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DsbM, a Novel Disulfide Oxidoreductase Affects Aminoglycoside Resistance in *Pseudomonas aeruginosa* by OxyR-Regulated Response

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A *Pseudomonas aeruginosa* mutant strain M122 was isolated from a Mu transposon insertion mutant library. In our prophase research, we have found that *PA0058*, a novel gene encodes a 234-residue conserved protein, was disrupted in the M122 mutant. In this study, the bacteriostatic experiment *in vitro* indicates that M122 has abnormally high aminoglycoside resistance. We expressed PA0058 in *E. coli* and found that PA0058 oxidizes and reduces disulfide. This biochemical characterization suggests that PA0058 is a novel disulfide oxidoreductase. Hence, the protein was designated as DsbM. Microarray analysis of the M122 mutant showed its unusual phenotype might be related to the bacterial antioxidant defense system mediated by the *oxyR* regulon. Meanwhile, we detected –SH content in the periplasm of M122 and wild strain and found a lower –SH/S–S ratio in M122. Therefore, we consider that the loss of *dsbM* function decreased the –SH/S–S ratio, which then prolongs the OxyR-regulated response, thereby conferring high aminoglycoside resistance to the M122 mutant strain. Our findings have important implications for understanding the mechanisms underlying aminoglycoside resistance in *P. aeruginosa*.

Keywords: aminoglycoside resistance, disulfide oxidoreductase, oxyR regulon, DsbM

Introduction

Pseudomonas aeruginosa is one of the most common opportunistic Gram-negative pathogens that often cause serious, persistent infections in hospitals (Hatano and Pier, 1998; Sadikot *et al.*, 2005). *P. aeruginosa* has a remarkable ability to adapt to a variety of environments, including those inside human hosts, and it is intrinsically resistant to a wide range of antibiotics. Indeed, the incidence of clinical multidrug-resistant *P. aeruginosa* infections is increasing every year (Bodey *et al.*, 1983; Rotschafer and Shikuma, 1986; Chang *et al.*, 2005; Salunkhe *et al.*, 2005). The occurrence of resistant *P. aeruginosa* limits current disinfection strategies and complicates the treatment of infections. Hence, investigating the mechanisms that underlie antibiotic resistance in *P. aeruginosa* has significant clinical value.

P. aeruginosa PA68 is a kanamycin-sensitive (minimum inhibitory concentration, MIC=2.5 μg/ml) strain of *P. aeruginosa* isolated from a patient with bronchiectasis in earlier research. In the present study, we investigated the mechanism of antibiotic resistance of *P. aeruginosa* PA68 using a combination of genetic and biochemical approaches. Shan *et al.* (2004) successfully introduced Mu transposition complexes into PA68 and established a library containing 6000 mini-Mu insertion mutants. A mutant strain designated as M122 was identified to have very high streptomycin resistance and was used in subsequent experiments. Our *in vitro* bacteriostatic experiments indicated that a mutation in *dsbM*, which encodes a novel disulfide oxidoreductase, contributes to a high level of resistance to several aminoglycosides in M122. The *dsbM* gene encodes a 234-residue protein of unknown function. The DsbM protein is considered a novel disulfide oxidoreductase. Disulfide oxidoreductases are necessary components of the protein folding pathway of many cell envelope proteins in the periplasmic space (Kurukawa *et al.*, 2000). Genetic and biochemical studies have led to the discovery of a series of Dsb proteins. At least five Dsb proteins (DsbA, DsbB, DsbC, DsbG, and DsbH) have been found in *P. aeruginosa* (Lee *et al.*, 2006; Mathee *et al.*, 2008; Winsor *et al.*, 2009). Furthermore, microarray analysis revealed that the expression levels of some members of the antioxidant defense system *oxyR* regulon are elevated by at least two-fold in M122. Additionally, glutathione (GSH) in the M122 periplasmic space was quantified, and a significantly lower –SH/S–S ratio was found. Based on these results, we conclude that the prolongation of the OxyR-regulated response conferred high aminoglycoside resistance to the M122 mutant strain.

Materials and Methods

Minimal inhibitory concentration of antibiotics for the PA68 and M122 strains

The minimal inhibitory concentrations (MICs) of antibiotics for the strains PA68 and M122 were determined using a microdilution assay (Firsov *et al.*, 1998). Overnight liquid cultures were diluted 1,000 times with Luria-Bertani (LB) broth, and 200 μl of the diluted culture was added to the first well of a 96-well plate. Antibiotics were then added at the desired concentrations to the same well and mixed. Then, 100 μl of the diluted liquid culture was added to the next

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well. Two continuous dilutions were performed to achieve a suitable concentration. The 96-well plate was incubated at 37°C, and the MICs were measured after 20 h.

RNA isolation

The effects of *dsbM* inactivation on the expression levels of other genes were assessed using a microarray analysis of *P. aeruginosa* strains PA68 and M122. The strains were initially grown in 20 ml of sterilized LB broth at 37°C with shaking at 200 rpm for 18 h. The overnight cultures were diluted 1:100 in LB broth and incubated at 37°C with shaking at 200 rpm for 4 h. The cells were harvested, quickly resuspended in TRIzol (Invitrogen, USA), and briefly lysed at room temperature. The cell lysate was extracted with chloroform and vigorously shaken. Isopropyl alcohol was then added to the water to precipitate the nucleic acids. The resulting pellet was washed with 75% ethanol and resuspended in RNase-free water. The RNA was purified using an RNeasy Kit (QIAGEN, USA) according to the manufacturer's instructions. Total RNA was treated with DNaseI to eliminate chromosomal DNA contamination. The total RNA yield was quantifiby measuring light absorption at 260 nm and 280 nm. All steps were carried out at 4°C. RNA was also intermittently cooled on ice during the RNA isolation procedure. The RNA samples were electrophoretically separated in 1.2% agarose with 2% formaldehyde as the denaturant.

Gene chip experiments and analyses

The resulting cDNA was synthesized from 10 μg of total RNA with random primers and reverse transcriptase using Affymetrix *P. aeruginosa* GeneChip arrays, as described by Affymetrix (Affymetrix, Inc., USA) and by Salunkhe *et al.* (2002). The cDNA was purified using a Qiagen MinElute PCR Purification Kit (QIAGEN, USA), and cleaved at 37°C for 10 min by adding DNaseI. Biotin-ddUTP was utilized to label the 3′ termini of the cDNA segments by terminal transferase. The labeling efficiency was assessed with a gel-shift assay. A hybridization solution was prepared with the fragmented and labeled cDNA, as well as a B2 control oligonucleotide. The arrays were hybridized, washed, stained, and scanned using an Affymetrix GeneChip Scanner 3000 7G4C.

Determination of -SH content in the periplasm

Ellman's Reagent [5,5′-dithio-*bis*-(2-nitrobenzoic acid); DTNB] was used to detect -SH compounds (Ames *et al.*, 1984). The strains PA68 and M122 were incubated at 37°C with shaking at 200 rpm until the OD_{600} reached 0.5. The Cells were harvested by centrifugation at 1100 rpm for 10 min, and then lysed in 30 μl of chloroform at room temperature for 15 min. The cell lysates were added to 200 μl of reaction buffer with 0.8 mM DTNB. After 5 min, the reaction mixture was centrifuged at 6,000 rpm for 20 min. The -SH content of the periplasm was quantified by measuring the light absorbed by the supernatant liquid at 412 nm.

Expression and purification

Full-length *P. aeruginosa dsbM* was amplified from the *P. aeruginosa* PAO1 genomic DNA by PCR using primers: *dsbM*Ex-F (5′-GGAATTCCATATGAACGACCTCACCC TT-3′) and *dsbM*Ex-R (5′-GGCGAATTCTTATCAGGCG ACGTCGAT-3′). PCR amplification was performed by incubation for 30 cycles at 98°C for 10 sec, 60°C for 15 sec, and 72°C for 45 sec. The PCR products were gel-purified using an Agarose Gel DNA Fragment Recovery Kit Ver. 2.0 (TaKaRa, Japan). The A-tailed PCR products were then ligated into the cloning vector pEASY-T3 using a pEASY-T3 Cloning Kit (Transgen, China). The *dsbM* fragment was cleaved from pEASY-T3 using *Nde*I and *Eco*RI, and then ligated into these sites in pET28a to create the recombinant plasmid pET28a-*dsbM*. This plasmid was used to transform *Escherichia coli* BL21 (DE3) for protein expression. Protein expression was induced by adding 1 mM isopropyl β-d-1-thiogalactopyranoside and further cultured for 4 h.

The cells were harvested by centrifugation at 6,000 rpm, 4°C for 10 min, and the resulting pellets were washed with binding buffer. The cells were suspended in the same buffer, sonicated thrice on ice for 10 min, and centrifuged at 12,000 rpm for 20 min. The supernatant liquid was loaded onto Ni-nitriloacetic acid resin previously equilibrated with the binding buffer and incubated at room temperature for 10 min. The protein-bound resin was washed twice with wash buffer by gravity flow until no protein could be detected in the flow-through. The protein was eluted by adding at least 10 ml of eluting buffer. The purified protein was dialyzed against Milli-Q water and subsequently freeze-dried.

After purification, the protein samples were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were run in 1× glycine buffer at 75 V for 45 min and 150 V for 90 min in stacking and separating gels, respectively. The sequence alignment and tertiary structure prediction of DsbM were performed using the online tool InterProScan.

Biochemical characterization

DTT oxidation activity: The oxidative activity of DsbM was determined by monitoring DTT oxidation. The reaction was performed in 100 mM formic acid/NaOH buffer (pH 4.0) with 1 mM EDTA at 25°C. The reaction system contained 5 mM each of DTT and glutathione disulfide (GSSG), and the final DsbM concentration was 100 μg/ml. Changes in absorbance at 287 nm were read and recorded (Wunderlich *et al.*, 1995).

Insulin reductase activity, optimal temperature, and optimal pH: The *in vitro* disulfide reductase activity of DsbM was measured based on its ability to catalyze insulin reduction by DTT, as previously described (Holmgren, 1979). Fresh reaction mixtures were prepared in 1 ml cuvettes with final concentrations of 0.13 mM insulin, 100 mM sodium phosphate, and 2 mM EDTA (pH 7.0). The protein was added to the cuvettes at 2 μM and 4 μM. A control cuvette contained only the buffer and insulin. The reaction was started with the addition of 0.4 mM DTT to all cuvettes at 25°C. Insulin reduction was monitored by measuring the optical density of the samples at 650 nm every 2 min for 30 min. The increase in turbidity from the precipitation of the insulin B chain was recorded.

The optimal temperature and pH of the catalytic reduction of insulin disulfide bonds were also determined. The

Table 1. MICs of antibiotics to *P. aeruginosa* PA68 and M122

Antibiotics	PA68 (μg/ml)	M122 (μg/ml)
Aztreonam	6.25	3.125
Tetracycline	25	12.5
Gentamycin	1.5625	6.25
Streptomycin	12.5	200
Carbenicillin	75	75
Cefuroxime Sodium	400	400
Ciprofloxacin	$5\times(0.5)^{7}$	$5\times(0.5)^{6}$
Chloromycetin	200	100
Kanamycin	2.344	600
Neomycin	100	200
Spectinomycin	3.125	400

reactions were started at various temperatures (5, 15, 25, 35, 45, 55, and 65°C) in 100 mM sodium phosphate buffer (pH 7.0) with 4 μM protein. The optimal pH was determined by measuring the activity at pH 4.0 to 9.0 using different buffers (pH 4.0, 100 mM formic acid/NaOH buffer; pH 5.0 to 7.0, 100 mM sodium phosphate buffer; pH 8.0 to 9.0, 50 mM Tris-HCl buffer) with 4 μM protein. The reactions were carried out at 25°C for 30 min. All measurements were compared with that obtained from a control cuvette containing 0 μM protein at comparable temperature and pH conditions.

Disulfide isomerase activity: The isomerase activity of DsbM was evaluated using a protein refolding assay. We chose horseradish peroxidase (HRP), which has four disulfide bonds, as the substrate. HRP was completely denatured and reduced in a buffer containing 6 M guanidine hydrochloride (GdnHCl) and 20 mM DTT at 4°C for 2 h. The denatured HRP was renatured in renaturation buffer (50 mM Tris-HCl, 1 mM EDTA, and 0.75 M GdnHCl; pH 8.5) at 4°C for 24 h. DsbM (4 μM/L) was added to the renaturation buffer to facilitate the refolding process. A control without DsbM was also established (spontaneous refolding). HRP enzyme activity was tested to determine the renaturation efficiency. HRP was found to catalyze 3,3′,5,5′-tetramethylbenzidine (TMB) to form a colored product and it has an absorbance peak at 450 nm.

State of expressed protein DsbM: The free thiol content in pure DsbM was assayed using DTNB to determine whether the cysteine residues were free or they formed disulfide bonds. DTNB reacts with free thiol and the resulting yellow product has an absorption peak of 412 nm.

Results

MIC of antibiotics for strains PA68 and M122

Our drug-sensitivity tests indicated that M122 has a higher resistance to several aminoglycosides (Table 1). M122 has 15-fold higher streptomycin resistance than the wild-type PA68. The Mu transposon carries a kanamycin resistance gene for selecting mutants using kanamycin; thus, the kanamycin resistance of M122 is very high. This very large increase is mainly caused by the resistance gene on the Mu transposon, not the loss of the *dsbM* gene. The resistance of M122 to gentamicin, neomycin, and spectinomycin also increased by 4-, 2-, and 168-fold, respectively. However, no significant difference was observed in β-lactam antibiotic resistance between PA68 and M122. Similar results were observed for other types of antibiotics such as tetracycline, quinolones, and chloramphenicol. Thus, the mutant strain M122 had significantly higher aminoglycoside resistance than PA68.

Microarray analysis

Differences in genetic expression between the wild-type PA68 and the mutant M122 strains were evaluated via cDNA microarray analysis. The gene upstream of *dsbM*, designated as *PA0057*, is a novel gene with unknown function in *P. aeruginosa*. The gene downstream of *dsbM* is *osmC* (*PA0059*), which encodes a peroxidase and shows homology with the organic hydroperoxide resistance gene *ohrR* (Gutierrez *et al.*, 1991). Compared with PA68, the level of *osmC* expression in M122 was slightly lower (by 0.87-fold).

Table 2. List of the changes of impotant *P. aeruginosia* genes in *dsbM* deleted strain M122

Gene (name)	Fold change	Protein name
PA0059(*osmC*)	0.87	Osmotically inducible protein OsmC
PA2018(*mexY*)	1	RND multidrug efflux transporter
PA2019(*mexX*)	1.74	RND multidrug efflux membrane fusion protein precursor
PA4119(*aph*)	0.15	Aminoglycoside 3-phosphotransferase type IIb
Pae_L06157cds2	9.85	aac(3)-Ib / PROD=aminoglycoside 3-N-acetyltransferase
Pae_M29695cds2	0.15	AAC (6)-II
Pae_L06161cds	0.31	aac(3)-IIIb / *P. aeruginosa* aac(3)-IIIb gene complete cds
Pae_L06160cds	1.07	aac(3)-IIIc / aminoglycoside 3-N-acetyltransferase
Pae_M98270cds2	0.76	ant(4)-IIa / aminoglycoside-4-adenyltransferase
PA2850(*ohr*)	4.29	Organic hydroperoxide resistance protein
PA4613(*katB*)	4.92	Catalase
PA0139(*ahpC*)	2	Alkyl hydroperoxide reductase subunit C
PA0140(*ahpF*)	3.73	Alkyl hydroperoxide reductase subunit F
PA0848(*ahpB*)	1.74	Probable alkyl hydroperoxide reductase
PA4236(*katA*)	1.32	Catalase
PA4366(*sodB*)	1.41	Superoxide dismutase

Table 3. The-SH content of PA68 and M122 in different growth conditions

Strains and growth conditions	PA68	M122	M122 (str50)	M122 (str100)
A412nm	0.481	0.434	0.391	0.356
SH-content (mM/ml)	0.039	0.035	0.032	0.029
changes	0	-10%	-18%	-26%

Strains with the multidrug efflux system MexXY-OprM reportedly tolerate aminoglycosides (Lomovskaya *et al*., 1999). Microarray analysis showed that *mex*Y did not change and *mex*X was upregulated by only 1.74-fold in the M122 strain (Table 2). Aminoglycoside-modifying enzymes (AMEs) are an important contributor to aminoglycoside resistance. AMEs are ubiquitous in *P. aeruginosa*. Six AME genes [*aac*(3′)-Ib, *aac*(3′)-IIIb, *aac*(3′)-IIIc, *aac*(6′)-II, *ant*(′4)-IIa, and *aph*(3′)-IIb] were found in M122. However, the signals of the AME genes were rather low and the changes in their expression level were not obvious.

Unusual aminoglycoside resistance is considered related to the bacterial antioxidant defense system. The loss of *dsbM* triggers the transcription of genes that encode superoxide dismutase (SOD), catalase, alkyl hydroperoxide reductase, and GSH peroxidase, which comprise the antioxidant defense system of *P. aeruginosa*. In the present study, the catalase gene PA4613 (*katB*) was remarkably increased by 4.92-fold in the M122 strain, whereas PA4236 (*katA*) was increased by 1.32-fold. The mRNA levels of several alkyl hydroperoxide reductase genes such as PA2850 (*ohr*), PA0848 (*ahpB*), PA0139 (*ahpC*), and PA0140 (*ahpF*), which are essential for resistance to oxidative stress, increased by 4.29-, 1.74-, 3.73-, and 3.73-fold, respectively.

Lower SH-content in the M122 periplasm

In animal and plant tissues, as well as in bacteria and yeast, GSH has the most abundant SH-. GSH has many functions, such as protection against the toxicity of biologically active oxygen and the maintenance of the SH- of proteins. To carry out these functions, GSH is converted into GSSG. However, GSSG is reduced by GSH reductase; hence, GSH is the main form in organisms. DTNB reacts with GSH to forms 2-nitro-5-thiobenzoic acid, which has an absorbance at 412 nm. A standard GSH concentration curve (mM/ml, x) was constructed, and absorbance at 412 nm (y) was determined. The standard curve had good linearity from 100 μM/ml to 6.25 μM/ml, and the linear equation was y=13.06x–0.029. The SH- content in the periplasm of the PA68 and M122 strains under different growth conditions are shown in Table 3. The SH-content of M122 cultured with 0 mg/L, 50 mg/L, and 100 mg/L streptomycin decreased by 10%, 18%, and 26%, respectively, compared with those of PA68.

Table 4. Renaturation efficiencies of spontaneous refolding HRP and catalyzed refolding HRP

	A450nm	Active HRP (ng)	Total HRP (ng)	Renaturation efficiencies
Denatrued HRP	0	0	100	0%
Spontaneous refolding	0.337	9.4	100	9.4%
Catalyzed refolding	0.472	13.2	100	13.2%
Undenatured HRP	0.358	10	10	100%

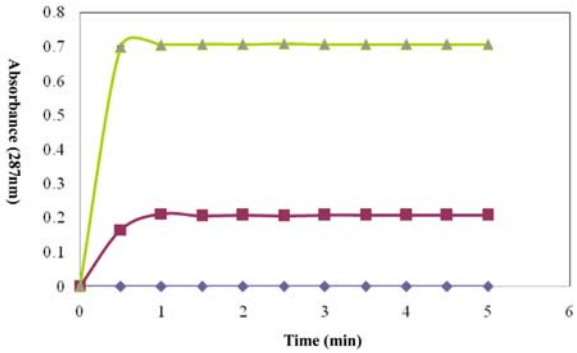

Fig. 1. The results of DTT oxidation activity. Assay of oxidative activity of DsbM by oxidation of DTT. The increase in absorbance at 287 nm is plotted against the reaction time in the absence (◆) or in the presence of pure DsbM: 4 μM/L protein with 5 mM/L GSSG and 5 mM/L DTT in reaction system (▲), 4 μM/L in the reaction system without GSSG and DTT (■) was used to eliminate the impact of protein precipitation.

Biochemical characterization

DsbM was successfully expressed as a soluble protein in *E. coli*. SDS-PAGE analysis showed that DsbM was about 25 kDa, which approximates the theoretical value of 24.93 kDa. The yield of the purified DsbM was 5 mg in 1 L culture. Our DTT oxidation experiments showed that DsbM catalyzes the oxidation of free thiols into disulfide bonds. The oxidized DTT formed by DsbM catalysis had strong absorption at 287 nm (Fig. 1). DsbM also exhibited insulin reductase activity. Reduction in the experimental groups, which contained the DsbM protein, was more rapid and intense than in the control group at 25°C and pH 7.0. The experimental group containing 6 μM protein showed a faster reaction rate than the control group with 4 μM protein (Fig. 2). Reductase activity was also assayed at pH rang-

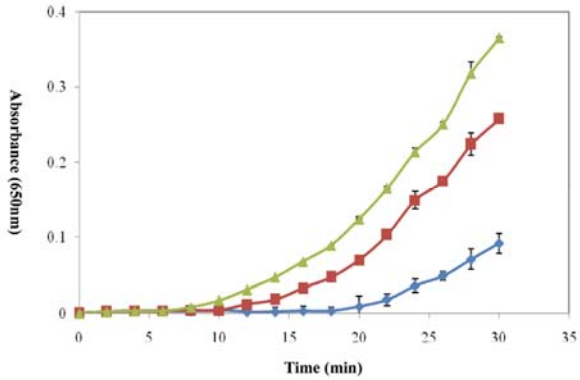

Fig. 2. The results of insulin reductase activity. Assay of reductive activity of DsbM by reduction of insulin. The DTT dependent reduction of insulin disulfide was carried out as described in 'Materials and Methods'. The increase in turbidity at 650 nm is plotted against the reaction time in the absence (◆) or in the presence of pure DsbM: 6 μmol/L (▲) and 4 μmol/L (■).

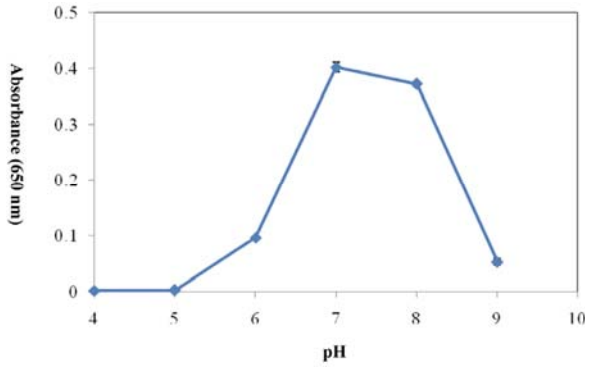

Fig. 3. Optimum pH for DsbM activity. The optimal pH of insulin reduction reaction catalyzed by DsbM. Reduction of insulin catalyzed by DsbM at various pH. The optimal pH is 7.0.

ing from 4.0 to 9.0 and at temperatures ranging from 5°C to 65°C. The optimal pH and temperature (at which DsbM had the highest activity) were found to be 7.0 and 35°C, respectively (Figs. 3 and 4).

Isomerase activity was measured using denatured HRP, undenatured HRP, spontaneous refolding HRP, and catalyzed refolding HRP. A variety of HRPs were allowed to react for 10 min with an ABC mixture from an EL-TMB Kit and solution D was used to stop the reactions. The renaturation efficiencies of denatured HRP, spontaneous refolding HRP, and catalyzed refolding HRP were 0%, 9.4%, and 13.2%, respectively (Table 4). In the experimental groups, the renaturation efficiency of catalyzed refolding was 3.8% higher than that of spontaneous refolding. DsbM has a relatively weaker disulfide bond isomerase activity than DsbC in *E. coli*, which reportedly has 8% higher renaturation efficiency in catalyzed refolding than that of spontaneous refolding (Xu *et al.*, 2008).

DTNB was used to assay the free thiol content of the expressed DsbM. The absorbance at 412 nm of 40 nM expressed DsbM and 40 nM reducing state DsbM were 0.007 and 0.922, respectively. Using the equation of the standard curve, the free thiol content of the expressed and reducing state DsbM were calculated to be 1.48 and 275 nM, respectively. Therefore, each expressed DsbM molecule had 0.07-SH groups, and each reduced DsbM molecule had 6.875-SH groups. Theoretically, one molecule oxidation state DsbM should has 0-SH groups and one molecule reducing state DsbM has 6-SH groups. This means that expressed protein DsbM is oxidation state DsbM.

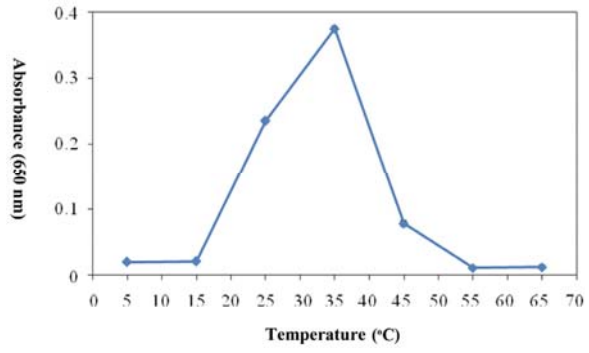

Fig. 4. Optimum temperature for DsbM activity. The optimal temperature of insulin reduction reaction catalyzed by DsbM. Reduction of insulin catalyzed by DsbM at various temperatures. The optimal temperature is 35°C.

Discussion

Earlier research found that Mu transposon was inserted into the novel gene *dsbM* of the M122 genome. Wang *et al.* (2012) reported, after transforming a plasmid containing the gene *dsbM* into M122, the resistance phenotype was partially restored. The MIC of streptomycin in the *dsbM* gene knockout strain Δ0058-PAK (64 μg/ml) was 4-fold higher than that of the standard strain PAK (16 μg/ml). Based on previous research results, in this study we hypothesized that the increased aminoglycoside resistance of the M122 strain is caused by *dsbM* inactivation. It is the first time the gene *dsbM* has been identified and reported to be involved in multidrug resistance of *P. aeruginosa*.

Compared with the PA68 strain, the *osmC* expression level did not change significantly in the M122 strain. In our prophase research, we also analyzed the *osmC* expression level using real-time quantitative PCR (qRT-PCR), and similar results were obtained. A yeast two-hybrid system was used to study the interaction between DsbM and OsmC, and no interaction was found between the two contiguous genes. Strains with the multidrug efflux system MexXY-OprM are able to tolerate aminoglycosides (Lomovskaya *et al.*, 1999). Therefore, we examined the expression MexXY-OprM level using qRT-PCR. *mexX* was only modestly upregulated (~20%) in the M122 strain.

Likewise, the AME gene expression levels were not significantly different. The gene Pae_L06157cds2(aac(3′)-Ib) encodes aminoglycoside 3-N-acetyltransferase, an AME. Although a 9.85-fold increase in gene expression was observed, it does not explain the high resistance to other aminoglycosides except kanamycin. The Mu transposon carries a kanamycin resistance gene, which accounts for the 9.85-fold upregulation of Pae_L06157cds2(aac(3)-Ib). This indicates that *dsbM* influences the aminoglycoside resistance through other ways aside from the enhanced expression of MexXY-OprM and AMEs.

In the M122 strain, the transcription of *katB*, *ahpB*, and *ahpCF* were upregulated. The expression of these oxidative stress defense genes are regulated by a highly specific OxyR-regulated response. This response is governed by a 34 kDa H_2O_2-responsive transactivator called OxyR in *P. aeruginosa* (Ochsner *et al.*, 2000). The tetrameric OxyR protein exists in two forms. Oxidized OxyR activates transcription, whereas reduced OxyR acts as a transcriptional autorepressor. The OxyR exists in reduced form under normal conditions. Under oxidative stress, OxyR changes into oxidized state by forming an intramolecular disulfide bond and acts as a transcriptional activator (Storz and Polla, 1990; Toledano *et al.*, 1994). Oxygen-dependent OxyR phenotypes have the ability to resist H_2O_2 and aminoglycosides, which are clinically and environmentally important antibiotics (Hassett *et al.*, 2000).

Table 5. CXXC motif of DsbM comparison of the other Dsb protein in *E. coli* and *P. aeruginosa*

Protein	Active sites	Subcellular localization and function
DsbM	CGWC	periplasm, thiol:disulfide interchange protein
DsbA (*E. coli*)	CPHC	periplasm, oxidize two-SH formed a disulfide bond
DsbB (*E. coli*)	CVLC	Inner membrane, reactive DsbA
DsbC (*E. coli*)	CGYC	periplasm,isomerize mis-paired disulfide bonds
DsbD (*E. coli*)	CVAC	Inner membrane,reduced DsbC
DsbE (*E. coli*)	CPTC	Inner membrane, participate in synthesis of cytochrome C
DsbG (*E. coli*)	CPYC	periplasm, isomerize mis-paired disulfide bonds
DsbA (*P. aeruginosa*)	CPHC	periplasm, thiol:disulfide interchange protein
DsbB (*P. aeruginosa)*	CSLC	cytoplasmic membrane, disulfide formation protein
DsbC (*P. aeruginosa*)	CPYC	periplasm, thiol:disulfide interchange protein
DsbG (*P. aeruginosa*)	CPYC	periplasm, thiol:disulfide interchange protein
DsbH (*P. aeruginosa*)	CPLC	cytoplasmic memberane, disulfide formation protein

In *E. coli*, OxyR is activated or oxidized only within a defined period, and oxidized OxyR is reduced by glutaredoxin 1 via GSH (Zheng *et al*., 1998). *In vivo*, a decreased GSH/GSSG ratio prolongs the OxyR-regulated response (Carmel-Harel and Storz, 2000). In the present study, we found the thiol/disulfide ratio in the periplasm of the M122 strain was significantly lower than in the wild-type PA68 strain. The conversion of OxyR from a reduced state to an oxidized state during oxidative stress occurs via post-translational modification. Therefore, the changes in the state of the protein are independent of OxyR expression level. Therefore, elevated OxyR expression does not directly cause increased resistance against aminoglycosides. However, the elevated expression of genes regulated by OxyR (such as *ahpB*, *ahpCF*, and so on) causes increased aminoglycoside resistance (Hassett *et al*., 2000).

DsbM is able to oxidize, reduce, and isomerize disulfide. It contains a CXXC (CGWC) motif that is conserved in the active sites of Dsb proteins (Martin, 1995) both in *E. coli* and *P. aeruginosa* (Table 5). Tertiary structure prediction demonstrated that the protein has a DsbA-like thioredoxin domain. These findings indicate that DsbM is a novel disulfide bond oxidoreductase in *P. aeruginosa*. The function of DsbM is likely related to the formation, reduction, and isomerization of protein disulfide bonds, especially when wrong disulfide bonds are formed. The relationship between DsbM and disulfide bond formation indicates that the deletion of DsbM disrupted the balance between GSH and GSSG in the periplasmic space, which caused the GSH/GSSG ratio to decrease in the M122 mutant strain. A decreased GSH/GSSG prolongs the OxyR-regulated response, thereby conferring a higher aminoglycoside resistance to the bacterial strain. Therefore, the higher aminoglycoside resistance in the M122 strain is caused by *dsbM* inactivation and the consequent decrease in the GSH/GSSG ratio in the periplasm.

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