JOURNAL OF PURE AND APPLIED MICROBIOLOGY, FEBRUARY 2014. Vol. **8**(1), p. 601-605

Detection of Interaction Between DsbM and OhrR by Yeast Two-hybrid System in *Pseudomona aeruginosa*

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(Received: 30 October 2013; accepted: 05 December 2013)

DsbM and OhrR proteins are encoded by the *P. Aeruginosa* genes *dsbM* and *ohrR*, respectively. We found a strong interaction between DsbM and OhrR proteins by yeast two-hybrid system. This result indicated the correlation between DsbM which affects the aminoglycoside resistance and the *ohrR* regulon in *Pseudomonas aeruginosa*. Our findings showed important implications for studying the aminoglycoside resistance in *Pseudomonas aeruginosa* in future.

Key words: DsbM; OhrR; Yeast two-hybrid.

Pseudomonas aeruginosa is an important opportunistic Gram-negative pathogen that usually causes persistent infections in hospitals (Hatano *et al*., 1998). The DsbM protein is a novel disulfide oxidoreductase which can catalytic the oxidation, reduction and isomerism of disulfide in *P. aeruginosa* (Wang *et al*., 2012). The expression of genome will be reduced once the *dsbM* gene is inactive. This also induces the over expression of some antioxidases and affects the the aminoglycoside resistance in *P. aeruginosa* (Chang *et al*., 2005).

Oxidation causes wide damage to bacterial components, including proteins, membrane lipids and genetic materials (Carmel-Harel *et al*., 2000). For the presence of reactive oxygen species (ROS), the detoxification enzymes are over expressed and the damaged proteins and DNA are intensively repaired in bacterium (Newberry *et al*., 2007). These responses are coordinately regulated by antioxidant regulons. Different kinds of conditioning systems are generated, including *oxyR*, s*oxR*, *perR* and *ohrR* (Ochsner *et al*., 2000; Panmanee *et al*., 2006). In *P. aeruginosa*, not only the OxyR system was intensively studied but also the OhrR system. The OhrR protein restrained an organic peroxide resistant protein and played derepressed role during response to the organic peroxide. A single conservative cysteine residue is necessary in this system and will be oxidized into a Cys-SOH derivative (Atichartpongkul *et al*., 2001).

The research on polymerization of proteins in vitro is not completely same as the activities in vivo. Yeast two-hybrid system is a novel approach and can be used in these studies (Philip *et al*., 2012). In the study, we found a strong interaction between DsbM and OhrR proteins in vivo. The findings show an important implication for studying the aminoglycoside resistance in *Pseudomonas aeruginosa* in future.

MATERIALS AND METHODS

Construction of the yeast two-hybrid vectors

Full-length *dsbM* and *ohrR* genes were amplified from PAOI genomic DNA by PCR. The following primers including forward primers with *Eco*R I restriction site sequences and reverse primers with *Nde* I restriction site sequences were

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used
dsbM forward primer
5'-GGAATTCCATATGAACGACCTCACCCTT-3'
dsbM reverse primer:
5'- GGCGAATTCTTATCAGGCGACGTCGAT-3'
ohrR forward primer:
5 ' -
GGAATTCCATATGTCCCGACTGCCCACCACCCCTT-3'
ohrR reverse primer:
5'-CGGAATTCTCAATCCGCGGATCC-3'

After amplification and recovery, the *dsbM* and *ohrR* gene fragments were cleaved using *Nde* I and *Eco*R I. pGADT7 and pGBKT7 were also cleaved by the same double digestion system and dephosphorylated. Then *dsbM* and *ohrR* fragments were ligated into pGADT7 and pGBKT7 and transferred into *E. coli* DH5±, respectively. The transformed cells were incubated on LB agar plates and got two-hybrid plasmids.

Best transformation condition of *Saccharomyces cerevisiae*

Preparation of *S. cerevisiae* competent cells

Preparation of competent cells was performed accord to an adapted method by Becker (Bai *et al*., 2006). Newly activated AH109 monoclone was introduced into 5mL YPDA liquid culture and incubated at 30°C, 230g overnight. When the concentration was 1×10s cells/mL, 0.5mL cells were introduced into number of bottles with 50 mL YPDA culture and incubated at 30°C, 230g. Incubation in different bottles was stopped when OD_{600} was between 0.5-1.5, respectively. Cells were collected at 4°C 4 000 g for 5 min, washed twice with 30 mL precooled sterile water, wash with 10 mL precooled sorbitol (1 mol L^{-1}), and resuspended in 500 µl sorbitol (1 mol L^{-1}). The competent cells (1×10 cells mL^{-1}) were kept on ice and transformed as soon as possible.

Electrotransformation of *Saccharomyces cerevisiae*

Competent cells 100µl and plasmids 400ng were mixed and added into precooled electroporation cuvettes. After electric shock under capacitance 25 µF, resistance 200 © and voltage 1.5 KV, the mixture were transferred into 900 µl sorbitol (1 mol L^{-1}) and incubated in ice for 5 min. Then 100µl strain cells were coated on plates with relevant culture.

Calculation of transformation efficiency

Transformation efficiency = (CFU/µg DNA) =

$$\frac{\text{amout of transformant (CFU)} \times \text{volume after transformation } (\mu L)}{\text{mass of DNA } (\mu g) \times \text{volume of coated cells } (\mu L)}$$

Yeast two-hybrid screens on reaction between DsbM and OhrR

Plasmids, pGADT7-*dsbM*, pGADT7-*ohrR*, pGBKT7-*dsbM* and pGBKT7-*ohrR* were transferred into yeast cells and identified on auxotrophic SD agar, respectively. Then combinations of plasmids, pGBKT7-*dsbM* + pGADT7-*ohrR*, pGBKT7-*ohrR* +pGADT7-*dsbM*, pGBKT7-53+ pGADT7-T (positive control), and GBKT7-*1am* + pGADT7- *T* (negative control) were transferred into yeast cells and identified on -Trp/-Leu SD agars. The transformants were transferred on -Trp/-Leu/-His/-Ade SD agars and cultured invertedly on 30°C for 3-4 d.

Measurement of β-galactosidase activity

Measurement of colony β-galactosidase

Clones from SD selective medium were activated, and those with diameters between 1-3mm were transferred to a piece of dry Whatman filter paper. The paper was submerged in liquid nitrogen for 10s, thawed immediately at room temperature and stacked on another filter paper pre-soaked by Z buffer/X-gal solution. The papers were cultured at 30°C for 30 min to 8 h and observed periodically. It was considered negative if there was no blue after 8h incubation. This indicated that there was no reaction between the measured proteins.

Measurement of liquid β-galactosidase

Fresh clones were transferred into 5 mL SD medium and cultured at 30°C, 230 g overnight. Culture 4mL was transferred into 16 mL YPDA medium and cultured at 30°C, 230 g for 3-5 h. Cells were collected from 1.5mL culture by centrifugation at 8,800 g for 30s when OD_{600} was about 0.6. The pellet was washed by 1.5 mL Z-buffer and resuspended with 300µL Z-buffer (concentration factor = 1.5mL/0.3mL = 5). Cells 100µL were transferred into a new 1.5mL tube and frozen/thawed in liquid nitrogen three times. A tube with 100 µl Z-buffer was taking as a negative control. After loading 700 µl Z-Buffer + β-Mercaptoethanol in the tubes, 160 µl ONPG was added immediately to start the reaction in water bath (30°C). When

the solution was yellow, 400µl Sodium Carbonate (1mol/L) was added to stop the reaction. The absorbance of supernatant (5000 g, 10min) at OD_{420}.

Every sample was measured three times and the activity of β-galactosidase was calculated.

β-galactosidase activation unit=

$$\frac{1000 \times OD_{420}}{t \times v \times OD_{600}}$$

where t indicates the reaction time (min), and v indicates 0.1 ml×a concentration factor or 0.1×5=0.5

RESULTS AND DISCUSSION

Best electrotransformation condition of *Saccharomyces cerevisiae*

Under the same cells concentrations (10^{10}cells mL^{-1}) and voltage of 1.5 KV, the transformation efficiency was attributed to the different growth stages of the cells being competent. When pGADT7-T was transferred into AH109 competent cells on SD/-Leu agars, the efficiency was highest (OD_{600} = 0.9, 2.8×10^3 CF μg^{-1} DNA). When pGADT7-*T* and pGBKT7-*53* were transferred into AH109 competent cells on SD/-Trp/-Leu agars, the efficiency was highest (OD_{600} = 0.9, 1.1×10^3 CFU μg^{-1} DNA) (Fig.1).

The detection of reaction between DsbM and OhrR by yeast two-hybrid

As shown in Table 1, cells transformed by single kind of plasmid with *dsbM* or *ohrR* did not self-activate; The negative control with plasmids pGBKT7-*lam* + pGADT7-*T* did not activate the reporter genes, while the positive control with pGBKT7-*53*+ pGADT7-*T* did. The transformants with combination of pGBKT7-*dsbM* + pGADT7-*ohrR* and pGBKT7-*ohrR* + pGADT7-*dsbM* could grow on -Trp/-Leu/-His/-Ade AD plates and turn 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) blue (Fig.2). This result indicated the direct reaction between DsbM and OhrR. To evaluate the strength of the reaction, we measured the activity of liquid β-galactosidase. The activity of β-galactosidase in transformants with pGBKT7-*dsbM* + pGADT7-*ohrR* and pGBKT7-*ohrR* + pGADT7-*dsbM* were similar to that in positive control.

It is important to transferred two-hybrid plasmids into the competent cells for the application of yeast two-hybrid system. The current methods are protoplast transformation, lithium acetate (LiAc) transformation and electrotransformation (Bai *et al*., 2006).. The classic protoplast transformation will produce polyploid cells and the protocol is complicated. It is also difficult to regenerate the transformants. PEG-LiAc transformation is handy and effective, but needs Carrier DNA. The efficiency of this method is associated with the relative molecular weight of polyethylene glycol (PEG) (Gietz *et al*., 1992). Electrotransformation is not only simple and easy to perform, but also effective. The

Table 1. Test of transformed yeast for His^+ phenotype and β-galactosidase activity

Transformation of yeast with plasmids	His^+ phenotype	β-galactosidase activity assay		
		color reaction	β-galactosidase activity unit	*n*[b]
pGADT7-*dsbM*	-	White	NT	
pGADT7-*ohrR*	-	White	NT	
pGBKT7-*dsbM*	-	White	NT	
pGBKT7-*ohrR*	-	White	NT	
pGADT7-*T*+ pGBKT7-*53*	+	Blue	9.5+0.06[a]	5
pGADT7-*dsbM*+pGBKT7-*ohrR*	+	Blue	6.7±0.23[a]	5
pGADT7-*ohrR*+pGBKT7-*dsbM*	+	Blue	6.6±0.31[a]	5
pGADT7-*T*+ pGBKT7-*lam*	-	White	0.06±0.02[a]	5

+: grow nornally on SD/-Trp/-Leu/-His/-Ade plate; -: can not grow on SD/-Trp/-Leu/-His/-Ade plate
Blue: with β-galactosidase activity; White: without β-galactosidase activity
NT: Not Tested
[a] standard deviations; [b] *n*, number of independent assays performed.

electrotransformation of *Pichia pastoris* has been reported a lot, but studies on *S. cerevisiae* are limited. We found the best electrotransformation condition of *S. cerevisiae* and evaluated the effect of different growth stages on transformation efficiency in the current study.

We found that there was reaction between DsbM and OhrR, which were resistance relative proteins in *P. aeruginosa*. The measurement of ²-galactosidase activity showed that the binding of the proteins was a little lower than that between protein P53 (pGBKT7-*53*) and T antigen SV40

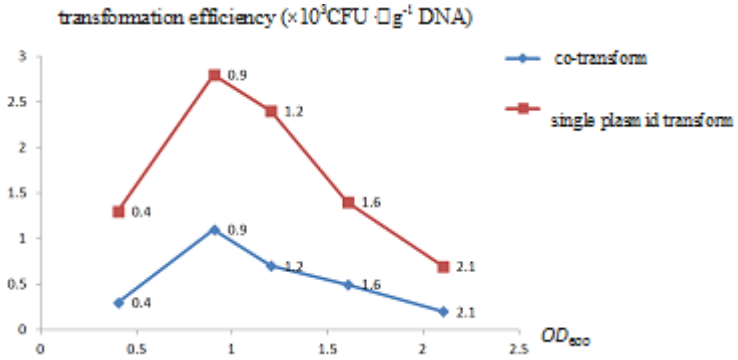
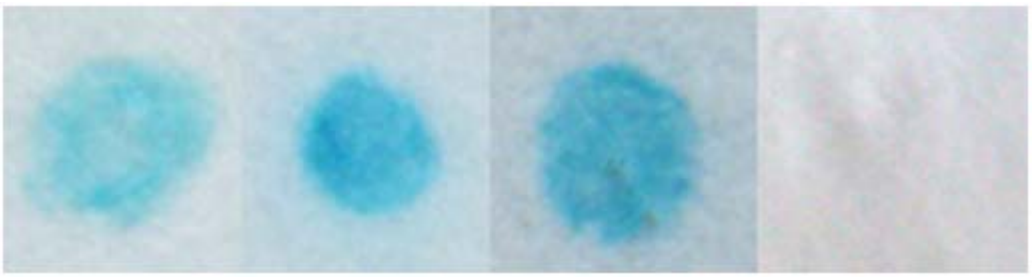


Fig.1. Effects of different growth phases of yeast on transformation efficiency



a. pGADT7-*T*+pGBKT7-*53* (positive control); b. pGADT7-*dsbM*+pGBKT7-*ohrR*;
c. pGBKT7-*dsbM*+pGADT7-*ohrR*; d. pGADT7-*T*+ pGBKT7-*lam* (negative control).

Fig. 2. β-galactosidase activities detected by filter assay

(pGADT7-*T*) (Li *et al*., 1993; Iwabuchi et al., 1993). This result indicated that there was a strong interaction between DsbM and OhrR in *P. aeruginosa*. But it is unclear that the relationship is how to affect the aminoglycoside antibiotics in *P. aeruginosa.*

There is a alkyl hydroperoxide reductase gene in the downstream of *ohrR* in *P. aeruginosa*, and this gene is homologous to *PA0059* (*osmC*). The *PA0059* (*osm*C) is in the downstream of *dsbM* and is belong to the same operon with *PA0060*. The expression of *PA2850* (*ohr*) in mutant strain missing *dsbM* is 4.3 times higher than that in a wild strain (Wang *et al*., 2012). The relationship among genes, *ohrR*, *ohr* and *dsbM* deserves further study in the future.

ACKNOWLEDGEMENTS

This work was supported in part by The National Natural Science Foundation of China (No. 30570089).

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