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EDGE ARTICLE

Chemical Insights into Dodecylamine Spore Lethal Germination

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Bacterial endospores can withstand common disinfection procedures and extreme environmental adversities. This tenacity for survival, coupled with pathogenicity, makes spores a major threat for the food and medical industries as well as national security. Though unsuitable for practical usage due to their high environmental toxicity, primary ammonium surfactants, dodecylamine (DDA) in particular, are the most potent antispore molecules known. However, over half a century after the initial discovery, the mechanism of DDA spore killing remains largely elusive and antispore compounds with practical utility are still greatly needed. Herein, we propose and provide evidence that DDA bioactivity may lie in its capacity to form hydrophobically stabilized salt bridges with carboxylate anions of the spore cortex, a structure critical in maintaining a low water content in the spore core. More importantly, the proposed mechanism of action was experimentally shown to be useful in guiding the design of potential antispore agents.

Introduction

Bacterial spores are known to survive under extreme environmental adversities, including heat, UV radiation, conventional antibiotics as well as strong acidic/basic conditions [1,2]. In contrast to this tremendous tenacity, spores show unexpectedly high susceptibility to primary ammonium surfactants: at as low as 10^{-4} M, dodecylamine (DDA) was reported to trigger *Bacillus megaterium* spore germination followed by inactivation at near physiological temperature [3,4]. However, more than half a century after its initial discovery, the molecular details of the DDA lethal germination process – its direct biological target(s) and the physiochemical nature of this interaction – remain largely unknown. This lack of fundamental understanding, together with the natural resistance of bacterial spores to conventional disinfection procedures and antibiotic compounds, makes the study of DDA-spore interaction of both scientific and practical importance.

The capability of spores to withstand harsh environmental conditions is mainly attributed to the low water content in the spore central core [1,2,5]. Besides the low permeability of the inner membrane [6], the high osmotic pressure originated from the electrostatic potential of the negatively-charged peptidoglycan matrices constituting the cortex layer has also been suggested to play a critical role in maintaining this low water content [7-9]. We hypothesize that DDA may initiate spore germination by quenching the negatively charged carboxylate groups within the spore cortex layer via salt bridge formation, a hybrid of hydrogen bonding and electrostatic interaction, thus annulling the osmoregulatory function of spore cortex, leading to core rehydration and finally the loss of viability after prolonged exposure to this cationic surfactant (Fig. 1).

This proposed mechanism of action has the following merits: 1), it is consistent with the known structure [10] and suggested function [7] of spore cortex; 2), the model is compatible with the high permeability of the outer membrane [11]; 3), the proposed interaction through salt-bridge formation neatly explains the almost qualitative difference in lethal germination potency between primary-ammonium-based DDA and quaternary ammonium surfactants [12].

In this work, we present experimental evidence supporting this hypothesis through the study of DDA structural analogues and germination inhibitors, a chemical approach proven useful not only for gaining insights of the fundamental mechanism but also in unveiling the structural determinants for its bioactivity. It was discovered that dodecylguanidinium (DDG), another surfactant molecule capable of forming salt bridges with carboxylate, has a lethal germination potency comparable to DDA, and both are at least two orders of magnitude more effective than dodecyl trimethylammonium chloride (DTAC) – a quaternary-ammonium-based cationic surfactant. Furthermore, our study also indicated that the main function of DDA alkyl tail is to stabilize the salt bridge through hydrophobic interactions with the cortex matrices rather than creating multivalent binding via self-assembly. Lastly, it is demonstrated through one example that the molecular understanding acquired in this study can be useful in the design and discovery of potential antispore agents that ideally share the high bioactivity of DDA while exempt from its severe environmental toxicity [13].
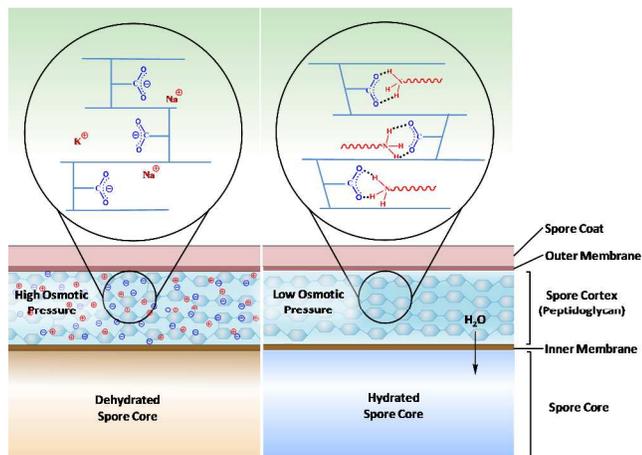
Figure 1. Proposed model of interactions between DDA and spores: prior to DDA exposure, the high osmotic pressure within the spore cortex helps maintain the core low water content; after DDA exposure, the primary ammonium head groups of DDA form hydrophobically stabilized salt bridges with carboxylate groups within the spore cortex, and this neutralization of the peptidoglycan layer nullifies the osmo-regulatory function of the cortex layer, causing the core to take up significant amounts of water.

Results and Discussion

DDA primary ammonium head group. Besides the primary ammonium, guanidinium is the most common cation in biological systems to form salt bridges with carboxylate anions [14]. We thus tested our hypothesis through the synthesis and characterization of DDG, a surfactant molecule with the same alkyl chain length as DDA but bearing guanidinium instead of primary ammonium as its head group. The efficacy of DDG in inducing germination and killing spores was tested with *B. megaterium* spores at 40°C and pH 8 in 100 mM NaCl. After a 30-min exposure, the cationic surfactants were diluted and quenched by sodium dodecyl sulfate (SDS) to stop the interaction [15]. The spore suspensions were then immediately spread onto nutrient agar plates. The drop in viable spore count, measured by the reduction in colony number compared to the control samples, was used as an indication for lethal germination potency of surfactant molecules. The results showed that both DDA and DDG at 10^{-4} M can cause significant reduction in *B. megaterium* viability: 0% and 11% survival respectively, compared to that of the negative control (Fig. 2a). In comparison, DTAC and SDS, two commonly used cationic and anionic surfactants, barely exhibited appreciable lethal germination activity even at 10^{-2} M under otherwise identical experimental settings.

According to the proposed model in Fig.1, strong binding between a cationic surfactant head group and a carboxylate is critical to the eventual spore core rehydration. Molecular simulations offer a way to isolate head group interactions and compare their binding free energies. To this end, molecular dynamics free energy calculations were performed for the head groups of three cationic surfactant molecules (DDA, DDG and DTAC) and results shown in Fig. 2b. A fixed volume simulation (NVT) was chosen over NPT ensemble for its considerably higher efficiency and shorter time needed for results to converge. The equivalent of 100 mM NaCl was also added to the simulation system to mimic the actual experimental conditions. The potential mean force free energy calculated from radial distribution function indicates that the binding of carboxylate anion with primary ammonium or guanidinium is much more energetically favoured in comparison with the quaternary ammonium, thus corroborating our previous experimental findings. (A more systematic study on the salt bridge interaction under high pressure and high ionic strength environments can be found in the supporting information of this manuscript.)
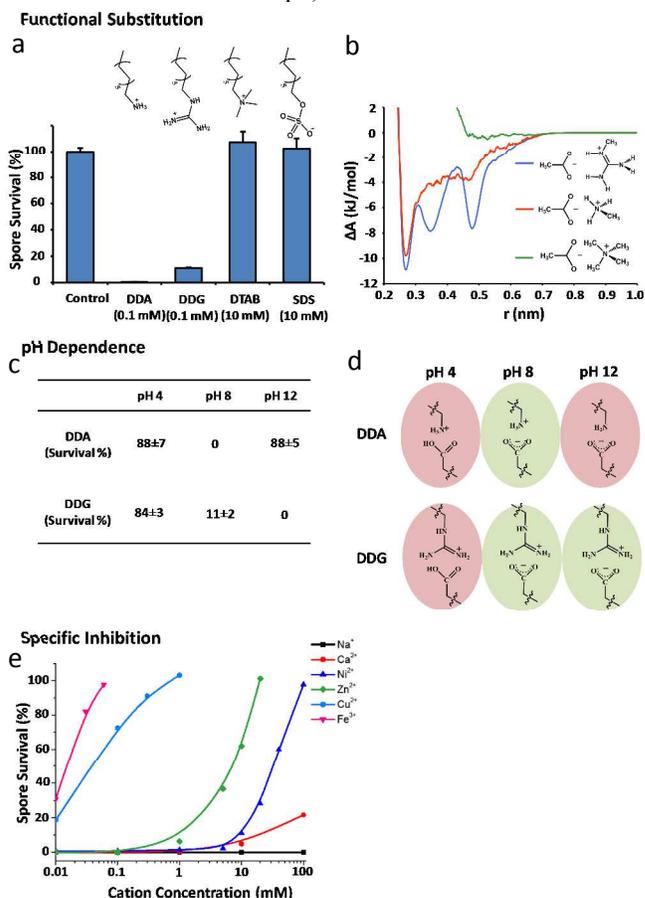
Figure 2. Primary ammonium head groups in DDA lethal germination. **a,** Survival percentage of *B. megaterium* spores after exposure to DDA or DDG at 0.1 mM and DTAB or SDS at 10 mM for 30 min at 40°C and pH 8. **b,** The potential mean force free energy between carboxylate anion binding with primary ammonium, guanidinium or quaternary ammonium cations. The three local energy minima in guanidinium-carboxylate interaction reflect multiple strong binding conformations. **c,** Compiled results of DDA and DDG lethal germination efficacy under different pH conditions. All experiments were performed at 40°C and 0.1 mM surfactant concentration for 30 min. **d,** Schematic illustrations of DDA and DDG forming salt bridges with carboxylate groups in response to the environmental pH. **e,** Multivalent cation inhibition of DDA lethal germination of *B. megaterium* spores as evaluated by supplementing 0.1 mM DDA with various metal cations at 40°C.

We further reasoned that if DDA and DDG indeed interact with spore cortex carboxylate groups via salt bridging, the change in their lethal germination potency in response to environmental pH should parallel the protonation states of the

participating head groups, as the coexistence of oppositely charged ionic groups is a prerequisite for salt bridge formation. This postulate was tested and the results are presented in Fig.2c and d. At pH 4, neither DDA nor DDG effectively induced *B.megaterium* spore lethal germination – though both surfactants were expected to bear positive charges under the acidic condition, the protonation of carboxylate groups (typical pKa 3.5~5) [16] made the interaction unfavourable. It is also worth noting that pH 4 is significantly higher than the intrinsic pKa values of most phosphonate anions (pKa<1) [17] found in membrane phospholipids, and the loss of DDA bioactivity at this pH thus suggests that the spore inner membrane itself is not the direct target for DDA salt bridge formation. At pH 8, salt bridges can be readily established for DDA as well as DDG, as was duly reflected in their antispore activities. At pH 12, most DDA molecules are unprotonated and charge neutral as a result of their comparatively lower pKa of 10.6 [16]. Meanwhile, the majority of DDG remains positively charged due to their higher pKa of 13.6 [16]. This difference in pKa corresponded to DDA and DDG's drastically different activities in initiating lethal germination at high pH. It is worth pointing out that the exact pKa values of various charged groups within the spore cortex are likely to differ from that measured in diluted aqueous solutions as a result of molecular crowding and environmental heterogeneity. However, the general tendency – the protonation of a carboxylate group under low pH and a much higher basicity of guanidinium over primary ammonium – is not expected to change because of this potential pKa deviation. (See supporting information for further discussions on ionic group pKa within the spore cortex region.)

Consistent with the effect of protonation of carboxylate groups at low pH preventing salt bridge formation, different multivalent metal cations were also observed to block lethal germination by DDA (**Fig. 2e**). By measuring the concentrations needed for various cations to effectively block the DDA lethal germination, the relative inhibition potency of these metal ions can be determined. This experimentally obtained ranking ($Fe^{3+} > Cu^{2+} > Zn^{2+} > Ni^{2+} > Ca^{2+} > Na^+$), a result of electrostatic attraction, hydrophobic interaction as well as stereochemical effect, matches exactly the relative affinity of these metal ions to a carboxylate-based anionic polymer matrix, thus suggesting an inhibition through competitive binding [18]. Another piece of noteworthy information supportive of cortex being the direct target of DDA is the observed higher inhibition potency of Zn^{2+} over Ni^{2+}: as previously reported, in the case of monomeric, polydentate ligand binding, Ni^{2+} is almost always more favoured than Zn^{2+}, while Zn^{2+} binds more strongly to an anionic polymer matrix due to its less strained tetrahedral stereochemical configuration [19].

DDA alkyl tail. In addition to the cationic head groups, the hydrophobic alkyl tail also played an important role in DDA bioactivity: for primary ammonium surfactants from octylamine (C8) to tetradecylamine (C14), the extension of every two carbon atoms in the alkyl tail lowers the minimal antispore effective concentration approximately 10-fold [4]. We reason that there are two plausible explanations for this observation: first, the increase in hydrophobicity favours the adsorption of DDA on to cortex polymeric matrices and stabilizes the salt bridges subsequently formed [20,21]. The magnitude of typical hydrophobic interactions was calculated to be around 30 cal/(mol*Å^2), corresponding to a 3.5-fold increase in binding constant for a single methyl group [22], which matches the change in the bioactivity of primary ammonium surfactants

with increasing tail length. Second, the long alkyl chain may also facilitate multivalent interactions via self-assembled micelle structures [23]. Multivalency here refers to the simultaneous interaction of multiple binding sites between two entities, and is often used to elevate the binding affinity in a competitive biological environment [24]. However, the fact that minimal antispore effective concentration of DDA is one order of magnitude lower than its reported critical micelle concentration (CMC) argues against the second explanation [25].
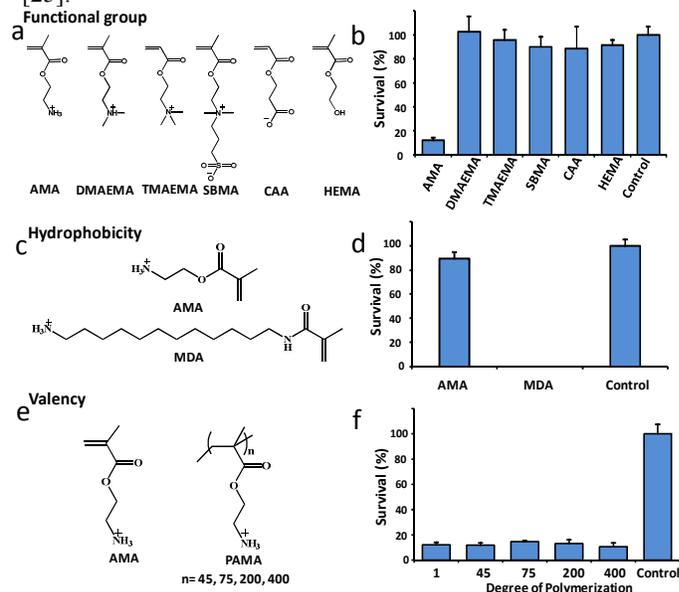

Figure 3. The role of DDA alkyl tail in spore germination studied using primary ammonium monomers and polymers. **a,** The chemical structures of six hydrophilic monomers. **b,** The loss of *B. megaterium* spore viability after exposure to various hydrophilic monomers (300 mM) at 80°C for 30 min in PBS. **c,** The chemical structures of two primary ammonium monomers: hydrophilic AMA and hydrophobic MDA. **d,** The loss of *B. megaterium* spore viability after exposure to AMA and MDA (3 mM) at 80°C for 30 min. **e,** The chemical structures of AMA monomer and PAMA polymers of different degrees of polymerization. **f,** The loss of *B. megaterium* spore viability after exposure to AMA monomer and PAMA polymers at 5% w/v (equivalent to 300mM for AMA), 80°C for 30 min.

To further understand and confirm the role of DDA alkyl chain in the chemical germination process, we tested the lethal germination properties of various primary ammonium monomers and polymers, as they offered an easy way to orthogonally manipulate the molecular hydrophobicity and valency. As a starting point, six types of hydrophilic monomers bearing primary ammonium, quaternary ammonium, tertiary ammonium, sulfobetaine zwitterion, carboxylate, and hydroxyl groups were incubated with *B. megaterium* spores at 300 mM and 80°C in PBS for 30 min. Among the six monomers, only the one having primary ammonium group, 2-aminoethyl methacrylate hydrochloride (AMA), gave a statistically significant reduction in spore viability, albeit with much decreased potency compared to DAA as evidenced by the elevated incubation temperature and high concentration of AMA needed (Fig. 3a, b). The results of this experiment suggested that the biological activity of primary ammonium group in DDA is functionally enhanced by but structurally separable from its alkyl tail. Based on this, we reason that if

simple hydrophobic interactions are responsible for the increased efficacy, a boost in bioactivity should be observed by switching to a hydrophobic but non-micelle-forming (critical packing parameter >1) primary ammonium monomer. If, on the other hand, multivalency plays a role in the process, a difference should be expected between a primary ammonium monomer and its corresponding polymers. To this end, we synthesized the hydrophobic primary ammonium monomer 12-methacrylamidododecan-1-aminium chloride (MDA) as well as poly(2-aminoethyl methacrylate hydrochloride) (PAMA) with varying degree of polymerization (DP) (Fig.3 c, e, Fig.3S and Fig.4S) [26,27]. Their effects on spore viability were similarly tested in PBS at 80°C for 30 min. 3 mM of monomers was used when comparing AMA and MDA, and 5% w/v (equivalent to 300mM for AMA) when AMA and PAMA were compared. Our results show that the bioactivity of the molecule correlates strongly with monomer hydrophobicity, but is largely independent of the degree of polymerization (Fig.3 d, f). This indicates that the chief role of the alkyl chain in DDA is to promote hydrophobic interaction and not to facilitate multivalent binding.

Structural determinants for antispore compounds. Taken together, the two essential structural features determining the potency of DDA-like molecules in inducing spore lethal germination are: 1) cationic head groups capable of forming salt bridges with carboxylate groups; 2) hydrophobic moieties, not necessarily unbranched alkyl chains that enhance surface adsorption and stabilize salt bridge interactions. Once spore germination is triggered, the germinated spore can then be killed by further exposure to the cationic compound initially used to induce germination (e.g. DDA or DDG) with or without the additional assistance of heating. To examine this assertion, we acquired a molecule with no previously reported bioactivity, N-[(3,5-dimethyl-1-adamantyl)methyl]guanidine hydrochloride (DMAG), from a chemical screening library. DMAG was chosen as it meets the aforementioned criteria of a salt-bridge-forming guanidinium portion and a hydrophobic dimethyladamantyl group, while bearing minimal structural resemblance to the original DDA molecule. At 1 mM, DMAG was found to reduce the surviving *B.megaterium* spore count by 57% upon a 30 min incubation at 50°C and pH 10, and completely eliminated viable spores when the temperature was increased to 60°C (Fig.4). A slightly basic condition was used as it was shown in previous experiments to favour guanidinium-based DDG lethal germination. In comparison, 10-fold higher concentration of guanidinium, 3,5-dimethyladamantane-1-acetic acid (DMAA) or a mixture of both showed no activity towards spores at 80°C at the same pH. The weaker bioactivity of DMAG compared to DDA and DDG is presumably a result of its relatively lower hydrophobicity (The calculated octanol-water partition coefficient is 1.6 for DMAG and 3.1 for DDA) [28].
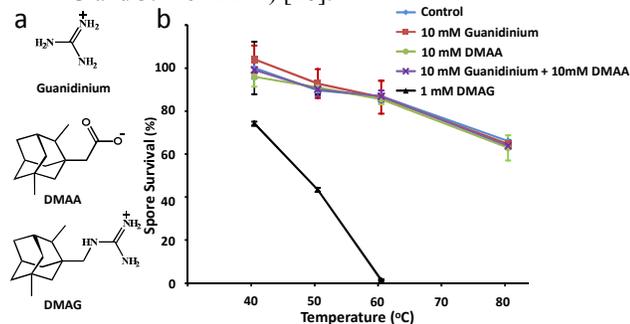
Figure 4. The loss of spore viability after exposure to DMAG. a, The chemical structures of guanidinium, DMAA and DMAG. Guanidinium and DMAA, each possessing the cationic and hydrophobic portions of DMAG, were included as controls in this experiment. b, The loss of *B. megaterium* spore viability after a 30-min exposure to guanidinium, DMAA, guanidinium/DMAA, or DMAG at different temperatures at pH 10. Note that the concentration of DMAG was 10 times lower than that of guanidinium, DMAA or guanidinium/DMAA.

The direct biological target of DDA has been the subject of significant speculation over the years. Past studies eliminated the spore coats and core from the list of possible targets, as the removal of the spore coats does not affect DDA bioactivity [29] and the accessibility of the core is blocked by the inner membrane permeation barrier [30]. Of the several remaining possibilities – mainly, the interactions between DDA and specific inner membrane proteins, the nonspecific interactions with inner membrane, or the nonspecific inter-actions with spore cortex – the chemical evidence presented in this study certainly favours the last possibility. The SpoVA proteins that are most likely constituents of an inner membrane Ca-DPA ion channel have been suggested to be involved in the DDA germination process [31], but several types evidence indicate that this channel likely plays a downstream biological function instead of interacting with DDA directly, since: 1) besides exposure to DDA, other experimental conditions, including the degradation of spore cortex by lysozyme [31], also activate this ion channel, making it a converging point of different biological inputs; 2) many DDA structural derivatives and primary ammonium polymers studied in this work can similarly decrease spore chemical/heat resistance to various degrees, displaying the nature of nonspecific interactions.

The spore core dehydration mechanism has been associated with the outer cortex layer ever since J. Lewis pointed out that a low permeability of inner membrane cannot really account for the unusually low water content within the spore core [32]. A later calculation further suggested that the osmotic pressure created in the cortex by carboxylate groups and their counter ions can easily exceed 30 atm [7], a condition almost certainly contributory to core dehydration. This potential biological function thus makes the spore cortex a viable target for chemically triggering core rehydration, as likely in the case of DDA lethal germination. It is interesting to note that while low pH and multivalent cations have also been known to affect the charge density on anionic polymer matrices, they themselves do not effectively trigger *ab initio* lethal germination at near physiological temperature as does DDA (though the reduction in spore resistance under acidic conditions or in the presence of mutltivalent cations is nevertheless well-documented [7,33]). This difference in bioactivity may stem from the difference in affinity of binding to carboxylate groups as well as the extent to which matrix osmotic pressure is altered after binding.

Conclusions

Through the study of various structural analogues and specific inhibitors, we have gained new chemical insights into DDA-spore interactions, suggesting that DDA head group interaction with spore cortex carboxylate via salt bridge formation and the hydrophobic stabilization from the alkyl tail are central to the exceptional antispore activity of DDA. We also demonstrated that this understanding enables one to make predictions of such bioactivities. The molecular understanding of DDA lethal germination acquired through this work will be

useful in guiding the design and screening of potential antispore molecules that ideally possess high efficacy but without the potent environmental toxicity that has so far limited broader applications of DDA [13].

Acknowledgements

Notes and references

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