

The effect of the disulfideisomerase domain containing protein in the defense against polyhexamethylene biguanide of highly tolerant *Acanthamoeba* at the trophozoite stage



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ABSTRACT

Acanthamoeba castellanii is a free-living protozoan pathogen capable of causing a blinding keratitis and fatal granulomatous encephalitis. Current treatment generally involves an hourly application of polyhexamethylene biguanide (PHMB) over a period of several days but this is not entirely effective against all strains/isolates. The tolerance mechanisms of PHMB in *Acanthamoeba* cells remain unclear. In this study, we found that the mRNA expression level of disulfideisomerase domain containing protein (PDI) increased rapidly in surviving cells of the highly PHMB-tolerant *Acanthamoeba castellanii* strain, NCKH_D, during PHMB treatment, but not in the ATCC standard strain. After PDI-specific silencing, NCKH_D was found to be more vulnerable to PHMB treatment. The results described above show that PDI is an important gene for PHMB tolerance ability in a highly PHMB-tolerant strain of *Acanthamoeba* and provide a new insight for more efficient medicine development for *Acanthamoeba* keratitis.

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1. Introduction

Acanthamoeba keratitis (AK) is a very painful, serious corneal infection caused by free-living pathogenic protozoans of the genus *Acanthamoeba* (McCulley et al., 1995; Visvesvara et al., 2007). *Acanthamoeba* species are ubiquitous, free-living protozoa that exist as motile trophozoites and dormant cysts in many environments, including swimming pools, hot tubs, lakes, soil, dust and drinking fountains (Brown et al., 1982). The trophozoite stage of *Acanthamoeba* feeds on organic particles and microbes and divides mitotically under optimal condition. With exposure to harsh conditions or during a period of stress, the cells can be transformed into double-walled cysts. The inner wall of the cyst possesses cellulose and the outer wall consists of proteins and polysaccharides.

During encystation, the largest increases in resistance are observed.

An increasing number of cases of severe ocular inflammation and visual loss have been caused by *Acanthamoeba* infection in the past two decades. This increased prevalence has been linked to the increasing use of contact lenses (Brown et al., 1982; Broxton et al., 1983; Auran et al., 1987; Allen et al., 2004). Clinical cases of AK are largely restricted to contact lens wearers who have experienced some sort of trauma to their corneal epithelium (CDC, 1987; Paszko-Kolva et al., 1991; van Klink et al., 1993; Morlet et al., 1997). Due to difficulty in early diagnosis and poor response to most antibiotics, therapeutic penetrating keratoplasty is often used to try to eradicate the pathogen in uncontrolled disease. If it is not diagnosed early and treated aggressively, extensive ocular damage include a ring-like corneal infiltrate, epithelial destruction, and disproportionately severe ocular pain, could occur and enucleation may be required (Auran et al., 1987).

The first two AK cases in Taiwan were reported in 1988, corneal biopsy yielded confirmative diagnosis (Chan and Chan, 1988). Medical records reviewed by all patients with clinically diagnosed microbial keratitis presenting at the National Taiwan University

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Hospital from January 1992 to December 2001, indicated that 4.4% of cases of microbial keratitis were caused by *Acanthamoeba* species (Fong et al., 2004). Currently, the treatment regimen in Taiwan involves hourly drug applications for prolonged periods (Gray et al., 1994; McDonnell and Russell, 1999; Visvesvara et al., 2007). Of these drugs, PHMB is a membrane-active agent with broad-spectrum antimicrobial efficacy that has been used as an anti-septic in infective keratitis (Gray et al., 1994; McDonnell and Russell, 1999; Donoso et al., 2002; Narasimhan et al., 2002), swimming pool sanitization, and the treatment of cooling systems (Kusnetsov et al., 1997). Preparations of PHMB are mixtures of polymeric biguanides with an approximate molecular weight range of 400–8000. The structure of PHMB is as follows: $[-(\text{CH}_2)_6\text{NH.C}(=\text{NH}).\text{NH.C}(=\text{NH}).\text{NH}-]_n$, where $n = 2-40$, with an approximate molecular weight range of 400–8000. In previous studies on prokaryotic cells, PHMB was shown to interact with cytoplasmic membranes to cause lethal action, nonspecific alterations in membrane integrity, loss of LPS, and loss of function in membrane proteins (Broxton et al., 1983, 1984; Gilbert et al., 1990). PHMB has also been shown to interfere with prokaryotic cell functions by binding to nucleotides (Allen et al., 2004), and Chindera et al. observed that PHMB not only disrupt the microbial membrane but also arrests cell division and the condensation of chromosomes in treated bacteria species (Chindera et al., 2016).

PHMB is further believed to cause the production of large quantities of phosphorus, which accumulate in the nuclei of *A. castellanii* cells as a result of reduced permeability and protein coagulation or the aggregation of phospholipids (Khunkitti et al., 1999). Trophozoites have been reported to absorb more biguanides than do cysts. Complex PHMB-nucleotide formations have been observed near the cell wall and nuclei following drug uptake by *Acanthamoeba*. Nonetheless, clinical outcomes tend to be poor, particularly in cases involving drug-resistant/tolerant strains. The mechanism underlying the resistance to PHMB by *Acanthamoeba* cells has yet to be elucidated for overcoming infection caused by these PHMB-resistant/tolerant strains.

To gain a deeper understanding of the mechanism underlying the first step of resistance to PHMB in *Acanthamoeba*, we established an RNA-seq database for *Acanthamoeba*, including the non-pathogenic ATCC strain and the highly PHMB-tolerant *Acanthamoeba castellanii* strain, NCKH_D (Huang et al., 2015). In our previous study, we have shown that *A. castellanii* clinical isolates NCKH_B and NCKH_D have higher tolerance toward PHMB than does the standard strain, ATCC 30010 (Huang et al., 2015). These two isolates differ in their response to PHMB stress: NCKH_B transforms into a pseudocyst to escape the drug, but NCKH_D does not. Especially, NCKH_D is collected from the combinational drug therapy failed patient. Therefore, NCKH_D utilized an alternative drug tolerance strategy rather than transform into pseudocyst/cyst.

In this study, we try to reveal the molecular mechanism of the PHMB resistance in NCKH_D trophozoites. Furthermore, we found that the mRNA expression level of the disulfideisomerase domain containing protein (PDI) had risen rapidly in surviving cells of the NCKH_D strain but not in the ATCC standard strain during PHMB treatment. After PDI-specific silencing, NCKH_D was found to be more vulnerable to PHMB treatment. The results of this study will help to characterize the potential PHMB resistance mechanisms of *Acanthamoeba* and will aid in the development of a more effective treatment regimen for AK.

2. Materials and methods

2.1. Culture of *Acanthamoeba* protozoa

Acanthamoeba species are axenically cultured in a protease

peptone-yeast extract-glucose (PYG) medium, pH 6.5, at 28 °C in cell culture flasks. Trophozoites are harvested at the logarithmic growth phase after cultivation for 3–5 days. The clinical isolates were isolated from the corneal ulcers of patients who were diagnosed with AK in the Cheng Kung University Hospital (Li et al., 2012), including the high PHMB tolerance pathogenic *Acanthamoeba castellanii* isolates NCKU_D which is used in this study (Huang et al., 2015).

2.2. Growth curves and survival rate

Detached cells will be centrifuged and the pellet will be suspended in a 0.5% trypan blue solution in sterile PBS for 1 min. Cells will be then counted in a Neubauer hemocytometer chamber, and the stained cells will be considered dead. Cell counting will be performed under a light microscope in the duration to get the numbers of alive and dead cells which will be differentiated with trypan blue dye exclusion viability method. The number of stained cells will be subtracted from the total, indicating the death ratio in each interaction condition and time.

2.3. Drug treatment

PHMB will be preparation in different concentration in Page's amoeba saline (PAS). To further clarify the appropriate amount of cells, drug concentration and duration of drug experiment; we will add the counted cells in drug solution and shake by 100 rpm.

2.4. Total RNA isolation and cDNA synthesis

We collected the RNA samples from the non-pathogenic isolate ATCC 30010 and the pathogenic *Acanthamoeba castellanii* isolates NCKU_D trophozoites. Pellet the cells by centrifugation, lyse the pellet of *Acanthamoeba* with TRIzol[®] (Invitrogen), add 100% Ethanol (Merck) as 1:1 vol, mix it well by vortexing. Extract the RNA by Direct-zol[™] RNA MiniPrep kit (Zymo Research). RNA was stored at –70 °C. The entire concentration and A260/A280 ratio of mRNA were measured with ND-1000 (NanoDrop). High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) were used in this study.

2.5. High-throughput RNA sequence reads collection

The RNA samples from the untreated/PHMB treated *A. castellanii* ATCC 30010 and clinical isolates NCKH_D were used for generating sequence reads with Illumina platform, by which more than 21 million of sequence reads. The samples were also used for reverse transcription PCR validation.

2.6. Reverse transcription PCR

One-step reverse transcription PCR was performed with SuperScript[™] One-Step RT-PCR with Platinum[®] Taq kit (Invitrogen) to investigate the gene expression of *Acanthamoeba*. All of the cDNA was synthesized from 1 µg of total RNA of *Acanthamoeba*. RT-PCR products were separated on the EtBr-stained gel after electrophoresis in 1.0–1.2% agarose. Primers were listed in Table 1.

2.7. Gene silencing methodology

Small interfering RNA (siRNA) targeting on the 1265th to 1283rd nucleotide in the exon coding region of the PDI (ACA1_189420) gene of *Acanthamoeba* was designed and synthesized by Dharmacon (GE Healthcare Dharmacon Inc.), based on the cDNA sequence of the PDI gene. The sense and the antisense sequences are

Table 1
Primer list used for PCR and reverse transcription PCR reactions.

Target region	Primers name	Primer sequence (5' – 3')	Amplicon (bp)	
18s rDNA	AcantF900	CCCAGATCGTTTACCGTGAA	180	Qvarnstrom et al., 2006
	AcantR1100	TAAATATTAATGCCCCCAACTATCC		
PDI gene		ACTTCGATGAGCACACTGCC	641	
ACA1_189420		AACGCCAGAGTAGTCCCTCA		
PDI gene		CAAGGCCGACCTCTTGACT	621	
ACA1_374120		CTGCCGAGCTCATAGTCCTG		
PDI gene		GACGTGTGGAACCCCATCAA	449	
ACA1_374090		TTGTCATTGTCCGCGAAACG		
PDI gene		TCGAAGACTTCACCGCCTTC	481	
ACA1_149830		AGCTCGTCTCTTTTCGGG		
TrXR		AGTACACAGAATGGTCATCGT	681bp	
ACA1_398900		TTGATCTTGAGGCCTGTGAC		

Table 2
Differential gene expression statistics from RNA-Seq.

Effective transcripts	DE transcripts ^a		
30010 non-treat VS treated PHMB 3HR	13,642	71	0.47%
D, non-treat VS treated PHMB 3HR	12,139	525	3.50%
D vs 30010, non-treat	13,583	3260	21.76%
Total references	14,980	(FDR ≤ 0.05)	

^a The effective transcripts defined as the count sum of two libraries for a transcript ≥ 1.

CCAAGAAGCCGGAGAACUAAU and 5'-PUAGUUCUCCGGCUU-CUUGGUU. The siRNA was transfected to *Acanthamoeba* trophozoites as described in a previous study (Kong and Pollard, 2002). As a negative control, siRNA with a scrambled sequence (Dharmacon) absent in *Acanthamoeba* was used.

3. Results

To characterize the potential PHMB resistance mechanisms of

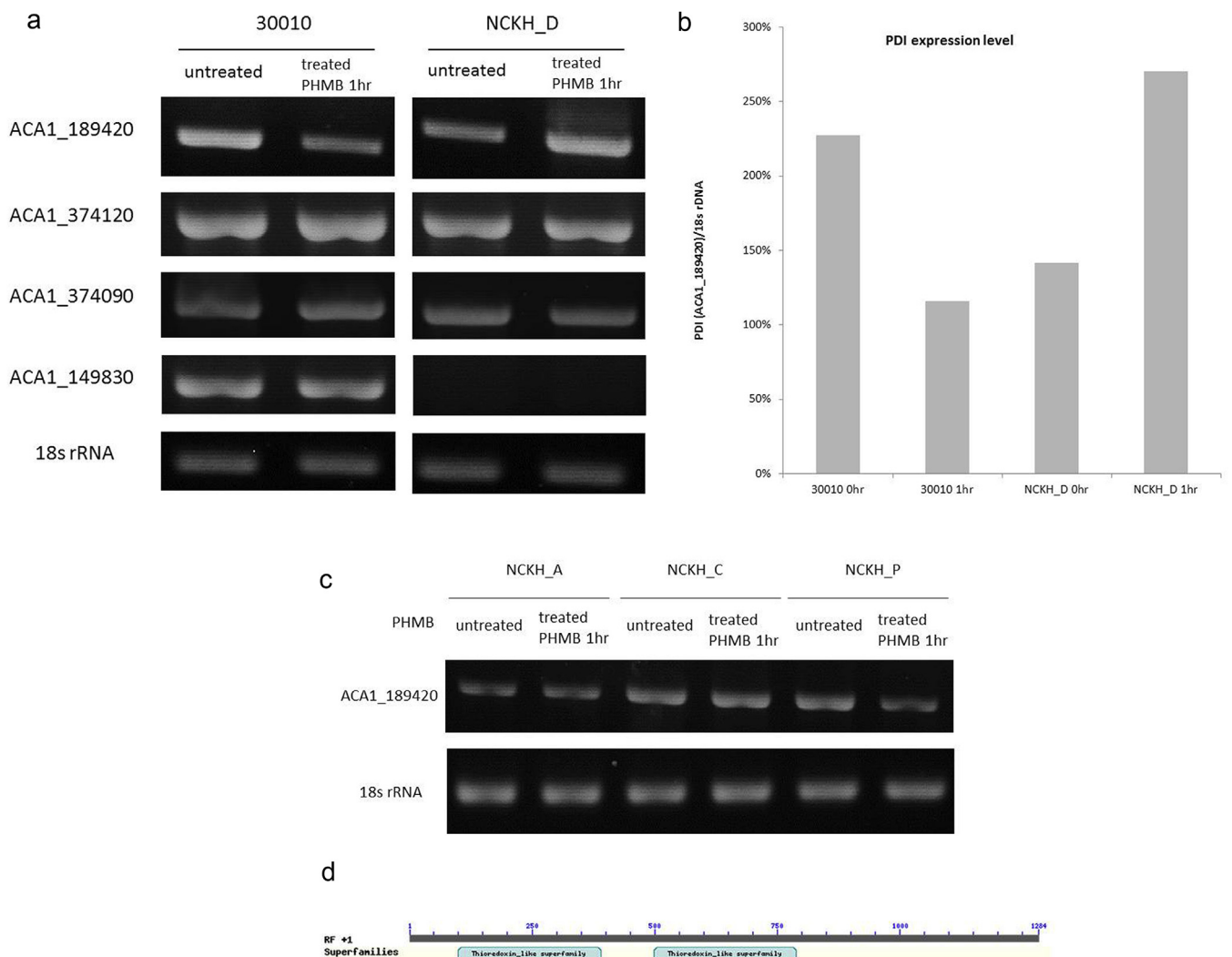


Fig. 1. The expression level of PDI genes in clinical isolate NCKH_D and ATCC 30010 with or without PHMB treatment. (A) and (C) Reverse transcription PCR analysis. The 18S rRNA expression is used as a control. (D) Functional domain prediction of PDI gene, ACA1_189420.

Acanthamoeba and to develop a more effective treatment for AK, we isolated the clinical strains NCKH_D with high PHMB tolerance and created the platforms for screening and elucidating the efficacy of treatment (Huang et al., 2015). To confirm that NCKH_D does not depend on transforming to a cyst to escape the drug, we used a differential interference contrast microscope to observe the morphology of NCKH_D. The result clearly indicates that NCKH_D did not transform to cyst during the 6 h PHMB treatment (Video 1). We proposed that *Acanthamoeba* could produce the stress proteins and regulate certain genes for responding to PHMB treatments. In this study, we established the RNA datasets of strains including NCKH_D and the standard strain, ATCC 30010, for examining the genes associated with PHMB resistance.

Supplementary video related to this article can be found at <http://dx.doi.org/10.1016/j.ijpddr.2016.11.001>.

3.1. The discovery of PDI by comparative analysis of RNA-seq

We analysed the RNA-seq data which were collected from treated and non-treated *Acanthamoeba* cells, with the software package edgeR (empirical analysis of DGE in R), which is part of the Bioconductor project (Gentleman et al., 2004; Robinson et al., 2010). The differential gene expression results from the comparative analysis are shown in Table 2. After combining the differential

gene expression results from RNA-seq databases, we noticed that a special gene, the disulfideisomerase domain containing protein (PDI, ACA1_189420), is up-regulated during PHMB treatment in NCKH_D but down-regulated during PHMB treatment in ATCC 30010.

3.2. The validation of PDI expression

From the comparative analysis of RNA-seq, we found that not only one isoform of PDIs, ACA1_189420, is upregulated during the PHMB-treated experiments but also three isoforms of PDI in the NCBI gene database, including ACA1_149830, ACA1_374090, and ACA1_374120, were up-regulated. To confirm the expression patterns of the PDI isoforms we analysed the RNA samples which were not only previous samples for establishing the database but also those repeated by reverse transcription PCR. The transcriptional expression patterns of the PDI proteins are shown in Fig. 1A and the quantified the expression patterns of ACA1_189420 are shown in Fig. 1B. Only the ACA1_189420 in surviving cells of the NCKH_D, a highly PHMB-tolerated isolate strain associated with keratitis infection, increased rapidly compared to the ATCC standard strain, and the three other *A. castellanii* isolates, NCKH_A, C, and P, which were collected from patients with PHMB treatment successes (Fig. 1C).

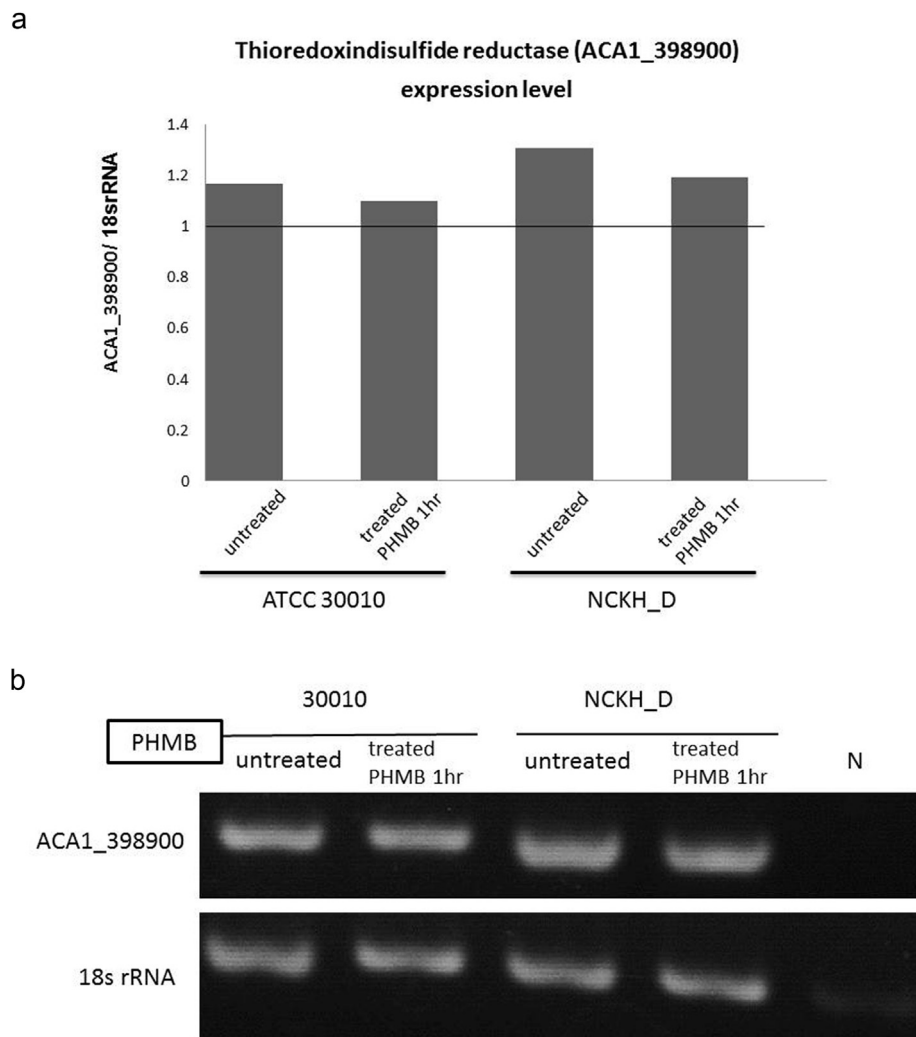


Fig. 2. The expression level of thioredoxindisulfide reductase in clinical isolate NCKH_D and ATCC 30010 with or without PHMB treatment. (A) and (B) Reverse transcription PCR analysis. The 18S rRNA expression is used as a control.

3.3. PDI (ACA1_189420) functional domain prediction

To understand the functions of PDI ACA1_189420, we predicted its functional domains via the NCBI Conserved Domain Search website (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). From the analysed prediction, there are two Thioredoxin-like superfamily domains in ACA1_189420 (Fig. 1D). Thioredoxins are proteins that act as antioxidants. Therefore, we proposed that PDI (ACA1_189420) may be involved in oxidative protein folding in the endoplasmic reticulum by acting as a catalyst and folding assistant.

3.4. The expression level of thioredoxindisulfide reductase (ACA1_398900) does not change during PHMB treatment

The proteins that act as antioxidants are usually maintained in their reduced form at steady state. So we were curious about the expression pattern of Thioredoxindisulfide reductase (ACA1_398900), which is the enzyme used to reduce Thioredoxindisulfide in *Acanthamoeba* cells during the PHMB treatment. From the results shown in Fig. 2A and B, the transcriptional expression level of ACA1_398900 does not change after PHMB treatment.

3.5. PDI (ACA1_189420) is important for NCKH_D PHMB tolerance ability

For elucidating the importance of PDI ACA1_189420 in the PHMB tolerance of NCKH_D, we knocked down the gene expression and used the PHMB treatment again. After PDI ACA1_189420-specific siRNA treatment, we found NCKH_D to be more vulnerable to PHMB (Fig. 3). The result revealed that PDI is an important gene for the PHMB tolerance ability.

3.6. PDI is not a general stress protein

To prove that ACA1_189420 is a general stress or a PHMB specific induced response protein, we tested physical and chemical stresses, such as heat shock and H₂O₂ treatments.

In heat shock experiments, we found that NCKH_D is more tolerant to high-temperature treatment than the ATCC standard strain 30010 (Fig. 4A). These results indicate that the PDI

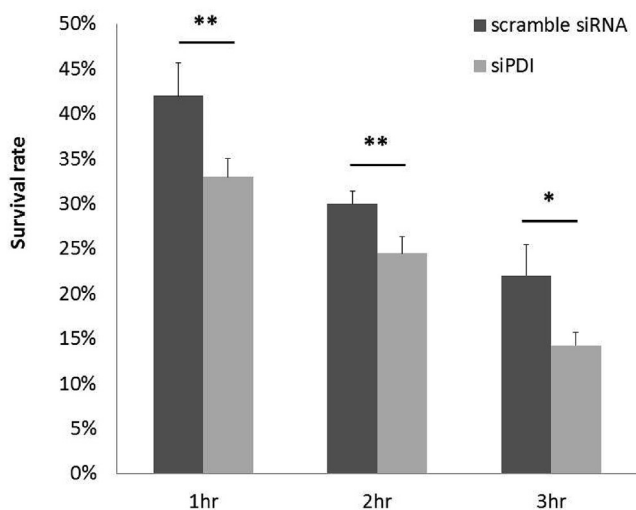


Fig. 3. Effects of ACA1_189420 silencing in the NCKH_D strain during the progress of PHMB treatment. **, the means are significantly different at a P value of <0.01; *, the means are significantly different at a P value of <0.05 by Student's *t*-test.

(ACA1_189420) expression of 30010 and NCKH_D is both down-regulated, which means PDI is not important for the high-temperature tolerance ability of NCKH_D (Fig. 4B). In H₂O₂ treatment experiments, NCKH_D was also more tolerant to hydrogen peroxide treatment than 30010, but the PDI expression of NCKH_D had no specific change (Fig. 5A and B). Thus, we have shown that PDI (ACA1_189420) is a PHMB induced stress protein but not a general stress protein.

4. Discussion

The broad-spectrum antimicrobial polymer PHMB kills bacteria, fungi, parasites and certain viruses. Presently, AK treatment involves hourly applications of PHMB over a prolonged period; however, even this aggressive approach is unable to ensure anything other than a poor prognosis. Further, the treatment is not entirely effective against all strains/isolates. Our previous work presented a PHMB highly tolerant isolate, NCKH_D, which does not depend on transforming to a cyst to escape the drug (Huang et al., 2015). However, the action and resistance mechanisms of PHMB in *Acanthamoeba* cells are still not clear. Knowledge of these mechanisms could be used to progress the current treatment for more effective use against the infection.

Based on the experimental results of the present study, we have for the first time provided solid evidence that PDI (ACA1_189420) is a PHMB resistance associated gene in *Acanthamoeba*. There are two Thioredoxin-like superfamily domains in PDI (ACA1_189420). Thioredoxin (Trx), an important factor for protein dithiol/disulfide homeostasis and is conserved from bacteria to man. However, the

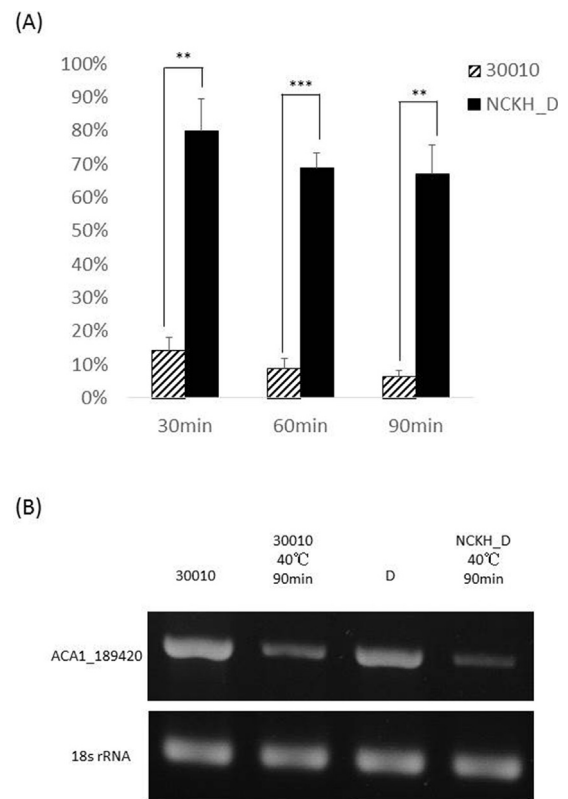


Fig. 4. Effects of the *Acanthamoeba* strains during the heat shock treatment. (A) Survival rate of *Acanthamoeba* strains during the 40 °C shock. (B) Reverse transcription PCR analysis of the PDI gene, ACA1_189420, expression level in clinical isolate NCKH_D and ATCC 30010 with or without 40 °C heat shock. The 18S rRNA expression is used as a control.

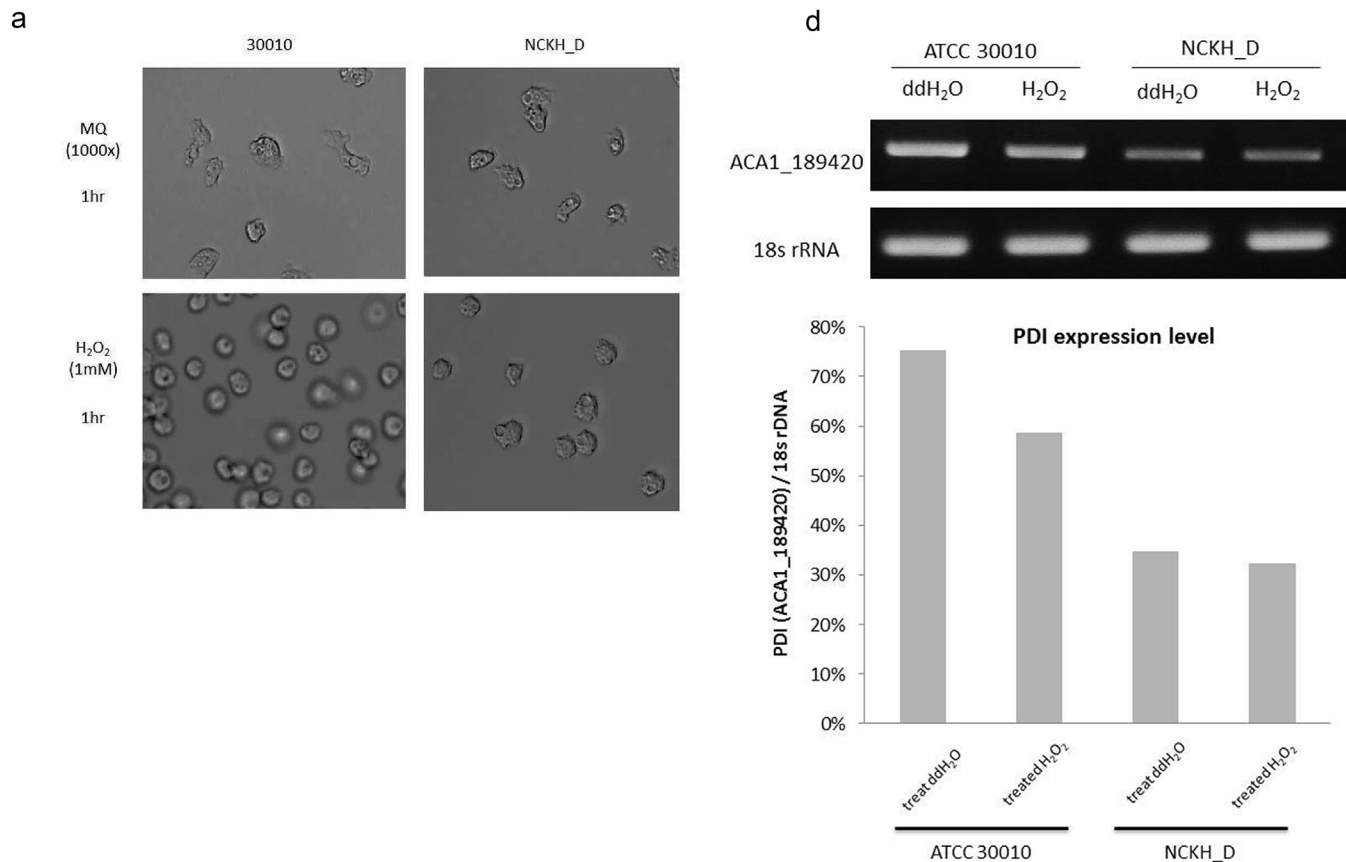


Fig. 5. The expression level of ACA1_189420 in *Acanthamoeba* strains during the H₂O₂ treatment. (A) The light observation for effect imaging of the addition of H₂O₂ to the live *Acanthamoeba* strains. (B) Reverse transcription PCR analysis of the PDI gene, ACA1_189420, expression level in clinical isolate NCKH_D and ATCC 30010 with or without H₂O₂ treatment. The 18S rRNA expression is used as a control.

role of the Thioredoxin reductase (TrxR)/Trx system is still poorly understood in *Acanthamoeba*. In other protists, there are alternative factors in the Trx system that have been reported as drug targets for parasites. In 2007, Leitsch et al. found that the anti-oxidative enzyme, TrxR, could reduce metronidazole and other nitro compounds in *Entamoeba histolytica* (Leitsch et al., 2007). TrxR has also been found to reduce metronidazole and other nitro-heterocyclic drugs in *Trichomonas vaginalis* (Leitsch et al., 2009). In *Giardia lamblia*, TrxR has been suggested to assist as a drug target (Leitsch et al., 2016).

In this study, we have elucidated the associated gene and possible mechanism of PHMB resistance, as described here with a highly PHMB-tolerant strain of *Acanthamoeba*. This study also provides novel insight for dissecting the PHMB resistant mechanisms in *Acanthamoeba* and possible improvements for PHMB treatment of *Acanthamoeba* keratitis.

5. Conclusion

These results described above show that PDI (ACA1_189420) is an important gene for PHMB tolerance ability in highly PHMB-tolerant *Acanthamoeba*.

Conflicts of interest

All authors declare no conflicts of interest.

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References

- Allen, M.J., Morby, A.P., White, G.F., 2004. Cooperativity in the binding of the cationic biocide polyhexamethylene biguanide to nucleic acids. *Biochem. Biophys. Res. Commun.* 318, 397–404.
- Auran, J.D., Starr, M.B., Jakobiec, F.A., 1987. *Acanthamoeba* keratitis. A review of the literature. *Cornea* 6, 2–26.
- Brown, T.J., Cursons, R.T., Keys, E.A., 1982. Amoebae from antarctic soil and water. *Appl. Environ. Microbiol.* 44, 491–493.
- Broxton, P., Woodcock, P.M., Gilbert, P., 1983. A study of the antibacterial activity of some polyhexamethylene biguanides towards *Escherichia coli* ATCC 8739. *J. Appl. Bacteriol.* 54, 345–353.
- Broxton, P., Woodcock, P.M., Heatley, F., Gilbert, P., 1984. Interaction of some polyhexamethylene biguanides and membrane phospholipids in *Escherichia coli*. *J. Appl. Bacteriol.* 57, 115–124.
- CDC, 1987. *Acanthamoeba* keratitis in soft-contact-lens wearers. *MMWR. Morb. Mortal. Wkly. Rep.* 36 (397–398), 403–394.
- Chan, M.Y., Chan, T.C., 1988. *Acanthamoeba* keratitis: report of the first two cases in Taiwan. *ACTA Soc. Ophthalmol. Sin.* 27, 172–178.
- Chindera, K., Mahato, M., Sharma, A.K., Horsley, H., Kloc-Muniak, K., Kamaruzzaman, N.F., Kumar, S., McFarlane, A., Stach, J., Bentin, T., Good, L., 2016. The antimicrobial polymer PHMB enters cells and selectively condenses bacterial chromosomes. *Sci. Rep.* 6, 23121.
- Donoso, R., Mura, J.J., Lopez, M., 2002. *Acanthamoeba* keratitis treated with propamidine and polyhexamethyl biguanide (PHMB). *Rev. Med. Chil.* 130, 396–401.
- Fong, C.F., Tseng, C.H., Hu, F.R., Wang, I.J., Chen, W.L., Hou, Y.C., 2004. Clinical characteristics of microbial keratitis in a university hospital in Taiwan. *Am. J. Ophthalmol.* 137, 329–336.

- Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., Zhang, J., 2004. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 5, R80.
- Gilbert, P., Pemberton, D., Wilkinson, D.E., 1990. Synergism within polyhexamethylene biguanide biocide formulations. *J. Appl. Bacteriol.* 69, 593–598.
- Gray, T.B., Gross, K.A., Cursons, R.T., Shewan, J.F., 1994. Acanthamoeba keratitis: a sobering case and a promising new treatment. *Aust. N. Z. J. Ophthalmol.* 22, 73–76.
- Huang, F.C., Shih, M.H., Chang, K.F., Huang, J.M., Shin, J.W., Lin, W.C., 2015 Dec 1. Characterizing clinical isolates of Acanthamoeba castellanii with high resistance to polyhexamethylene biguanide in Taiwan. *J. Microbiol. Immunol. Infect.* pii: S1684-1182(15)00908-1.
- Khunkitti, W., Hann, A.C., Lloyd, D., Furr, J.R., Russell, A.D., 1999. X-ray microanalysis of chlorine and phosphorus content in biguanide-treated Acanthamoeba castellanii. *J. Appl. Microbiol.* 86, 453–459.
- Kong, H.H., Pollard, T.D., 2002. Intracellular localization and dynamics of myosin-II and myosin-1C in live Acanthamoeba by transient transfection of GFP fusion proteins. *J. Cell Sci.* 115, 4993–5002.
- Kusnetsov, J.M., Tulkki, A.I., Ahonen, H.E., Martikainen, P.J., 1997. Efficacy of three prevention strategies against legionella in cooling water systems. *J. Appl. Microbiol.* 82, 763–768.
- Leitsch, D., Kolarich, D., Binder, M., Stadlmann, J., Altmann, F., Duchene, M., 2009. Trichomonas vaginalis: metronidazole and other nitroimidazole drugs are reduced by the flavin enzyme thioredoxin reductase and disrupt the cellular redox system. Implications for nitroimidazole toxicity and resistance. *Mol. Microbiol.* 72, 518–536.
- Leitsch, D., Kolarich, D., Wilson, I.B., Altmann, F., Duchene, M., 2007. Nitroimidazole action in Entamoeba histolytica: a central role for thioredoxin reductase. *PLoS Biol.* 5, e211.
- Leitsch, D., Muller, J., Muller, N., 2016. Evaluation of Giardia lamblia thioredoxin reductase as drug activating enzyme and as drug target. *Int. J. Parasitol. Drugs Drug Resist* 6, 148–153.
- Li, M.L., Shih, M.H., Huang, F.C., Tseng, S.H., Chen, C.C., 2012. Treatment of early Acanthamoeba keratitis with alcohol-assisted epithelial debridement. *Cornea* 31, 442–446.
- McCulley, J.P., Alizadeh, H., Niederkorn, J.Y., 1995. Acanthamoeba keratitis. *CLAO J.* 21, 73–76.
- McDonnell, G., Russell, A.D., 1999. Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev.* 12, 147–179.
- Morlet, N., Duguid, G., Radford, C., Matheson, M., Dart, J., 1997. Incidence of acanthamoeba keratitis associated with contact lens wear. *Lancet* 350, 414.
- Narasimhan, S., Madhavan, H.N., K, L.T., 2002. Development and application of an in vitro susceptibility test for Acanthamoeba species isolated from keratitis to polyhexamethylene biguanide and chlorhexidine. *Cornea* 21, 203–205.
- Paszko-Kolva, C., Yamamoto, H., Shahamat, M., Sawyer, T.K., Morris, G., Colwell, R.R., 1991. Isolation of amoebae and Pseudomonas and Legionella spp. from eyewash stations. *Appl. Environ. Microbiol.* 57, 163–167.
- Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139–140.
- van Klink, F., Alizadeh, H., He, Y., Mellon, J.A., Silvano, R.E., McCulley, J.P., Niederkorn, J.Y., 1993. The role of contact lenses, trauma, and Langerhans cells in a Chinese hamster model of Acanthamoeba keratitis. *Investig. Ophthalmol. Vis. Sci.* 34, 1937–1944.
- Visvesvara, G.S., Moura, H., Schuster, F.L., 2007. Pathogenic and opportunistic free-living amoebae: acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri, and Sappinia diploidea. *FEMS Immunol. Med. Microbiol.* 50, 1–26.