. To construct pJCO, pJRO, and pJC104, *copC*, *copR*, and *C104* were inserted into pBT at the *Sma*I site, and the recombinant plasmids were digested with *Swa*I; the fragments carrying *copC*, *copR*, and *C104* were inserted into pJT at the *Swa*I site, resulting in pJCO, pJRO, and pJC104, respectively. To construct pJT601 and pJT210, a *Xba*I linker (5'-GATCCATTTAAATCCCGGG TCTAGAATTTAAAT-3') was inserted into pACYC177 (New England BioLabs, USA) between the *Bam*HI/*Sma*I sites, re-

**Table 2.** Primers used in this study

| Primer | Sequences (5'→3')[a] |
|---|---|
| 322F4 | ATTTCTATGCGCACCCGTT |
| 322R4 | GCGCTCATGAGCCCGAA |
| F2 | TACCACCGTAGCTGTTGCG |
| F11 | GGTCTTTATAGCCTCAGGGG |
| F19 | ATCATGAAATTGGCTGGTTTGG |
| F32 | GGGCTCCTCAAACGCAGG |
| F49 | TAATTTTCCTGATTAGCTTGGC |
| F56 | TATTCGAGAGCCATCCCCATG |
| F57 | GTCTTGCATGACTCAGTTCTCTGGTGCA |
| F58 | CGCGATTTAAATATCAGTCTCTGTGGAGAA (*Swa*I) |
| F60 | GCATTTAAATCAACCATGGCGTCGTGT (*Swa*I) |
| F61 | TCACGGCGGAATTGCTGCTCCGTC |
| R6 | CAACTCGCCATAAGTGC |
| R9 | GATATCTTATTCCGACCCCGTC |
| R12 | GGAAAGCGCTTGTTCAGGG |
| R32 | GATATCTTACAGCATGTACCCCATG |
| R51 | CTGGAGAATGATAGAATGAG |
| R61 | CGCCGTGAGGAAAAGCACA |
| R62 | GAACTGAGTCATGCAAGACTCCTGACA |
| R63 | GCGCATTTAAATCGCGCCACAGACA (*Swa*I) |
| R65 | CAGCAATTCCGCCGTGAGGAAAAGCA |
| R66 | GCGCATTTAAATAGGTCGTAGTCCACT (*Swa*I) |

[a] Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends

sulting in p180, which was digested with *Xba*I and ligated to the promoterless luciferase gene (*luc*) of pGL28 (Zhang *et al*., 2008), resulting in p181; D601 (the 601 bp DNA upstream of the coding sequence of *copC*) and D210 (the 210 bp DNA upstream of the coding sequence of *copR*), respectively, were inserted into p181 at the *Sma*I site, resulting in p181D6 and p181D2, respectively, which were digested with *Swa*I and the fragments carrying *D601-luc* and *D210-luc* fusions were ligated to the *Swa*I-linearized pJT, resulting in pJT601 and pJT210, respectively. pS601 was created by inserting D601 into pSC11 (Wang *et al*., 2008) at the *Swa*I site. p704T was created by inserting the tetracycline resistance determinant of pACYC184 into pGP704 between the *Sma*I/*Sca*I sites. To construct p7TS, the *sacB* gene of pKO3 (Link *et al*., 1997) was inserted into pGM-T (Tiangen) at the *Eco*RV site, resulting in pGSB, which was digested with *Sma*I/*Pst*I, and the *sacB*-containing fragment was inserted into pBS-T (Tiangen) at the compatible sites, resulting in pBSB, which was digested with *Sma*I/*Eco*RV, and the *sacB*-containing fragment was inserted into p704T at the *Sma*I site, yielding p7TS.

**Strain construction**

To construct TSSRM and TSSCM, the DNA fragments containing *copR* and *copC* in-frame deletions were generated by overlap extension PCR as follows: the overlapping PCR was performed with the primer pairs F60/R65 and F61/R66, and F58/R62 and F57/R63, respectively; the fusion PCR was performed with the primer pairs F60/R66 and F58/R63, respectively. The PCR products were inserted into p7TS at the *Sma*I site, resulting in p7TRM and p7TCM, respectively. S17-1λpir was transformed with p7TRM and p7TCM, and the transformants were conjugated with TSS. The transconjugants were selected first on LB plates supplemented with tetracycline and kanamycin and then on LB plates supplemented with 5% sucrose and kanamycin. The colonies that were resistant to sucrose and sensitive to tetracycline were analyzed by PCR; the PCR products were subsequently subjected to DNA sequencing to confirm the deletion.

TSSDM and 342M were constructed as follows: the internal fragments of *copD* and *orf342* (amplified by PCR with the primer pairs F11/R6 and F49/R51, respectively) were inserted into p704T at the *Sma*I site; the resulting recombinant plasmids were introduced into strain S17-1*λpir* by transformation, and the transformants were mated with TSS. Chromosomal insertions of the plasmids in these strains were verified by PCR and subsequent sequencing of the PCR products.

**Cloning of the *cop* cluster**

TSS genomic DNA was digested with Sau3A1 and the fragments between 4 and 6 kb were ligated into pBU (Zhang and Sun, 2007) at the *Bam*HI site. DH5α was transformed with the ligation mix, and the transformants were plated on LB agar plates and selected for crater-forming colonies as described previously (Zhang and Sun, 2007). One of the crater-forming colonies contained the recombinant pBU carrying partial *copC*. The complete sequence of the *cop* cluster was subsequently obtained by genome walking as described previously (Zhang and Sun, 2007).

**Purification of the recombinant CopR**

The *E. coli* strain BL21(DE3) was transformed with pETCR and the recombinant CopR was purified from BL21(DE3)/pETCR with nickel-nitrilotriacetic acid columns as described previously (Zhang and Sun, 2007).

**Reverse transcriptase PCR and quantitative real-time reverse transcriptase PCR**

Total RNA was extracted from cells grown in LB medium to $OD_{600}$ of 0.8 by using the SV total RNA isolation system (Promega, USA). Six hundred nanograms of total RNA was reverse-transcribed to cDNA using Moloney murine leukaemia virus reverse transcriptase and random primers (Promega). Reverse transcriptase PCR (RT-PCR) was carried out using the cDNA and primers specific to *copC*, *copD*, and, as an internal control, 16S rRNA gene. Quantitative real-time RT-PCR (qRT-PCR) was carried out in an ABI 7300 Real-time Detection System (Applied Biosystems, USA) by using the SYBR ExScript RT-PCR kit (TaKaRa) as described previously (Zhang *et al*., 2008). Each assay was performed in triplicate with the 16S rRNA gene as a control. All data were given in terms of relative mRNA expressed as means plus or minus standard errors of the means (SE). Statistical analyses were performed by using the two-tailed *t*-test.

**β-Galactosidase assay**

Cells were grown in LB medium to $OD_{600}$ of 1.2 and used for β-galactosidase assay, which was performed as described previously (Zhang *et al*., 2008).

**Electrophoresis mobility shift assay**

DNA fragments (D601 and D210) were generated by PCR and labeled with carboxyfluorescein (Sangon, China). The labeled DNA (100 ng) was mixed with CopR (100 ng) and incubated at 25°C for 30 min in 20 μl binding buffer [10 mM bis-Tris; pH 7.5, 0.1 mM $MnCl_2$, 40 mM KCl, 0.5 mg/ml bovine serum albumin, 15% (v/v) glycerol] containing or not containing the negative control DNA, a 246 bp DNA fragment (generated by PCR with primers 322F4/322R4) of pBR322. The samples were then separated by electrophoresis in nondenaturing 8% polyacrylamide gels. For competition assays, unlabeled D601 and D210 were added at increasing concentrations (100 to 400 ng) into the assay buffer.

**MIC determination**

Cells were grown in LB medium to mid-logarithmic phase and diluted to $1\times10^6$ CFU/ml. Cells (100 μl) were plated on LB plates supplemented with $CuSO_4$ at various concentrations. The plates were incubated at 28°C for 48 h and then inspected for growth. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of $CuSO_4$ that prevented visible growth.

**Luciferase assay**

Cells that had been grown in LB medium to mid-logarithmic phase were lysed and assayed for luciferase activity by using the Luciferase Assay System (Promega). Light production was measured on a Glomax luminometer (Promega).

**Bacterial conjugation/mating**
pJT and its derivatives were introduced into the *E. coli* strain S17-1*λpir* by transformation. The transformants and TSS were grown in LB medium to $OD_{600}$ of 1 and mixed in 1:1 ratio. The mixed cells were washed and resuspended in 10 mM $MgSO_4$ and dropped onto a LB plate. After incubation at 28°C for 12 h, the growth on the plate was scraped off and resuspended in 2 ml LB, from which 100 μl were taken and spread onto a LB plate supplemented with kanamycin and tetracycline. The plate was incubated at 28°C for 48 h and the colonies that appeared were verified to be authentic transconjugants by PCR and sequence analysis of the PCR products.

**Biofilm assay**
Biofilm formation on polystyrene microtitre dish (Costar, USA) was determined as described previously (Zhang *et al.*, 2008).

**Experimental infection and bacterial recovery from the livers of the infected fish**
Japanese flounder (*Paralichthys olivaceus*; ~12 g) were randomly divided into several groups (five fish per group). Each group was injected intraperitonealy (i.p.) with $7\times10^6$ CFU of TSS, TSSRM, TSS/pJRO, TSS/pJC104, and TSS/pJT, respectively, that had been cultured in LB medium, washed, and resuspended in phosphate-buffered saline (PBS) to $OD_{600}$ 0.6. To examine bacterial recovery from the liver, the livers of the infected fish were taken under aseptic conditions at 24 and 48 h post-infection. The livers were homogenized in PBS and the homogenates were plated on LB agar plates supplemented with kanamycin (marker for TSS) or kanamycin plus tetracycline (marker for plasmid-harboring TSS). After incubation at 28°C for 48 h, the colonies that appeared on the plates were enumerated. The nature of these colonies was verified by PCR analysis of 20 colonies randomly selected from each plate using primers specific to TSS and the plasmid; one of the 20 PCR products was subsequently analyzed by DNA sequencing. Statistical analysis was performed by using the *t*-test.

**Examination of plasmid stability**
To examine the stability of pJRO and pJT in TSS/pJRO and TSS/pJT, respectively, during infection, Japanese flounder were infected with TSS/pJRO and TSS/pJT as described above. The livers of the infected fish (five for each infection) were taken at 24 h post infection and homogenized in PBS; the homogenates were plated on LB agar plates supplemented with kanamycin (for the selection of TSS harboring or not harboring plasmid) or kanamycin plus tetracycline (for the selection of plasmid-harboring TSS). After incubation at 28°C for 48 h, the colonies that appeared on the plates were enumerated. The nature of these colonies was verified as described above.

**Database searching and nucleotide sequence accession numbers**
Nucleotide and amino acid sequence searching was conducted using the BLAST programs at the National Center for Biotechnology Information (NCBI). Subcellular localization predictions were performed using the CELLO v.2.5 and PSORTb v.2.0 servers. The nucleotide sequence of the *cop* cluster has been deposited in GenBank database under the accession number EU927287.

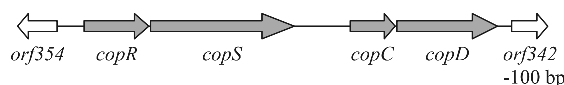


**Fig. 1.** Schematic presentation of the *cop* cluster (shaded arrows) and the surrounding regions. The arrows indicate the directions of transcription.

## Results

**Organization of the *copRSCD* (*cop*) cluster**
The *cop* cluster was cloned from TSS by using the pBU system, a signal sequence trap developed in our laboratory for the identification of proteins with functional secretion domains (Zhang and Sun, 2007). Sequence analysis revealed that the *cop* cluster consists of four open reading frames (ORF) with the lengths of 696, 1389, 384, and 939 bp, respectively, which were named *copR*, *S*, *C*, and *D* (Fig. 1). Immediately upstream of and divergent from *copR* is a 354 bp *orf* (named *orf354*) that encodes a hypothetical protein of unknown function; immediately downstream of *copD* is a 342 bp *orf* (named *orf342*) encoding another hypothetical protein. CopR and CopS share the highest sequence identities (84 and 69%, respectively) with the two-component heavy metal response transcriptional regulator and the heavy metal sensor histidine kinase of *P. mendocina* (GenBank accession nos. ABP84993 and ABP84994, respectively). CopC and CopD share the highest sequence identities (76 and 54%, respectively) with the copper resistance CopC and CopD peptides of *P. syringae* (GenBank accession nos. AAA25808 and AAA25809, respectively). By using the PSORTb and CELLO servers, CopS and CopD were predicted to be located in the cytoplasmic membrane while CopC was in the periplasm. Conserved domain searching revealed two functional domains in CopR, one being the signal receiver domain (residues 4~116) and the other the effector domain (residues 128~221).

To determine whether *copC* and *copD* belonged to the same transcriptional unit, RT-PCR was performed using total RNA extracted from TSS grown in the presence of 100 μM $CuSO_4$ (so that the expressions of *copC* and *copD* were induced; described below) and the primers F2 and R12. F2 covers the 18~36 region of the *copC* coding sequence and R12 covers the 335~353 region of the *copD* coding sequence. The result showed that positive PCR products were produced (data not shown), suggesting that *copC* and *copD* are most likely co-transcribed into a single mRNA species.

**The Cop cluster is involved in copper resistance**
To investigate whether CopRSCD played any role in copper resistance, *copR* and *copC* were mutated by in-frame deletion, whereas *copD*, being at the end of the operon, was mutated by insertion mutagenesis. In addition, *orf342* also was mutated by insertion mutagenesis. The resulting mutant strains, TSSRM (*copR* mutant), TSSCM (*copC* mutant),

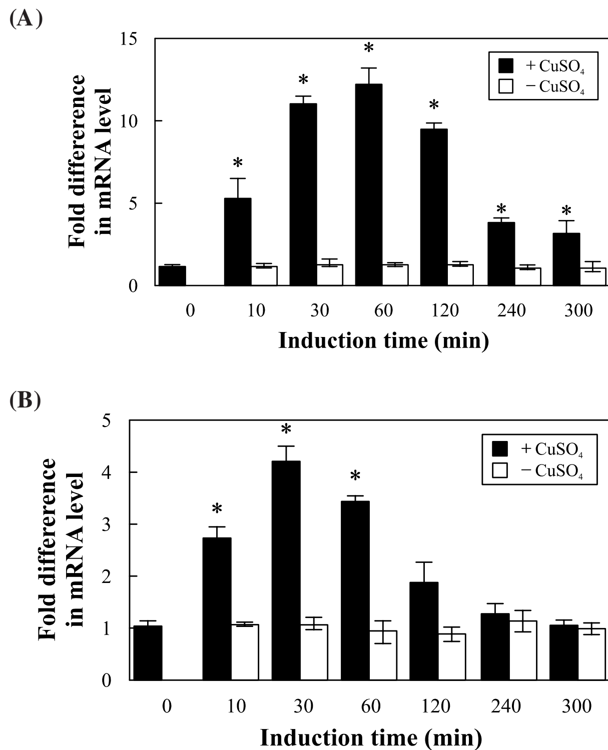


**Fig. 2.** Expression of *copC* (A) and *copR* (B) in response to copper. Cells were grown in LB medium to $OD_{600}$ 0.4; one half of the cell culture was then removed and grown separately in the presence of 100 μM $CuSO_4$ (black bar) while the other half of the cell culture was grown continuously in the absence of $CuSO_4$ (white bar). Total RNA was extracted from the cells harvested at different time points and used for quantitative real-time PCR. Data are the means of three independent experiments and presented as the Means±SE. *, $P<0.001$.

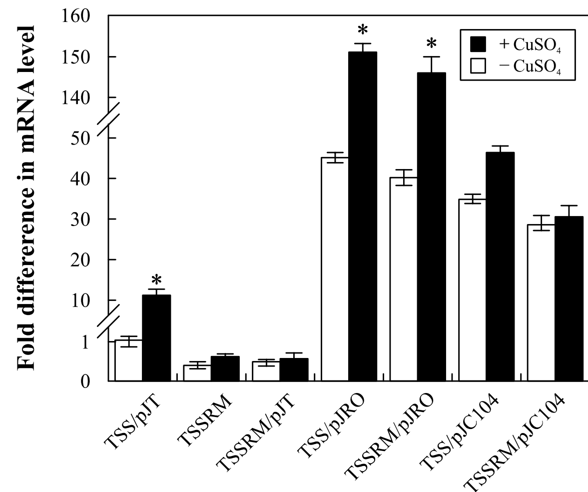


**Fig. 3.** Effect of CopR and C104 on the expression of *copC*. Quantitative real-time PCR was performed using total RNA extracted from cells that had been grown in LB medium to $OD_{600}$ 0.8 in the presence (black bar) or absence (white bar) of 100 μM $CuSO_4$. Data are the means of at least three independent experiments and presented as the Means±SE. *, $P<0.001$.

TSSDM (*copD* mutant), and 342M (*orf342* mutant), were compared with the wild-type strain TSS for alterations in copper resistance. The results showed that, while 342M exhibited the same level of copper resistance as that exhibited by TSS (MIC of 1.8 mM), TSSRM, TSSCM, and TSSDM exhibited reduced tolerance to copper (MICs of 1.45, 1.3, and 1.5 mM, respectively), suggesting that CopR, CopC, and CopD, but not ORF342, were required for full copper resistance.

To determine whether Cu had any effect on the expression of the *cop* cluster, qRT-PCR was carried out to analyze *copR* and *copC* expression in relation to Cu. The results showed that the presence of $CuSO_4$ (100 μM) enhanced *copC* expression, which peaked (12.4-fold increase) at 60 min after the addition of Cu (Fig. 2A). Similarly, Cu also enhanced *copR* expression, which peaked (4.2-fold increase) at 30 min after Cu induction (Fig. 2B). The potential effect of other metal ions, i.e., silver, cadmium, cobalt, mercury, manganese, and zinc, on *copC* and *copR* expression was also examined by qRT-PCR, and the results showed that none of these metals had any apparent effect on *copC* or *copR* expression (data not shown).

To further examine the effect of Cu on *copC* and *copR* expression, the conjugative plasmids pJT601 and pJT210 were constructed. In pJT601, the 601 bp DNA (named D601) immediately upstream of the translational start of *copC* is fused to a promoterless luciferase gene (*luc*) that serves as a reporter of the promoter activity in D601. Similarly, in pJT210, the 210 bp DNA (named D210) immediately upstream of the translational start of *copR* is fused to the *luc* reporter gene. pJT601 and pJT210 were conjugated into TSS and the resulting transconjugants TSS/pJT601 and TSS/pJT210, respectively, were assayed for luciferase activity in relation to Cu. The results showed that the presence of $CuSO_4$ (100 μM) caused, respectively, 6.9- and 1.9-fold increase in the luciferase activities of TSS/pJT601 and TSS/pJR201, suggesting that D601 and D210 contain functional promoters that can be activated by Cu. Since, considering the positions of D601 and D210 in the *cop* cluster, the promoters of D601 and D210 are most likely the promoters of *copC* and *copR*, respectively, these result suggested that Cu induced transcription from both the *copC* and the *copR* promoters.

Taken together, the above results demonstrate that the *cop* cluster is required for full copper resistance in TSS and the expressions of *copR* and *copC* are responsive to Cu.

## CopR regulates the expression of *copC*

To examine whether CopR had any effect on *copC* expression, qRT-PCR was conducted to analyze *copC* expression in strains TSSRM (defective in *copR*) and TSS/pJRO, which is TSS transformed with the plasmid pJRO that expresses *copR*. The results showed that in the absence of Cu, *copC* expression decreased 2.5-fold in TSSRM and increased 45-fold in TSS/pJRO compared with *copC* expression in TSS harboring the control plasmid pJT (Fig. 3); in the presence of 100 μM $CuSO_4$, *copC* expression decreased 18.3-fold in TSSRM and increased 14.5-fold in TSS/pJRO. These results indicated

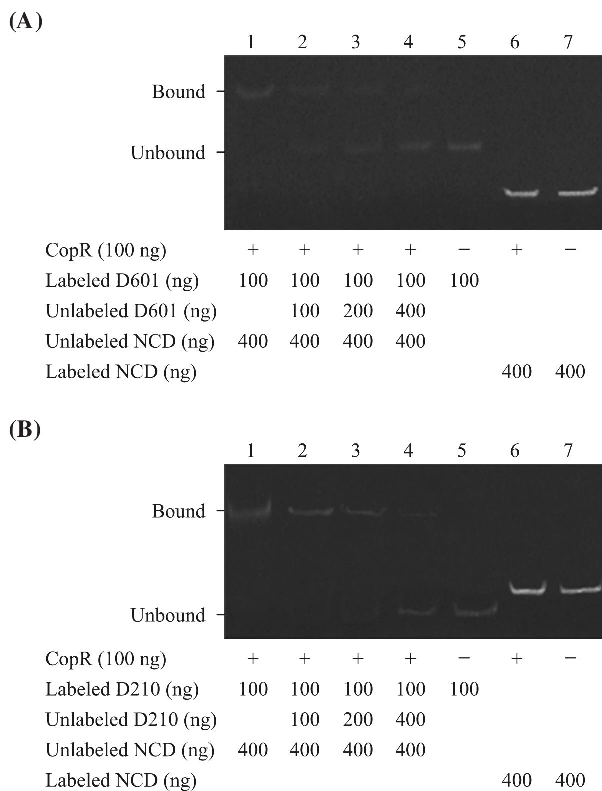


**Fig. 4.** Interaction between CopR and the promoter regions of *copC* (A) and *copR* (B). Electrophoresis mobility shift assay was performed in the binding buffer containing CopR, unlabeled or carboxyfluorescein-labeled negative control DNA (NCD), carboxyfluorescein-labeled D601 (A) or D210 (B), and different concentrations of unlabeled D601 (A) or D210 (B). The negative control DNA (NCD) is derived from the plasmid pBR322. The assays were repeated three times.

that CopR had a positive effect on *copC* expression and this effect of CopR was modulated by Cu.

To determine whether CopR could interact directly with the *copC* promoter region, electrophoresis mobility shift assay was performed, which showed that the purified recombinant CopR could bind to D601, and this binding was blocked by unlabeled D601 but not by an unrelated DNA fragment derived from pBR322 (Fig. 4A, data not shown). These results suggest that CopR regulates *copC* expression by binding directly to the *copC* promoter region.

## CopR regulates its own expression

To examine whether CopR regulated its own expression, qRT-PCR was performed to analyze the expression of the chromosomal *copR* in TSS/pJRO by using primers F56 and R61, which cover, respectively, the -18 to +3 and the +222 to +240 regions relative to the translation start of *copR*. Since pJRO contains only the *copR* coding sequence and has no F56 sequence, when the primer pair F56/R61 was used in qRT-PCR analysis of *copR* expression in TSS/pJRO, positive PCR products could only be produced from the mRNA transcribed from the chromosomal *copR* and not from the *copR* carried by the plasmid pJRO. The result of qRT-PCR showed that the chromosomal *copR* expression in TSS/pJRO was 26.8-fold more than that in TSS/pJT, suggesting that CopR autoregulates its own expression in a positive manner. Consistently, electrophoresis mobility shift assay showed that the purified recombinant CopR could bind to D210, and this binding was blocked by unlabeled D210 but not by an unrelated DNA fragment (Fig. 4B, data not shown), suggesting that CopR can interact specifically with its own promoter region.

## Disruption of *copR* and *copC* expression affects bacterial growth

Since *copC* and *copR* expressions in TSS are regulated, we determined the effect of interference with these regulations on bacterial growth. For this purpose, the plasmids pJCO and pJRO, which constitutively express *copC* and *copR*, respectively, were conjugated into TSS and its derivatives. The growth patterns of TSSRM (defective in *copR*), TSSCM (defective in *copC*), TSS/pJRO (overexpressing *copR*), TSS/pJCO (overexpressing *copC*), TSSCM/pJCO (TSSCM complemented with *copC*), and TSSRM/pJRO (TSSRM complemented with *copR*) were then compared with those of TSS and TSS harboring the control plasmid pJT (TSS/pJT). The results showed that in the absence of Cu, TSSCM, and TSSRM exhibited growth patterns similar to that exhibited by TSS (Fig. 5A), whereas TSS/pJRO and, especially, TSS/pJCO displayed much slower growth rates and lower maximum cell densities than TSS/pJT (Fig. 5C). In the presence of Cu, TSS/pJRO and TSS/pJCO displayed growth patterns similar to that displayed by TSS/pJT (Fig. 5D), whereas TSSCM and TSSRM exhibited reduced growth rates and maximum cell densities compared with TSS (Fig. 5B). In contrast, TSSCM/pJCO and TSSRM/pJRO displayed growth profiles comparable with that displayed by TSS in the presence of Cu (Fig. 5B), suggesting that the growth defects of TSSCM and TSSRM could be rescued by CopC and CopR, respectively. These results demonstrate that expression of *copR* and *copC* is required for optimal growth under copper stress and that constitutive expression of *copR* and *copC* in the absence of Cu is detrimental to the cells.

Given the above results, we wondered whether aberrant expression of *copR* would have any effect on biofilm formation, which is one form of growth. To investigate this question, we compared the abilities of TSSRM, TSS, TSS/pJRO, and TSS/pJT to form biofilm on polystyrene surface. The results showed that in the presence of $CuSO_4$ (100 μM), the amounts of biofilm formed by TSSRM and TSS/pJRO were, respectively, 1.5-fold more and 2-fold less than that formed by TSS and TSS/pJT, the latter two strains producing comparable amounts of biofilm. Hence, it appeared that CopR had an inhibitory effect on biofilm development. Since in the presence of copper, optimal planktonic growth requires *copR* expression, this observation suggests that under copper stress, TSS tends to grow in the planktonic, rather than the biofilm, form. In line with this hypothesis, biofilm production by TSS was 1.8-fold less in the presence than that in the absence of $CuSO_4$ (100 μM).

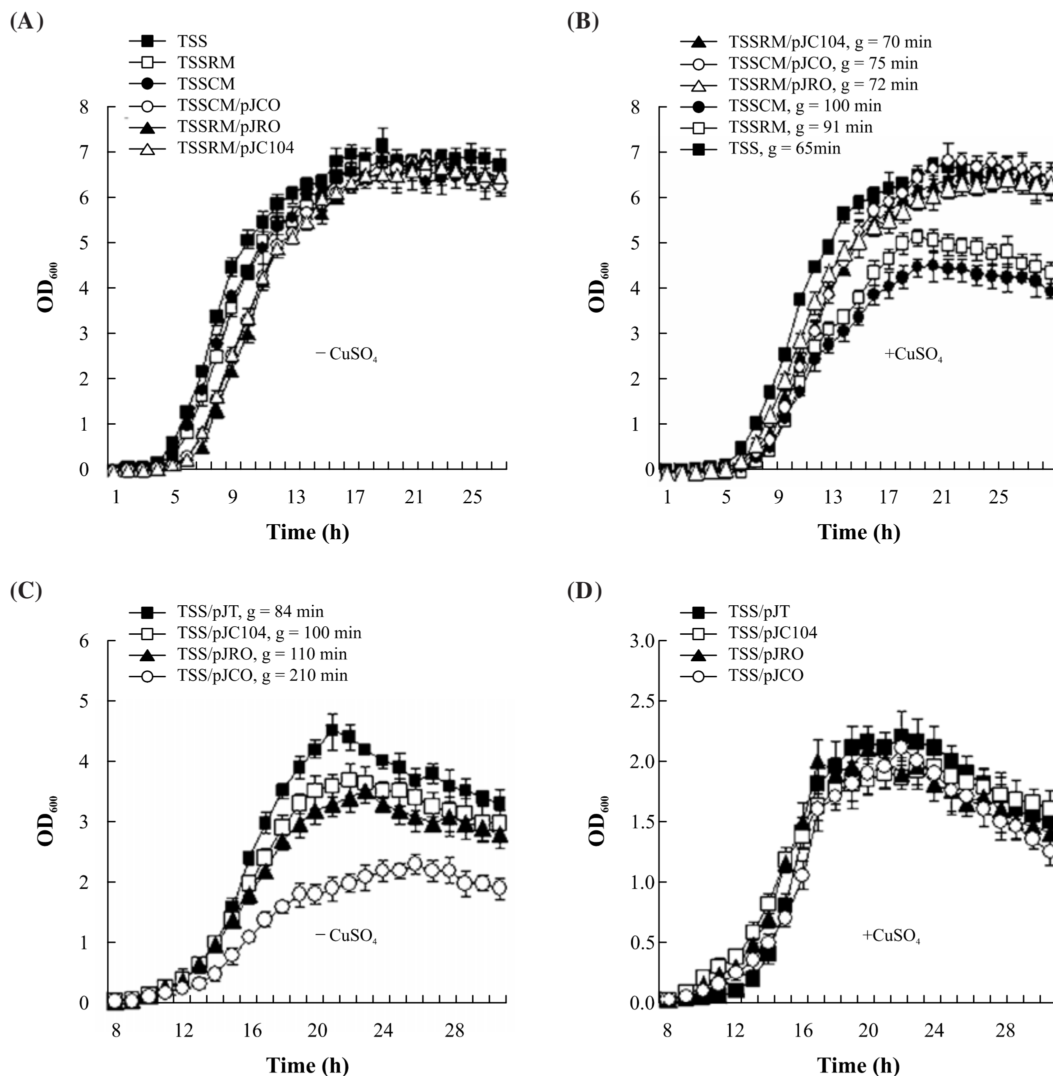


**Fig. 5.** Growths of TSS and its derivatives in response to Cu. Cells were cultured at 28°C in LB medium supplemented with (B and D) or without (A and C) 0.5 mM $CuSO_4$. The culturing medium of TSS/pJT, TSS/pJRO, and TSS/pJCO were also supplemented with tetracycline (15 μg/ml) for plasmid maintenance. g, mean generation time. Data are the means for three to four independent experiments and are presented as the Means±SD.

### Disruption of *copR* expression attenuates bacterial virulence

Since TSS is a fish pathogen, we examined the effect of disrupted expression of *copR* on bacterial virulence. For this purpose, Japanese flounder were infected with the same dose of TSS, TSSRM, TSS/pJRO, and TSS/pJT, respectively, and bacterial recoveries from the livers of infected fish were determined. The results showed that the numbers of bacteria recovered from the livers of TSS-infected fish were comparable with those recovered from the livers of TSS/pJT-infected fish, but the numbers of bacteria recovered from the livers of TSSRM- and TSS/pJRO-infected fish were significantly lower than those recovered from TSS/pJT-infected fish (Fig. 6).

To determine whether the reduced bacterial recovery from TSS/pJRO-infected fish was due to the loss of pJRO as a result of selective pressures, the stabilities of TSS/pJRO and TSS/pJT were analyzed. The results showed that with TSS/pJRO-infected fish, the average amount of bacteria that appeared on the kanamycin plates was 10.8% higher than that appeared on the kanamycin plus tetracycline plates; PCR analysis indicated that 86.7% (78/90) of the colonies that appeared on the kanamycin plates were TSS/pJRO. With TSS/pJT-infected fish, the average amount of bacteria that emerged on the kanamycin plates was 8% higher than that emerged on the kanamycin plus tetracycline plates; PCR analysis indicated that 90% (81/90) of the colonies that appeared on the kanamycin plates were TSS/pJT. These results indicate that the plasmid loss rate of TSS/pJRO is at most 3.3% higher than that of TSS/pJT, which is apparently far too low to account for the more than 50-fold difference (Fig. 6) in the amount of bacteria recovered from TSS/pJRO-

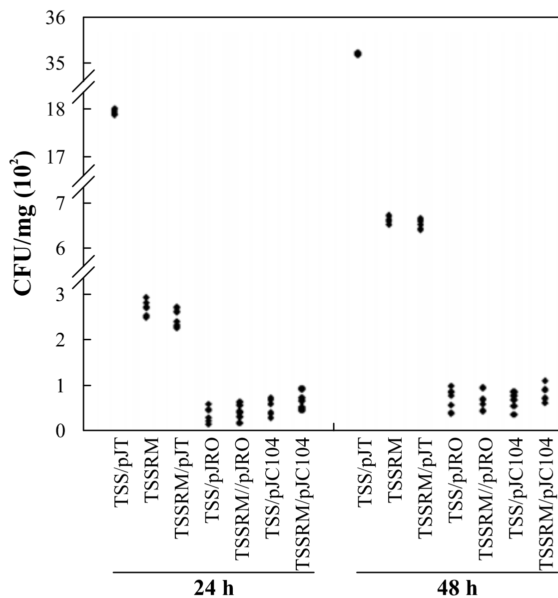


**Fig. 6.** Bacterial dissemination in the livers of fish infected with TSS variants. Japanese flounder were administered with the same dose of TSS or its derivatives and the livers of the infected fish (five from each infection) were taken at 24 and 48 h post-infection. The livers were homogenized in PBS, and the homogenates were plated on selective LB plates. The plates were incubated at 28°C for 48 h, and the bacteria that appeared on the plates were enumerated. Data of each time points are the results obtained from five fish.

and TSS/pJT-infected fish. Taken together these results demonstrate that interference with the expression of *copR* vitiates the tissue dissemination and survival ability of TSS.

### Identification and analysis of a constitutively active CopR mutant

As mentioned in the introduction, it is generally known that CopR is activated by phosphorylation as a result of response to environmental signals such as Cu. Given our above observation that aberrant expression of *copR* had an attenuating effect on infection, we speculated that a self-active CopR mutant, whose activity was independent on external stimuli, may have some effect on bacterial virulence. To investigate this idea, we wanted first to select a constitutive active CopR mutant. For this purpose the plasmids pC85, pC94, and pC104 were created, which constitutively express, respectively, the truncated CopR mutants encoding the 137~221, 128~221, and 128~231 regions. All these CopR mutants lack the N-terminal receiver domain involved in phosphorylation-mediated activation. pS601, a promoter probe plasmid in which D601 is fused to a promoterless *lacZ* reporter gene so that the promoter activity of D601 (i.e., the *copC* promoter activity) could be measured by β-galactosidase assay, was introduced into DH5α by transformation. DH5α/pS601 was then transformed separately with pC85, pC94, pC104, and pBTR, which expresses the wild type *copR*. Subsequent β-galactosidase assays showed that, compared with DH5α/pS601/pC85 and DH5α/pS601/pC94, DH5α/pS601/pC104 exhibited the highest β-galactosidase activity, which was 42% of that exhibited by DH5α/pS601/pBTR. Hence, the CopR mutant (named C104) expressed from pC104 retains partial regulator activity compared with the wild-type CopR.

To examine the effect of C104 on growth, the conjugative plasmid pJC104, in which C104 is constitutively expressed as *copR* is in pJRO, was introduced into TSS and TSSRM. Growth studies showed that the transconjugants TSS/pJC104 and TSSRM/pJC104 exhibited growth profiles similar to those of TSS/pJRO and TSSRM/pJRO, respectively, in both the presence and the absence of Cu (Fig. 5).

To examine the effect of C104 on *copC* expression, TSS/pJC104 and TSSRM/pJC104 were compared with TSS/pJRO and TSSRM/pJRO for *copC* expression by qRT-PCR. The results showed that, in the absence of Cu, *copC* expressions in TSS/pJRO, TSSRM/pJRO, TSS/pJC104, and TSSRM/pJC104 were comparable, which were 45- to 32-fold higher than that in TSS/pJT (Fig. 3), suggesting that constitutive expression of C104, like constitutive expression of *copR*, enhanced *copC* expression. The presence of Cu significantly increased *copC* expression in TSS/pJRO and TSSRM/pJRO but had no significant effect on *copC* expression in TSS/pJC104 or TSSRM/pJC104 (Fig. 3). These results suggest that (i) in TSS/pJC104, as far as *copC* expression is concerned, the effect of the chromosomal *copR* is negligible compared with that of C104; and (ii) the activity of C104, unlike that of the wild-type CopR, is not inducible by Cu. In other words, C104 is constitutively active.

We next examined the effect of C104 on bacterial virulence. For this purpose, Japanese flounder were infected with the same dose of TSS/pJC104, TSS/pJRO, and TSS/pJT, respectively. The livers were taken from the infected fish at 24 and 48 h post-infection, and bacterial recoveries were determined as described above. The results showed that the amounts of bacteria recovered from TSS/pJC104-infected fish were comparable with those recovered from TSS/pJRO-infected fish, which were significantly lower than the amounts of bacteria recovered from TSS/pJT-infected fish (Fig. 6).

Taken together, these results demonstrate that although C104 is impaired in activity compared with the wild-type CopR, constitutive expression of C104 has an attenuating effect on bacterial virulence that is comparable with that caused by the constitutive expression of the wild-type *copR*.

## Discussion

*copABCD* homologues have been identified in a number of bacterial species, including *P. syringae* (Lim and Cooksey, 1993; GenBank accession no. CP000075), *P. putida* (Adaikkalam and Swarup, 2005), and *P. fluorescens* (GenBank accession no. CP000094). In most of the cases the four functionally related genes, *copA*, *B*, *C*, and *D*, form a genetic cluster. Exceptions are found in the *P. putida* strain KT2440 and the *P. aeruginosa* strain PAO1, both of which possess *copAB* but not *copCD* (Nelson *et al.*, 2002; Canovas *et al.*, 2003; Winsor *et al.*, 2005). In TSS, we found that the *cop* cluster consists of only *copCD* that is immediately preceded, not by *copAB*, but by the two-component signal transduction system-encoding genes *copRS*. In the classical CopABCD system, CopR regulates the expression of *copABCD* by di-

rectly interacting with the *copABCD* promoter (Mills *et al*., 1994). In TSS, CopR directly regulates the expression of *copCD* by binding specifically to the *copC* promoter region. Therefore, the expression of *copCD* in TSS is modulated by CopR in a fashion resembling that observed with the regulation of *copABCD* in the classical *copABCD* system. In *P. syringae copA* and *copB* are essential to fundamental copper resistance while *copC* and *copD* are required for full copper resistance (Mellano and Cooksey, 1988). Similarly, in TSS, mutation of *copC* and *copD* reduces but does not abolish the copper resistance ability of the bacterium. The fact that in TSS *copCD* expression is well regulated and required for full copper resistance suggests the possibility that CopCD may exist independently of CopAB as a functional system.

In line with the observation that TSS bearing *copC* and *copR* mutations (i.e., TSSCM and TSSRM) exhibited reduced copper tolerance, growth studies indicated that the growths of these two mutant strains were unaffected in the absence of copper but much impaired in the presence of copper. In contrast, the growths of TSS/pJCO and TSS/pJRO, which constitutively express *copC* and *copR*, respectively, were similar to that of the wild-type strain in the presence of copper but impaired when copper was absent. These results demonstrate that the Cop system is required for coping with situations of copper stress and also suggest that CopR, when overproduced, is active (although not as much as when Cu is present) in the absence of copper, which is consistent with the observation that *copC* expression was enhanced in TSS/pJRO under conditions of copper absence. A similar observation of CopR activity in the absence of copper has also been reported in *P. aeruginosa* (Caille *et al*., 2007). It is possible that in addition to CopS, the recognized cognate kinase for CopR activation, there exist other protein kinases that can also activate CopR either nonspecially or specifically as a signal transduction partner; another possibility is that CopR in the underphosphorylated or unphosphorylated state possesses certain residual activity that may become accumulated when CopR is overproduced (such as in the case of TSS/pJRO) and hence account for the heightened *copC* expression in TSS/pJRO in the absence of copper.

Recent studies have shown that copper stress can induce profound changes in the transcription profiles of bacteria (Yamamoto and Ishihama, 2005; Barre *et al.*, 2007; Magnani *et al*., 2008). In *P. aeruginosa*, transient or prolonged copper stimulation can alter the expression levels of a large number of genes (Teitzel *et al*., 2006). Some of these copper-responsive genes are controlled by CopR, which has lately been found to participate in the modulation of systems other than CopABCD. In *P. aeruginosa*, CopR is involved in the regulation of porin production, antibiotic resistance, and Zn homeostasis (Perron *et al.*, 2004; Caille *et al*., 2007). In TSS, disruption of the temporal expression of *copR* affects not only copper resistance but also bacterial growth and pathogenicity. The growth defect may account for or contribute to the impaired infectivity observed with TSSRM, TSS/pJRO, and TSS/pJC104. Alternatively, mutation or constitutive expression of *copR* may have a direct effect on bacterial virulence. Links between copper response and bacterial pathogenicity have been documented previously in *P. aeruginosa*, in which it has been shown that CopR can activate the expression of a repressor of the type III secretion system and that copper, alone or in combination with quaternary ammonium compounds, can prevent biofilm formation (Ha *et al.*, 2004; Harrison *et al*., 2008). Given these precedents, it is reasonable to speculate that in TSS, CopR may, via the regulation of certain yet unknown genes, participate in cellular processes that operate during the course of infection; interruption of the temporal expression of *copR* may disrupt these processes and, as a result, attenuate the virulence capacity of TSS.

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