Nucleoid clumping is dispensable for the Dps-dependent hydrogen peroxide resistance in Staphylococcus aureus

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Dps family proteins have the ferroxidase activity that contributes to oxidative stress resistance. In addition, a part of Dps family proteins including Escherichia coli Dps and Staphylococcus aureus MrgA (metallo regulon gene A) bind DNA and induce the structural change of the nucleoid. We previously showed that a mutated MrgA with reduced ferroxidase activity was unable to contribute to the hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and UV resistance in S. aureus, suggesting that the nucleoid clumping by MrgA is not sufficient for the resistance. However, it remained elusive whether the nucleoid clumping is dispensable for the resistance. Here, we aimed to clarify this question by employing the E. coli Dps lacking DNA-binding activity, Dps\textsubscript{Δ18}. Staphylococcal nucleoid was clumped by E. coli Dps, but not by Dps\textsubscript{Δ18}. H\textsubscript{2}O\textsubscript{2} stress assay indicated that Dps and Dps\textsubscript{Δ18} restored the reduced susceptibility of S. aureus ΔmrgA. Thus, we concluded that the staphylococcal nucleoid clumping is dispensable for the Dps-mediated H\textsubscript{2}O\textsubscript{2} resistance. In contrast, Dps was unable to complement S. aureus ΔmrgA in the UV resistance, suggesting the MrgA function that cannot be compensated for by E. coli Dps.

INTRODUCTION

Bacterial genome is packed in a cell as a form of nucleoid, being associated with hundreds of proteins and RNAs (Rimsky & Travers, 2011; Robinow & Kellenberger, 1994). The nucleoid-associated proteins are composed of not only the factors for genome functions (e.g. machineries for gene expression, replication, DNA repair, etc.) but also a variety of factors with accompanying functions such as fatty acid synthesis enzymes and oxidoreductases (e.g. sodA, ahpC, trxA) that might contribute to the oxidative stress resistance (Ohniwa et al., 2011).

Dps family proteins are broadly conserved in bacteria. Escherichia coli Dps is induced toward stationary phase and by oxidative stress. On the other hand, Staphylococcus aureus MrgA (Dps orthologue) is specifically induced under oxidative stress conditions and not in the stationary phase (Morikawa et al., 2006). E. coli Dps and S. aureus MrgA are nucleoid-associated proteins that share the basic characteristics conserved in the Dps family proteins (Almirón et al., 1992; Grant et al., 1998; Ushijima et al., 2014). They form dodecamer with inter-subunit ferroxidase centres. The oxidation of ferrous iron to ferric iron by ferroxidase activity contributes to oxidative stress resistance by preventing the hydroxyl radical formation via Fenton reaction. They also bind DNA, like some other Dps family proteins, but the modes of DNA binding are distinct among Dps family proteins (Ingmer, 2010). E. coli Dps requires N-terminal 18 amino acid residues for the DNA-binding activity. In S. aureus MrgA, which lacks E. coli-type N-terminal region, the portion responsible for DNA binding is not known. Irrespective of this difference, both E. coli Dps and S. aureus MrgA can cause structural changes in nucleoid. E. coli Dps compacts the nucleoid toward the stationary phase but not under oxidative stress (Ohniwa et al., 2006), and this compaction is believed to contribute to the stress tolerance. The compacted E. coli nucleoid can be a crystal-like structure under starved conditions (Grant et al., 1998). S. aureus MrgA induced by oxidative stress also causes nucleoid clogging or clumping.

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Abbreviation: PQ, 9,10-phenanthrenequinone.
We previously showed that \textit{S. aureus} mrgA-null mutant is sensitive to oxidative stresses (Morikawa et al., 2006). MrgA* (MrgA<sup>Asp56Ala/Glu60Ala</sup>) that is impaired in the ferrioxidase activity but sustains DNA-binding/nucleoid-clumping ability was unable to complement the ΔmrgA strain in the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) resistance, suggesting that MrgA-induced nucleoid clumping alone is not sufficient for the resistance (Ushijima et al., 2014). However, whether the nucleoid clumping by MrgA would be essential to the oxidative stress resistance was elusive.

In order to answer this question, we tested here if a Dps family protein without DNA-binding activity can compensate for MrgA function in \textit{S. aureus}. We employed \textit{E. coli} Dps because its DNA-binding activity can be eliminated by deletions of its N-terminal 18 amino acids (DpsΔ18) (Ceci et al., 2004). MrgA, Dps and DpsΔ18 were expressed in \textit{S. aureus} ΔmrgA. Dps and MrgA, but not DpsΔ18, clumped staphylococcal nucleoid. Both Dps and DpsΔ18 complemented the \textit{S. aureus} ΔmrgA in terms of the H<sub>2</sub>O<sub>2</sub> resistance, demonstrating that staphylococcal nucleoid clumping is dispensable for the Dps-mediated H<sub>2</sub>O<sub>2</sub> resistance.

**METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1.

**Table 1. Strains, plasmids and primers used in this study**

<table>
<thead>
<tr>
<th>Strains and Plasmids</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>\textit{S. aureus}</td>
<td></td>
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<tr>
<td>N315v</td>
<td>N315 carrying pRIT5H</td>
<td>Morikawa et al. (2003)</td>
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<tr>
<td>N315M</td>
<td>N315 carrying pRIT5H-mrgA</td>
<td>Morikawa et al. (2006)</td>
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<tr>
<td>N315-ΔmrgA</td>
<td>mrgA-null mutant derived from N315</td>
<td>Morikawa et al. (2006)</td>
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<td>ΔmrgAv</td>
<td>N315-ΔmrgA carrying pRIT5H</td>
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<td>N315-ΔmrgA carrying pRIT5H-dps</td>
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<td>\textit{E. coli}</td>
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<tr>
<td>W3110</td>
<td>K-12 strain</td>
<td>Bachmann (1972)</td>
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<tr>
<td>BL21(DE3)</td>
<td>Strain for the expression of protein</td>
<td>Phillips et al. (1984)</td>
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<tr>
<td>Plasmid</td>
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<td>pRIT5H</td>
<td>Shuttle vector, expresses cloned gene under the control of spa promoter, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Inose et al. (2006)</td>
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3 min incubation at 37 °C, 30 µl of 10 % SDS was added. After further addition of 20 units of DNase (Promega) and 20 µg of RNase followed by 1 h incubation at 37 °C, the cells were sonicated with Bioruptor for 15 min and the resultant clear suspension was used as the whole cell extract. The proteins were quantified by using DC protein assay kit (Bio-Rad). One or five micrograms of total protein per lane was subjected to 15 % SDS-PAGE.

Western blot analysis was carried out as previously described with some modifications (Morikawa et al., 2003). The fractionated proteins were blotted onto PVDF membranes (Bio-Rad). The membrane was soaked in the blocking solution [5 % skimmed milk and 0.1 % human serum in TBST (Tris-buffered saline with Tween 20)] at 25 °C for more than 1 h and washed with TBST at 25 °C for 5 min for three times. The membrane was further incubated in the presence of a first antibody [1000-fold diluted anti-MrgA IgY or anti-Dps IgG (Azam et al., 2000)] at 4 °C overnight. After washing three times with TBST at 25 °C, the membrane was incubated in TBST containing a second antibody (4000-fold diluted goat anti-chicken IgY or goat anti-rabbit IgG) conjugated with alkaline phosphatase (Promega) at 25 °C for 45 min. After washing three times with TBST at 25 °C, the membrane was subjected to the signal detection by using Western blue stabilized substrate (Promega). Purified MrgA (Ushijima et al., 2014) and Dps (described in ‘Purification of recombinant Dps and DpsΔ18’) were used to make standard curves. Signal intensities of target proteins were measured by using the National Institutes of Health ImageJ software (https://imagej.nih.gov/ij/). In order to quantify the signal intensity of DpsΔ18 that is close to the non-specific signal, we quantified the signal intensity of the region including both the non-specific signal and the DpsΔ18 signal, and the background signal intensity (corresponding region in ΔmrgAv) was subtracted.

**Purification of recombinant Dps and DpsΔ18.** *E. coli* dps and dpsΔ18 were amplified by PCR from W3110 genome DNA using primer sets, Ecdps-S and Ecdps-A5 and EcdpsD18-S and Ecdps-A5, respectively. These fragments were cloned into the Ndel and Xhol sites of pET-23b (Novagen) to generate pET23b-dps and pET23b-dpsΔ18, respectively. These vectors express Dps or DpsΔ18 without histidine tag in BL21 (DE3).

Glycerol stocks of BL21 (DE3) pET23b-dps and BL21 (DE3) pET23b-dpsΔ18 were inoculated into LB media supplemented with 50 µg ml\(^{-1}\) of ampicillin (LB/amp) and cultured at 37 °C with shaking at 180 r.p.m. (BR-15, TAIITEC). Five hundred microlitres of the overnight culture was inoculated into 100 ml of fresh LB/amp medium and grown until OD\(_{600}\) reached 0.5. The expression of Dps was induced by 100 µM IPTG at 37 °C for 4 h. Cells were collected by centrifugation at 5000 g for 10 min at 4 °C and washed once with the buffer containing 50 mM Tris/Cl (pH 8.0) and 50 mM NaCl. The cells were suspended in the same buffer followed by the addition of 1 mM phenylmethylsulfonyl fluoride. Cells were disrupted using Fast Prep (MP Biomedicals) and cell debris was removed by centrifugation at 15000 r.p.m. for 10 min at 4 °C. The resultant supernatant was filtered through 0.45 µm membrane and loaded onto a HiTrap Q HP column 1 ml (GE Healthcare Bionsciences) pre-equilibrated with 20 mM Tris/HCl (pH 9.0). Dps was harvested in a flow-through fraction (10 ml), while DpsΔ18 was absorbed in the column. To recover the DpsΔ18, the column was washed with the same buffer and eluted with 20 ml of 10–500 mM gradient of NaCl in 20 mM Tris/HCl (pH 9.0). Three millilitres of the DpsΔ18-containing fractions was pooled. Following the concentration to 2 ml by Amicon 3K and filtration through the 0.45 µm membrane, each protein was further fractionated using a HiPrep 16/60 Sephacyr S-200 column with 200 µl injection per one fractionation, with the buffer system of 20 mM Tris/HCl (pH 8.0) and 200 mM NaCl. Dps was eluted at distinct sizes corresponding to some multimers including dodecamer, while DpsΔ18 was eluted as monomer, possibly due to the effect of the deletion on the multimer assembly or stability, as was reported in *Deinococcus radiodurans*.
AFM analyses. The nucleoid of lysed S. aureus was examined in air at 25°C by AFM using a Bruker Nanoscope VIII (Bruker) AFM instrument. The systems were operated in tapping mode with a 125 μm scanner (I scanner). Probes made of a single silicon crystal with a cantilever length of 240 μm and spring constant of 1.7 N m⁻¹ (OMCL-AC240TS-C3; Olympus) were used for imaging. Data were collected in the height mode with a scanning rate of 0.5–1.0 Hz. Images were captured in a 512×512 pixel format and the captured images were flattened and plane fitted before analysis. The numbers of nucleoids with (dispersed) or without (clumped) fibrous structures spreading out of the cell were counted to calculate the percentage of the dispersed nucleoid.

Lyses procedure to examine nucleoid clumping. Lyses procedure to observe the nucleoid structure was described previously (Ushijima et al., 2014). Briefly, the cells were collected from 100 μl of the culture prepared as described in the section 'Quantitative Western blot'. The cells were suspended in 250 μl of PBS, and the 50 μl aliquot was placed on a round cover glass (15 mm in diameter). The extracellular liquid was removed by nitrogen gas blow. Each sample was immersed in 2 ml of detergent buffer containing 1 % of solid sodium deoxycholate. After 30 min of incubation at 25°C, 2 ml of detergent buffer containing 1 % of Brij 58 (polyoxyethylene hexadecyl ether) and 0.4 % of sodium deoxycholate was added. After 30 min, the cover glass was dried under the nitrogen gas. The surface of the cover glass was washed with distilled water and dried again for atomic force microscopy (AFM) analyses. For the observation by fluorescent microscopy (Axiovert 200, Olympus), the sample was stained by DAPI.

Ferroxidase activity. Ferroxidase activity was measured as described previously (Ushijima et al., 2014). Reactions contained 400 μM ferrous iron and 19.2 μM monomer equivalent of MrgA, Dps or DpsΔ18. The oxidation of Fe²⁺ in air was monitored spectrophotometrically at 310 nm at 25°C.

H₂O₂ stress assay. Oxidative stress assays were performed as described previously (Ushijima et al., 2014). Cell cultures were prepared as described in the 'Quantitative Western blot' section. Cells equivalent to 400 μl culture of OD₆₀₀ 1.0 were harvested by centrifugation at 10 000 r.p.m. for 3 min at 4°C. Cells were washed once with ice-cold PBS and were suspended in 400 μl ice-cold PBS. Fifty microlitres of cell suspension was mixed with 450 μl of PBS with or without 400 mM (final concentration) H₂O₂ incubated at 25°C for 3 min and was serially diluted with ice-cold BHI. The viable cells were counted as c.f.u. on BHI agar plates.

UV stress assay. Fifty microlitres of cell suspension prepared as described in 'H₂O₂ stress assay' was 1000-fold diluted with cold BHI and 10 μl of the cell suspension was plated on BHI agar plates. The plates were placed under a germicidal lamp (254 nm; National GL-15) and exposed for 0–30 s.

Fig. 3. The nucleoid structures of N315v (PQ), ∆mrgAv, ∆mrgA+MrgA, ∆mrgA+Dps and ∆mrgA+DpsΔ18. N315v (PQ) served as a control that shows clumped nucleoid. All other samples were grown under the same conditions without PQ. (a) The frequency of dispersed nucleoids. The number of nucleoids with dispersed fibre structures in the AFM images was divided by the total cell number and indicated as percentage. Mean values with SD of at least two independent experiments are shown. (b), (d), (f), (h) and (j): AFM images. (c), (e), (g), (i) and (k): DAPI staining. Bars, 2 μm.
RESULTS AND DISCUSSION

Dps family proteins with distinct modes of DNA binding

Dps family proteins have different DNA-binding modes (Ingmer, 2010), but it is not known for staphylococcal MrgA. The surface on the tertiary structure of \textit{S. aureus} MrgA does not show the positively charged characteristics (data not shown), which is important for the DNA binding in \textit{Helicobacter} Dps (Ceci et al., 2007). Fig. 1 shows the sequence alignment of Dps family proteins with DNA-binding activities, and it indicates the existence of N-terminal or C-terminal extensions in some members. Importantly, the DNA binding requires the extended N-terminal regions in \textit{E. coli} Dps (Ceci et al., 2004) and \textit{Deinococcus radiodurans} Dps1 (Grove & Wilkinson, 2005), while the DNA binding of \textit{Mycobacterium smegmatis} Dps1 depends on the C-terminal regions (Roy et al., 2007). These regions are not conserved in \textit{S. aureus} MrgA. Here, we considered that such Dps family proteins with known DNA-binding region would be useful to test the requirement of the nucleoid clumping in the Dps-dependent oxidative stress resistance in \textit{S. aureus}.

Expression of \textit{E. coli} Dps, but not Dps\text{\textsubscript{18}}, can clump the nucleoid in \textit{S. aureus}

The deletion of N-terminal amino acids of \textit{E. coli} Dps (Dps\text{\textsubscript{18}}) impairs the DNA-binding activity (Ceci et al., 2004) and, in turn, affects the nucleoid clumping. Therefore, we expressed the recombinant Dps\text{\textsubscript{18}} in \textit{S. aureus} and tested whether it could clump the nucleoid. The results showed that Dps\text{\textsubscript{18}} could not clump the nucleoid, whereas Dps could. This indicates that the N-terminal region is important for the DNA binding and nucleoid clumping activity of Dps in \textit{S. aureus}.

Fig. 4. Quantitative Western blot analysis for Dps, Dps\text{\textsubscript{18}} and MrgA. (a) Left: standard curve made by using the purified recombinant Dps protein. Right: signals of Dps (lane 2) and Dps\text{\textsubscript{18}} (lane 3), in \textDelta mrgA\text{\textsubscript{+Dps}} and \textDelta mrgA\text{\textsubscript{+Dps\text{\textsubscript{18}}}}, respectively. N315\textDelta mrgAv is the negative control (lane 1). Five micrograms of protein of the whole cell lysate was subjected to the Western blot analysis. The positions of the Dps (18.7 kDa) and Dps\text{\textsubscript{18}} (16.7 kDa) are shown by arrows. (*) Non-specific signal. (b) Left: standard curve by the purified recombinant MrgA protein. Right: MrgA signals in N315v treated with PQ (lane 2) and in \textDelta mrgA\text{\textsubscript{+MrgA}} (lane 3). N315\textDelta mrgAv treated with PQ serves as the negative control (lane 1). One microgram of protein of the whole cell lysate was subjected to the Western blot analysis. The position of the MrgA (16.7 kDa) is shown by an arrow.
et al. (2006), but it was not known whether the ferroxidase activity is sustained in DpsΔ18. To test the ferroxidase activity of DpsΔ18, we purified recombinant proteins expressed in E. coli as described in Methods. Ferroxidase activity of DpsΔ18 was comparable with that of Dps (Fig. 2). Thus, DpsΔ18 was considered to be a useful molecule that can confer the ferroxidase activity but would not affect nucleoid structure when expressed in S. aureus cells. Then, we constructed the expression vectors of E. coli dps and dpsΔ18, as well as S. aureus mrgA, as a control. They were transformed into the mrgA-null S. aureus strain, N315∆mrgA, to generate ∆mrgA+Dps, ∆mrgA+DpsΔ18 and ∆mrgA+MrgA. The nucleoids were observed as described in Methods (Fig. 3a–k). N315v (PQ) is a positive control, showing the clumped nucleoids by the endogenous MrgA expression induced by the oxidative stress PQ (Fig. 3b, c). The nucleoids in the mrgA-null mutant were not clumped in line with the previous study (Fig. 3d, e) (Morikawa et al., 2006; Ushijima et al., 2014). Expression of E. coli Dps, as well as S. aureus MrgA, clumped the nucleoid (Fig. 3f–i), while DpsΔ18 expressing strain did not (Fig. 3j, k).

We also confirmed that Dps and DpsΔ18 were expressed at similar levels in ∆mrgA+Dps and ∆mrgA+DpsΔ18, respectively, by quantitative Western blot analysis (Fig. 4a, b). Dps was about 1.8 ng per 1 µg of total protein, and DpsΔ18 was about 2.2 ng. Thus, the expression of quantitatively equivalent amounts of Dps and DpsΔ18 allowed us to obtain two strains that are distinct in the nucleoid statuses.

E. coli DpsΔ18 can compensate for S. aureus mrgA in H₂O₂ resistance

S. aureus mrgA mutant (ΔmrgA) exhibited increased susceptibility to H₂O₂ treatment (Fig. 5) as reported previously (Morikawa et al., 2006). The expression of Dps and MrgA recovered the susceptibility (∆mrgA+Dps, ∆mrgA+MrgA), suggesting that E. coli Dps is functionally compatible with S. aureus MrgA in terms of H₂O₂ resistance.

We found that DpsΔ18 complemented the H₂O₂ susceptibility (∆mrgA+DpsΔ18). Since DpsΔ18 does not clump the nucleoid (Fig. 3a, j and k), we conclude that the nucleoid clumping is dispensable for the Dps-dependent H₂O₂ resistance in S. aureus cells. It is likely that ferroxidase activity alone is sufficient to confer the H₂O₂ tolerance under the employed experimental conditions in S. aureus. In E. coli, on the other hand, Meyer’s group recently showed that a point mutation in the DNA-binding motif, Lys10Ala, but not Lys5Ala, increased H₂O₂ sensitivity in E. coli (Karas et al., 2015). Although the susceptibilities to H₂O₂ are different between E. coli and S. aureus [E. coli viability is 1.5×10⁻² in 15 min treatment with 10 mM H₂O₂ (Karas et al., 2015), while S. aureus N315 is 9.2×10⁻¹ in 15 min treatment with 10 mM H₂O₂], these findings might imply the species-specific roles of nucleoid structures.

E. coli Dps did not complement staphylococcal mrgA in UV resistance

H₂O₂ produces hydroxyl radicals via Fenton reaction (Fe²⁺ + H₂O₂ → Fe³⁺ + OH⁻ + *OH), while UV irradiation involves electron transition to produce unpaired electron and produces reactive molecular species that damage a series of biomolecules including DNA.

**Fig. 5.** Susceptibilities to H₂O₂. Each strain was treated with 400 mM H₂O₂ for 3 min. Viability was calculated as the ratio of c.f.u. values before and after the treatment. Mean values with SD of at least three independent experiments are shown. Statistical significance was evaluated by Student’s t-test. *P<0.01.

**Fig. 6.** Susceptibilities to UV irradiation. Each strain was spread on BHI agar plate and exposed to UV for the indicated time. Surviving cells were visualized as colonies after overnight incubation.
As reported before, the mrgA mutant has increased susceptibility to UV irradiation (Morikawa et al., 2006) (Fig. 6; N315v vs AmrgAv). MrgA expression restored the UV resistance. However, we found that the expression of Dps did not recover the UV resistance, and thus we could not test the importance of the nucleoid clumping in the MrgA-dependent UV resistance. This might be attributed to the difference in the expression levels of Dps and MrgA. Western blot analysis showed that endogenous MrgA is 86.1 ng per µg lysate protein in the PQ-treated N315 and 84.3 ng when MrgA was expressed by the plasmid (ΔmrgA+MrgA) (Fig. 4b), which are about 40-fold higher levels than the exogenously expressed Dps and DpsΔ18 (Fig. 4a). Alternatively, there may be yet-to-be clarified MrgA function that cannot be compensated for by E. coli Dps, such as the affinity of Dps/MrgA to DNA, the mode of DNA packaging or the electron supplementation (Arnold & Barton, 2013), as discussed in our previous paper (Ushijima et al., 2014).

In conclusion, the nucleoid clumping was firstly shown to be dispensable for the MrgA-dependent H₂O₂ resistance in S. aureus. This study further substantiates the critical role of ferroxidase centre of MrgA in the survival strategy of this important human pathogen.

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