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Action of Disinfectant Quaternary Ammonium Compounds against *Staphylococcus aureus*▽

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Mode-of-action studies concluded that alkyldimethylbenzylammonium chloride (ADBAC) (a blend of C_{12}, C_{14} and C_{16} alkyl homologues) and didecyldimethylammonium chloride (DDAC) are both membrane-active agents, possessing subtly different modes of action reflecting early cell interactions against *Staphylococcus aureus*. ADBAC and DDAC exhibited similar MIC behaviors from 0.4 ppm to 1.8 ppm over an inoculum range of 1×10^5 to 1×10^9 CFU/ml at 35°C. For ADBAC and DDAC, an increased rapidity of killing against *S. aureus* (final concentration, 2×10^9 CFU/ml) was observed at 35°C compared to 25°C. Concentration exponents (η) for killing were <2.5 for both agents, and temperature influenced the η value. Examination of leakage and kill data suggested that a single leakage marker was not indicative of cell death. ADBAC and DDAC possessed Langmuir (L4) and high-affinity (H3/4) uptake isotherms, respectively. ADBAC molecules formed a single monolayer of coverage of cells at the end of primary uptake, and DDAC formed a double monolayer. Rapid cell leakage occurred at bactericidal concentrations, with total depletion of the intracellular potassium and 260-nm-absorbing pools released in this strict order. Autolysis was observed for ADBAC and DDAC at concentrations of 9 μg/ml (0.0278 mM and 0.0276 mM, respectively) and above, together with the depletion of approximately 30% of the internal potassium pool. Autolysis contributed to ADBAC and DDAC lethality, although high biocide concentrations may have inhibited autolytic enzyme activity.

There continues to be concern regarding the frequent occurrence of methicillin-resistant *Staphylococcus aureus* within hospital settings (37, 52) and reports on the lowered efficacy and the limited number of last-resort antibiotics at the disposal of the physician (36). Correct practice of disinfectant use within hospitals has long been considered the most appropriate first line of defense to curb infection outbreaks and to minimize antibiotic prescription (14, 33, 34, 40). Hospital staff should be made aware of correct disinfectant practices and the adverse consequences of a failure to appreciate the effects of temperature and dilution on disinfectant performance. The thorough understanding of antimicrobial action is necessary for adequate advice to be given to hospital infection control teams in order to formulate appropriate disinfection protocols and policy guidelines.

Some investigations have implied that there is disinfectant cross-resistance with antibiotics (6, 9, 29, 43). Bacteria, and in particular *S. aureus*, can acquire plasmid-encoded multidrug resistance genes that negate bactericidal properties of a number of antimicrobial agents. It is known that some quaternary ammonium compounds (QACs) are subject to resistance mediated by the QacA efflux pump (32).

Increasing regulatory hurdles have prompted closer scrutiny of established chemical biocides such as the QACs used in household disinfectants in preference to the development of novel agents (4, 10, 36, 54). However, despite widespread use, there is still much to learn about the modes of action of these compounds, and such information is vital for the rational design of optimized formulations (38, 56).

The structural functionality of QACs, especially the role of chain length on activity against different bacteria, has been previously observed (50). Ahlström et al. (3) previously reported that QACs with a C_{16} hydrophobic tail length affected the outer membrane of gram-negative bacteria more extensively than shorter-chain compounds, possibly due to the C_{16} chain interacting strongly with the fatty acid portion of lipid A. It was also reported previously that monoalkyl QACs bind by ionic and hydrophobic interactions to microbial membrane surfaces, with the cationic head group facing outwards and the hydrophobic tails inserted into the lipid bilayer, causing the rearrangement of the membrane and the subsequent leakage of intracellular constituents. Hamilton (20) previously reported that a common feature of QACs is their ability to cause cell leakage and membrane damage, primarily due to their adsorption to the bacterial membrane in large amounts.

A systematic approach may be used to identify differences between QAC agents, particularly their modes of action, beginning with an initial focus on bactericidal, bacteriostatic, and uptake isotherm properties followed by an assessment of membrane sensitivity. Some biocides may possess a multiplicity of action, while others may simply target a specific structure or metabolic process in a cell. In order to single out a prime lesion of biocide attack, low concentrations of biocide may be more appropriate than higher concentrations, which may elicit multiple effects (13). The affinity of a biocide to a given cell may be determined by uptake isotherms, which may also provide information on the availability of target sites. At a specific exposure time point, uptake isotherms record the amount of

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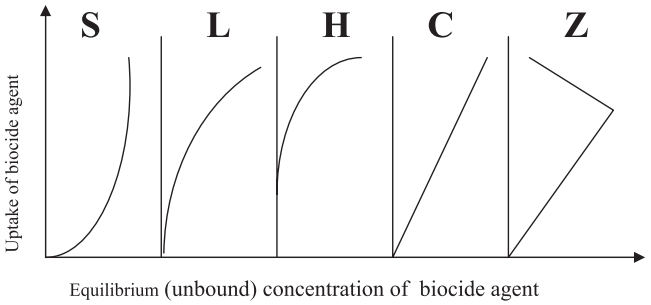

FIG. 1. S-shaped, Langmuir (L), high (H), C-shaped, and Z-shaped uptake isotherm profiles. Subclass groups 1 to 4 are not shown.

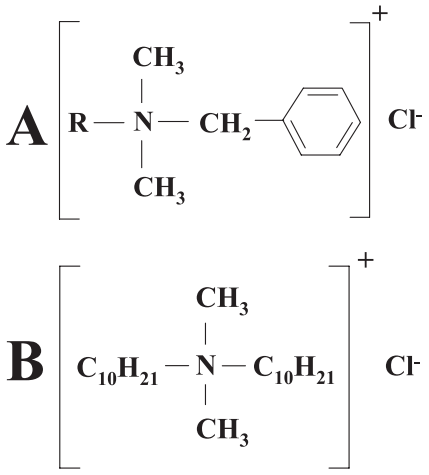

FIG. 2. Structures of ADBAC, where R is a monoalkyl chain length varying in carbon number (50% C_{14}, 40% C_{12}, and 10% C_{16}) (A), and DDAC (B).

biocide bound to cells versus the residual level of biocide (equilibrium concentration). A classification system developed previously by Giles et al. (19) divided sorbing agents into different classes depending on their uptake isotherms: Langmuir (L), high (H), C-shaped, S-shaped, and Z-shaped uptake profiles (Fig. 1). If uptake plateaus are identified, the S, L, and H isotherms may be further divided into subclass groups 1 to 4. For L curves, the biocide molecules are believed to be adsorbed flat or perpendicular to the cell surface. The H curves are related to the L curve, with the difference being that maximum adsorption at less concentrated biocide doses occurs, which is reflected in the vertical uptake pattern at the initial stages of the isotherm.

Additional numerical indicators of biocide behavior can be calculated from bactericidal studies. The concentration exponent, η, describes the relationship between dilution and activity of a biocide and is calculated by determining the time to killing of a specified proportion of the population at a particular concentration (23). The concentration exponent may predict agent activity when diluted or when the concentration is increased in a given environment. The activity of a biocide is usually increased when the temperature at which it acts is increased. Q_{10} values may be used to describe the biocide activity change through a 10°C change in temperature. A Q_{10} numerical value can be quoted for different experimental endpoints based on death times recorded at two temperatures. Q_{10} values can be used to predict the implication of biocide delivery at different temperatures.

Measurements of specific cell leakage markers such as potassium and 260-nm-absorbing materials are indicative of membrane sensitivity to specific biocide doses in relationship to unexposed cells (12, 25, 42). Cell leakage may be induced by monolayer or supramonolayer deposition of biocide on the cell surface.

Longer biocide exposure times may also detect additional breakdowns of intracellular material, which are indicative of autolysis (7, 13, 22, 51). Autolysis is an intracellular event where the cell enters a self-destruct state and becomes committed to death. Induction of autolysis is an important characteristic of a biocide that offers an additional level of security when a biocide is used in a given environment, especially when the contact dose is compromised due to biocide dilution (23), adsorption of biocide by debris, or quenching of biocide by biofilms. This may result in a sublethal dose with a subsequent reliance on triggering autolysis in cells to elicit killing rather than bactericidal action per se (13).

In this study, the gram-positive bacterium *Staphylococcus aureus* (ATCC 6538) was used to explore the modes of action and performance parameters of a quaternary ammonium blend, alkyldimethylbenzylammonium chloride (ADBAC), and a dialkyl agent, didecyldimethylammonium chloride (DDAC) (Fig. 2).

MATERIALS AND METHODS

Preparation of QACs. *N*-Alkyldimethylbenzylammonium chloride (consisting of a blend of C_{14} [50%], C_{12} [40%], and C_{16} [10%] homologues) and didecyldimethylammonium chloride were obtained from the Stepan Company, IL. Hexadecyltrimethylammonium bromide (Cetrimide) was obtained from Sigma, Poole, Dorset, England. Biocides were prepared in sterile 0.05 M HEPES buffer (adjusted to pH 7.4) to give a final stock concentration of 200 ppm. Biocide preparations were maintained in a dark, cool storage cabinet and prepared daily. All glassware was pretreated with Decon^{90} (BDH, Poole, Dorset, England) using the following procedure: immersion in 5% (vol/vol) Decon^{90} for 24 h, washing in deionized water, and immersion for a further 24 h in 0.1 M HCl followed by a wash in deionized water.

Organism employed. *Staphylococcus aureus* (ATCC 6538) was obtained as a freeze-dried ampoule, and cells were rehydrated in nutrient broth (ICN Biomedicals, Aurora, OH). Stock *S. aureus* cells were conditioned for freezing (55) and maintained at −70°C (model no. MDF-U71V; Sanyo Electric Company).

Culture maintenance and growth. Frozen bacterial samples were reconstituted monthly in 100 ml sterile nutrient broth and incubated at 37°C for 18 h. From the suspension, tryptone soya agar (TSA) (Oxoid, Basingstoke, Hampshire, England) isolation plates were incubated at 37°C for 24 h and stored at 4°C. The purity of the culture was verified by microscopic examination and Api STAPH (bioMerieux, Marcy l'Etoile, France). Single colonies removed from the isolation plates were used to inoculate 10 ml of nutrient broth, which was incubated at 37°C for 18 h. After further subculturing, the suspension was used to inoculate an AOAC (Becton Dickinson, Sparks, MD) agar plate (supplemented with 1.2% [wt/vol] agar granules [Oxoid technical agar no. 3]), which was maintained at 37°C for 18 h.

Following incubation, 20 ml sterile HEPES buffer was added to the surface of the AOAC plate, and the lawn of bacterial cells was gently scraped off. The cell suspension was then diluted further with HEPES buffer and washed three times by centrifugation at 1,508 × *g* for 20 min (Sorvall RT6000B; DuPont, Stevenage, Hertfordshire, England) before finally being resuspended in 30 ml HEPES buffer. Stock suspensions of *S. aureus* at 2.0×10^{10} CFU/ml were prepared by reference to a standardized calibration graph.

Estimation of MICs using the agar dilution method. Final biocide concentrations ranging from 0 to 3.2 μg/ml (increasing in 0.1-μg/ml increments) in HEPES buffer were added to separate sterile petri dishes to which 19 ml of molten TSA

(Oxoid, Basingstoke, Hampshire, England) was added at 50°C, mixed, and allowed to set.

The surface of each agar plate was inoculated in triplicate with 5 μl of challenge inoculum containing 10-fold dilutions from 1×10^9 to 1×10^5 CFU/ml in HEPES buffer. The plates were incubated at 35°C for 48 h. At 24 and 48 h, the presence of growth was examined visually on each plate, and the MIC was recorded. The MIC was determined as the concentration where no growth or <10 colonies were detected for each inoculation spot within a triplicate. Those cells growing in an inoculation spot where the MIC was recorded were subcultured and retested for MIC determinations. They exhibited no further resistance.

Bactericidal testing. Final biocide concentrations studied were 35, 45, and 55 μg/ml, and these were prepared in HEPES buffer. Reaction vessels (100-ml wide-necked conical flasks) contained 36 ml biocide, and 4 ml of bacterial stock suspension was added (2×10^{10} CFU/ml). Experiments were conducted at 25°C and 35°C (shaking water bath at 100 oscillations min^{-1} and an amplitude of 10 cm). Upon exposure of cells to biocide, 1-ml samples were removed and transferred to 9 ml AOAC letheen neutralizer broth (Becton Dickinson, Cockeysville, MD) every 30 s for up to 5 min inclusive. At the end of the experiment, a 10-fold dilution of the stock suspension into HEPES buffer followed by a further 10-fold dilution into neutralizer was performed to confirm the original viable count. A Bioscreen (Labsystems, Helsinki, Finland) was employed to estimate viable counts of all samples by recording the time taken for an unknown inoculum to grow to the threshold optical density, subsequently converting this value to a defined inoculum size by reference to a calibration curve. A sterile 100-well plate consisting of 10 columns, each containing 10 wells filled with 360 μl of nutrient broth into which samples were inoculated, was used according to the method described previously by Lambert et al. (27).

Uptake isotherm measurements. The spectrophotometric determination of quaternary ammonium surfactants with orange II dye (44) was modified to observe uptake isotherms for ADBAC and DDAC. Final concentrations of 4.5, 9, 12, 15, 25, and 35 μg/ml ADBAC and DDAC were prepared in 9 ml HEPES buffer. In addition, a 9-ml volume containing HEPES buffer only was subjected to the test. One milliliter of *S. aureus* stock suspension was added to each 9-ml preparation to give a final concentration of 2×10^9 CFU/ml. The mixture was vortexed every 20 min for a total contact time of 1 h. After the contact period, the suspension was centrifuged (using glass centrifuge tubes) at $1,430 \times g$ (Sorvall RC-5B; Dupont, Stevenage, England) for 15 min. Four milliliters of supernatant liquid was then removed and mixed with 1 ml orange II dye (4×10^{-4} M prepared in 0.1 M NaCl), followed by the addition of 5 ml chloroform. The mixture was vortexed for 30 s to ensure that the chloroform and dye were adequately mixed. Three milliliters from the bottom layer containing the dye-biocide complex (orange color) was removed into a UV silica cuvette (Hellma precision synthetic far-UV quartz, 200 to 2,500 nm; light path, 10 mm), and the absorbance was measured at 485 nm. A HEPES buffer control extracted in a similar way was used to blank the spectrophotometer (lambda 2; Perkin-Elmer, CT). Variables such as orange II concentration, absorption maxima for dye salts, extractability of dye salt using chloroform, and pH were validated by methods described previously by Scott (44). The assay was unaffected by changes in pH, buffer, and the presence of cell exudates.

Biocide uptake was calculated using calibration graphs of biocide concentrations versus the absorbance at 485 nm. Isotherm profiles comparing the equilibrium concentration (μg/ml) with the uptake concentration (μg/2×10^9 cells) were plotted.

Biocide-induced leakage and autolysis. Final biocide concentrations of 4.5, 9, 12, 15, and 18 μg/ml in addition to a HEPES control were investigated against a final *S. aureus* challenge suspension of 2×10^9 CFU/ml. Reaction vessels (glass 100-ml wide-necked conical flasks) contained 45 ml biocide plus 5 ml bacterial cells. Experiments were conducted at 25°C and 35°C in a shaking water bath (100 oscillations min^{-1}, with an amplitude of 10 cm). Before the introduction of cells to the biocide, a 10-fold dilution of the stock inoculum in HEPES buffer was made, and 5 ml of that suspension was passed through a 0.45-μm filter (Acrodisc [32 mm] with a Supor membrane; Pall Gelman, MI) into a 15-ml glass Bijou bottle. This filtrate represented the level of leakage from *S. aureus* at time zero; this procedure was performed before the inoculation of each reaction vessel.

After the addition of bacterial cells to the reaction vessel, 4-ml samples were removed and filtered into 15-ml glass screw-cap Bijou bottles at 0.5, 1.5, 3, 6, 9, 15, 30, 45, 120, and 240 min (the time points refer to the midpoint of filtration, which could take up to 20 s). Each sample was removed using a 10-ml sterile plastic syringe attached to a sterile plastic filling tube to enable easy access to the reaction mixture suspension. After each sample removal, the syringe was washed in sterile deionized water and reused. A separate syringe was employed for each reaction vessel. All filtrates were stored at −70°C until analysis.

Two additional vessels containing 2×10^9 CFU/ml *S. aureus* suspended in 1 mM cetrimide (prewarmed to 37°C) and HEPES buffer were prepared and maintained for 1 h shaken (120 rpm) at 37°C (Innova incubator 4230 and platform shaker 2000; New Brunswick Scientific, Edison, NJ). These reaction vessels were established to determine the maximum intracellular potassium pool and to monitor leakage from cells in the absence of biocide, respectively.

Determination of the intracellular pool of 260-nm-absorbing material. To determine the soluble 260-nm-absorbing pool, treatment of cells with trichloroacetic acid was employed according to the protocol described previously by Gale and Folkes (15). The UV/VIS spectrophotometer (lambda 2; Perkin-Elmer, CT) was blanked with a 5% (wt/vol) trichloroacetic acid sample (exposed to the same conditions as the test samples). In the following experiments, material that leaked following biocide treatment was recorded as a percentage of the intracellular pool.

Potassium analysis. The potassium ion concentration in filtrate samples was determined using a Perkin-Elmer 1100B atomic absorption instrument in flame emission mode (wavelength, 766.5 nm; slit, 0.7 nm high; air-acetylene flame) (21). Before calibration and measurement of samples, the instrument was autozeroed with HEPES buffer, and this was repeated periodically throughout the analysis. The instrument was calibrated using potassium standards (analytical grade; Sigma-Aldrich, Poole, United Kingdom) of 100, 200, 300, 400, 500, and 600 μg/liter (final concentration) prepared in HEPES buffer. There was a linear relationship between emission and potassium concentration.

The filtrate samples were diluted in HEPES buffer to give potassium levels that could be detected at the midpoint on the calibration graph. The potassium standards were remeasured periodically during the experiment to verify instrument accuracy. Filtration and the presence of ADBAC, DDAC, and HEPES buffer did not interfere with potassium analysis.

Analysis of 260-nm-absorbing material. For each filtrate sample, 260-nm-absorbing material was detected using a Perkin-Elmer lambda 2 UV/VIS spectrophotometer at a wavelength of 260 nm (42). A matched pair of UV silica cuvettes (Hellma precision synthetic far-UV quartz, 200 to 2,500 nm; light path, 10 mm) was used to measure each filtrate sample against HEPES buffer. After each measurement, the cuvette was washed in HEPES buffer.

Survival levels and 260-nm leakage from cells demonstrating autolysis. Three separate reaction vessels containing an 18-ml volume of ADBAC, DDAC, and HEPES buffer were prepared. The final reaction concentration of each biocide was 9 μg/ml, and both reaction vessel and stock suspension (2×10^{10} CFU/ml) were maintained at 35°C. Two milliliters of the stock suspension was added to each reaction vessel to give a final concentration of 2×10^9 CFU/ml. The reaction vessels were then maintained at 35°C for 4 h (Mickle shaking water bath at 100 oscillations min^{-1}, with an amplitude of 10 cm) to initiate autolysis. After 4 h, 20 ml of double-strength neutralizer (HEPES buffer consisting of 0.14% [wt/vol] lecithin and 1.0% [wt/vol] Tween 80) was added to each reaction vessel for 10 min. The reaction vessel contents were then centrifuged at $1,508 \times g$ for 20 min (Sorvell RT6000B; DuPont, Stevenage, Hertfordshire, England) at room temperature. The pellet was then resuspended in 40 ml HEPES buffer, centrifuged, and finally resuspended in 20 ml HEPES buffer. The harvested cells were then held at 35°C for the duration of the experiment. Sample volumes of 3 ml were removed at 0, 2, 4, 18, 22, and 24 h and filtered (Acrodisc [32-mm, 0.45 μm] filter with a Supor membrane; Pall Gelman, MI), and the absorbance at 260 nm was measured.

To assess survivor levels, the procedure described above was followed, except that at 0, 2, 4, 6, 18, 22, 24, and 48 h, a 1-ml sample was removed from each reaction vessel and serially diluted in HEPES buffer, and 100 μl was plated onto TSA and incubated at 37°C. After 18 h of incubation, colony counts were recorded and converted to CFU/ml survivors.

RESULTS

MIC. The MIC remained stable after 48 h of incubation, and the MIC versus inoculum concentration was recorded (Table 1) to evaluate trends. The growth recorded within the triplicate inoculation spots was consistent under the defined conditions.

As shown in Table 1, MICs for ADBAC and DDAC appeared to be unchanged between 1×10^5 and 1×10^7 CFU/ml. Upon increasing the inoculum to 1×10^8 CFU/ml, the MIC rose by 0.2 to 0.3 μg/ml. A sharp rise by almost double the MIC (1×10^8 CFU/ml) occurred for ADBAC when the inoculum was increased to 1×10^9 CFU/ml, and more than double the MIC was witnessed for DDAC.

TABLE 1. MIC behavior for ADBAC and DDAC against increasing *S. aureus* concentrations after 48 h of incubation using TSA medium

Biocide	Biocide MIC (μg/ml) for inoculum test concn (CFU/ml) of:				
	10^5	10^6	10^7	10^8	10^9
ADBAC	0.6	0.6	0.7	1.0	1.8
DDAC	0.4	0.4	0.4	0.6	1.6

Bactericidal activity. Concentrations of 35, 45, and 55 μg/ml, when challenged with 2×10^9 CFU/ml *S. aureus*, provided kill profiles for each biocide at 25°C and 35°C. A general overview shown in Fig. 3A and B indicates that DDAC was less temperature sensitive than ADBAC. ADBAC and DDAC profiles suggested an initial rapid interaction of biocide with cells inflicting killing, which eventually slowed.

After 5 min of contact time, the ADBAC concentrations appeared to have profoundly different endpoints, which were also affected by temperature. In comparison, the dialkyl quaternary ammonium compound (DDAC) suggested rapid killing in a short contact time at both 25°C and 35°C (Fig. 3B).

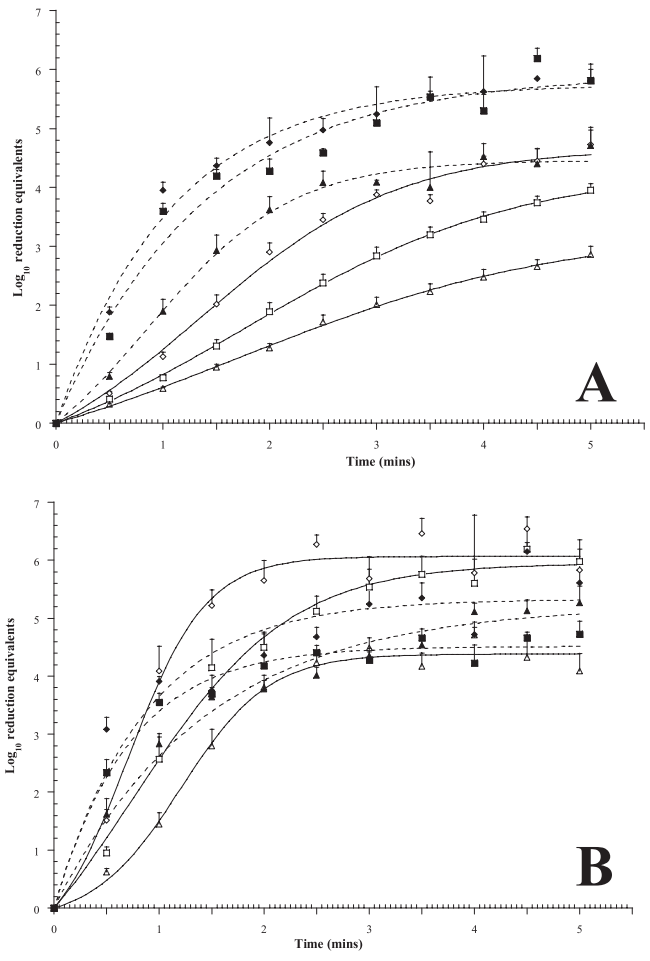

FIG. 3. Time-kill ($\log_{10}$ reduction equivalents) studies for ADBAC (A) and DDAC (B) versus 2×10^9 CFU/ml *S. aureus* at 25°C (—) and 35°C (---) in a HEPES buffer medium. Final challenge concentrations studied were 35 μg/ml (▲ and △), 45 μg/ml (■ and □), and 55 μg/ml (◆ and ◇), and each symbol indicates the means ± standard errors (SEs) for five observations.

TABLE 2. Q_{10} values for ADBAC and DDAC

Biocide	Concn (μg/ml)	Q_{10} value for $^0{}_2D$ $\log_{10}$ reduction range[a]
ADBAC	55	3.3
	45	3.7
	35	2.9
DDAC	55	1.4
	45	2.0
	35	1.7

[a] $^0{}_2D$ $\log_{10}$, the 0 to 2 $\log_{10}$ decimal endpoints used to calculate the Q_{10} values.

The two-sample *t* test assuming equal variance analysis at 25°C [*t* STAT = 8.58; *P* ($T \Leftarrow t$) two tail = 0.00035] and 35°C [*t* STAT = 2.51; *P* ($T \Leftarrow t$) two tail = 0.046] suggested that at both temperatures, a significant difference in killing between ADBAC and DDAC occurred, with DDAC being the more potent agent.

Concentration exponents and Q_{10} values. The concentration exponent values derived from a 2-$\log_{10}$ (99%) reduction endpoint (23) were 1.6 and 1.8 for ADBAC and 1.6 and 1.2 for DDAC at 25 and 35°C, respectively (for the values at 25°C for ADBAC and DDAC, the r^2 values were 0.05). Hugo and Denyer (23) previously suggested that the concentration exponent should be largely independent of the level of killing. The concentration exponents for both ADBAC and DDAC were the same at 25°C. At 35°C, the concentration exponent for DDAC decreased, and that for ADBAC increased.

The Q_{10} (Table 2) values suggested that upon increasing the temperature from 25°C to 35°C, the level of killing inflicted could be almost four times greater. The Q_{10} appeared to be influenced by the biocide challenge concentration at 25°C and 35°C. The Q_{10} value for DDAC confirms that it was less sensitive to temperature changes than ADBAC.

Uptake isotherms. Fig. 4 indicates the uptake isotherms for ADBAC and DDAC when exposed to 2×10^9 CFU/ml *S. aureus*. Upon applying the uptake classification scheme developed previously by Giles et al. (19), the ADBAC and DDAC isotherm profiles obey the L4 and H3/4 patterns, respectively.

ADBAC (Fig. 4A) showed Langmurian uptake to the cells with a possible subtle inflection point between 6 and 7 μg/2×10^9 cells (marked A and B) and a final plateau (marked C) with a maximum uptake of around 14 μg/2×10^9 cells. Upon increasing the biocide concentration beyond point C, little additional biocide was bound to cells. DDAC (Fig. 4B) possessed a very strong initial uptake, representing high-affinity binding, and over the biocide concentration range studied, the majority of biocide was bound to cells. An inflection point was clearly observed between 9 and 14 μg/2×10^9 cells (marked A and B). Beyond point B, a further rapid uptake of DDAC by cells was observed. The inflection points for both ADBAC and DDAC may be due to additional binding sites arising by cell fragmentation or critical membrane damage exposing additional inner target regions.

Hugo and Longworth (24) previously described a procedure for calculating the approximate number of molecules required to form a biocide monolayer on the surface of a bacterial cell.

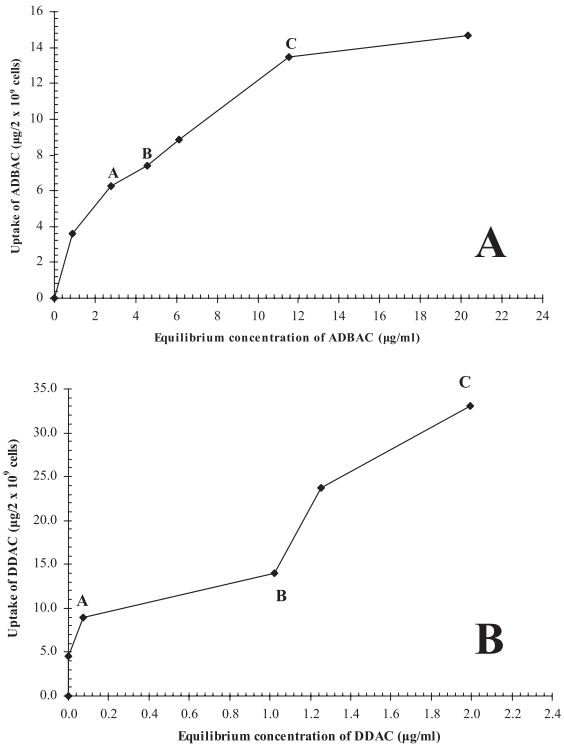

FIG. 4. ADBAC L4 (A) and DDAC H3/4 (B) uptake isotherms against 2×10^9 CFU/ml *S. aureus* in HEPES buffer. Primary uptake completion (marked B) and possible secondary uptake (marked C) are indicated. Symbols indicate means for two observations.

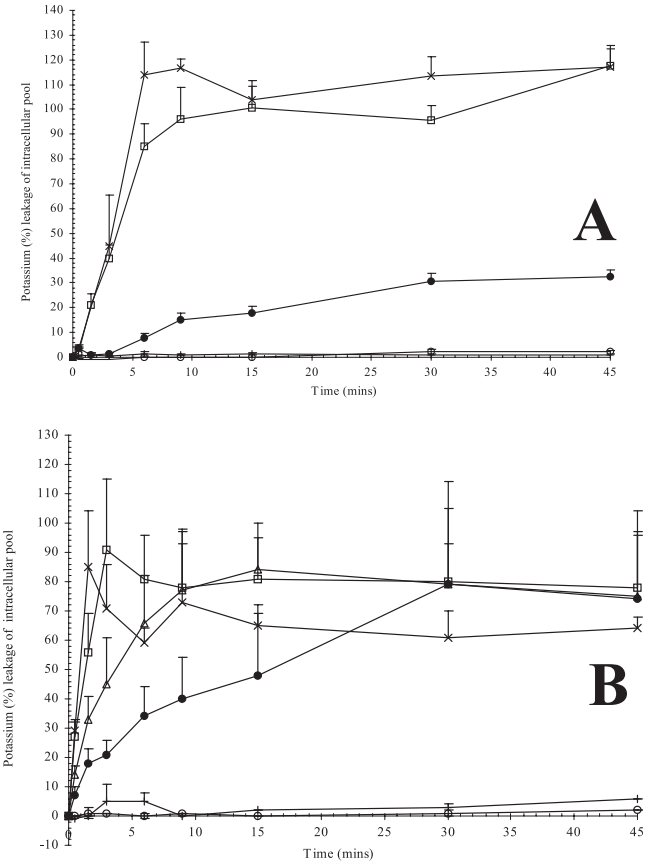

FIG. 5. Time versus percent potassium leakage from the *S. aureus* (2×10^9 CFU/ml) intracellular pool in HEPES buffer (○) and treatments with ADBAC at 25°C (A) and DDAC at 25°C (B) at final concentrations of 4.5 μg/ml (+), 9 μg/ml (●), 12 μg/ml (DDAC only) (△), 15 μg/ml (□), and 18 μg/ml (×). Each symbol indicates the means ± SEs for at least three observations.

The number of ADBAC monolayers per cell was calculated to be 1.2 at the completion of primary uptake (marked B in Fig. 4A). It appears that the ADBAC biocide could exercise its effect through monolayer or supramonolayer absorption. The ADBAC plateau sorption at point C (Fig. 4A) would represent approximately a two-monolayer uptake. The completion of primary uptake for DDAC occurred at the binding of 14 μg/2×10^9 cells (marked B in Fig. 4B). The number of DDAC monolayers at the completion of primary uptake was calculated to be 1.9, and the sorption plateau at point C (Fig. 4B) represented approximately a 4.4-monolayer uptake.

Leakage of intracellular material. Potassium leakage for both ADBAC (Fig. 5A) and DDAC (Fig. 5B) at concentrations of 15 μg/ml (0.0463 mM and 0.0460 mM, respectively) and above reached maximum pool levels within approximately 5 min of contact at 25°C. For DDAC, a 9-μg/ml (0.0276 mM) dose appeared to release potassium levels to completion over 45 min, compared to 9 μg/ml (0.0278 mM) ADBAC, which depleted the internal potassium pool by only 30%. Based on the concentrations studied, 9 μg/ml may be an important concentration differentiator for both ADBAC and DDAC; concentrations above 9 μg/ml (0.0278 mM and 0.0276 mM) for both agents appeared to rapidly release the entire internal potassium pool, and a 4.5-μg/ml (0.0139 mM and 0.0138 mM) dose induced leakage similar to that induced by the HEPES control. For ADBAC and DDAC at 35°C (data not shown), the leakage profiles were very similar to those obtained at 25°C, although leakage appeared more rapid at 35°C.

ADBAC- and DDAC-generated 260-nm-absorbing material leakage was monitored over 45 min at 25°C (Fig. 6A and B); it was rapid and reached maximum pool levels at concentrations above 9 μg/ml. Leakage at 9 μg/ml was gradual, and leakage at 4.5 μg/ml followed the leakage profile of the HEPES control. Upon increasing the ADBAC and DDAC reaction temperature to 35°C (data not shown), there was a rapid leakage of 260-nm-absorbing material compared to that at 25°C, and there was only modest leakage above the 100% pool level. This suggested that only largely unbound 260-nm-soluble pool material was detected at both temperatures.

The mean percent leakage of intracellular material at 30 min for both ADBAC and DDAC at 25°C (Fig. 7A and B) was compared to the biocide concentration (μg/ml). This approach was used in an attempt to determine the approximate concentrations where the initial membrane integrity is lost and subsequent catastrophic membrane collapse then occurs (11). A loss of membrane integrity occurs when the membrane loses conformity and leakage exceeds that of untreated cells. Catastrophic membrane collapse occurs when a complete loss of membrane integrity occurs upon exposure to biocide, and high levels of intracellular material are rapidly released. Figure 7 indicates concentrations where membrane damage of various severities may be assumed to occur. Upon examining this fig-

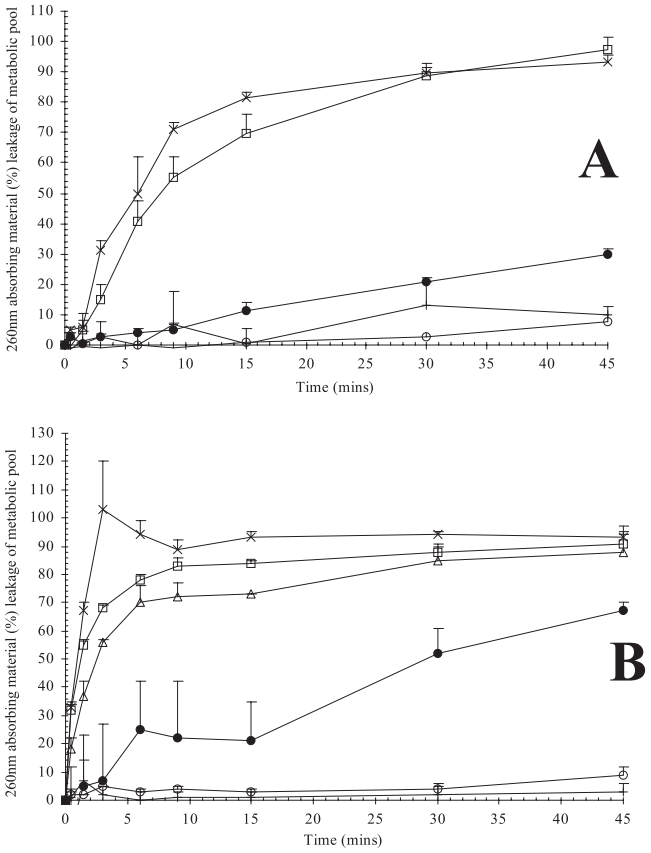

FIG. 6. Time versus percent leakage of 260-nm-absorbing material from the *S. aureus* (2×10^9 CFU/ml) metabolic pool in HEPES buffer (○) and treatments with ADBAC at 25°C (A) and DDAC at 25°C (B) at final concentrations of 4.5 μg/ml (+), 9 μg/ml (●), 12 μg/ml (◇), 15 μg/ml (□), and 18 μg/ml (×). Each symbol indicates the means ± SEs for at least three observations.

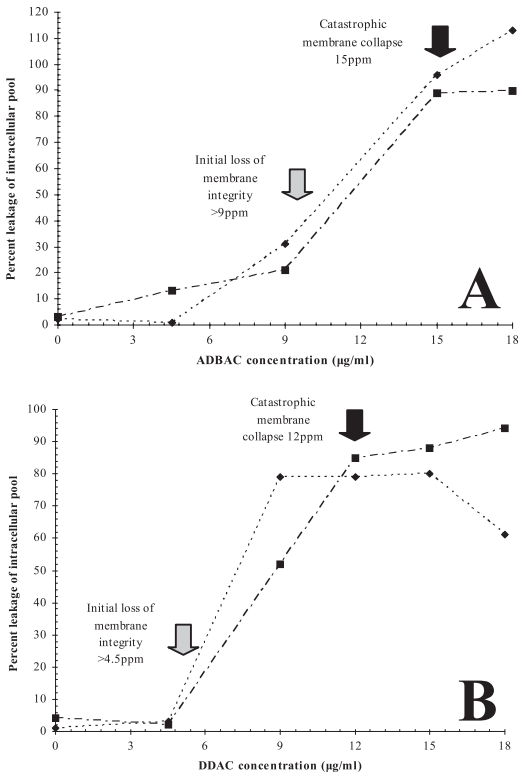

FIG. 7. Leakage of potassium (◆) and 260-nm-absorbing (■) materials with increasing ADBAC (A) and DDAC (B) concentrations (μg/ml) at 30 min (25°C). Assumed membrane integrity is indicated by the respective arrows. Symbols indicate means for at least three observations.

ure, the critical difference between the ADBAC and DDAC agents is the release of material induced at a concentration of 9 μg/ml (0.0278 mM and 0.0276 mM). For ADBAC, a loss of membrane integrity was induced at approximately 9 μg/ml (0.0278 mM), as opposed to DDAC, where the range lies just above 4.5 μg/ml (0.0138 mM). The catastrophic membrane collapse for both ADBAC and DDAC occurred above concentrations of 15 μg/ml (0.0463 mM) and 12 μg/ml (0.0368 mM), respectively. Figure 7 suggests that the ADBAC and DDAC concentrations studied do not cause major intracellular coagulation (39), although this may become evident at higher, uninvestigated concentrations.

Effect of temperature, biocide agent, and potassium loss on autolysis. Schematics representing the percent leakage of potassium (at 45 min) and 260-nm material (at 240 min) at 25°C and 35°C for both ADBAC and DDAC are presented in Fig. 8A to D. Figure 8A and B focus on data for ADBAC and DDAC at 25°C, respectively. Leakage above the cells' internal 260-nm pool material (100%) (indicated by a dotted line in the figures), suggestive of autolysis, was not convincing for either agent. A marked difference in 260-nm leakage was induced by a 9-μg/ml dose of either agent compared to 4.5 μg/ml, and this concentration boundary also had a mirrored effect on potassium leakage.

Figure 8C and D show that a temperature of 35°C encourages the autolytic processes. Figure 8C indicates that greater levels of autolysis are induced by ADBAC than by DDAC over a concentration range of 9 to 18 μg/ml. This also coincided with a release of >60% of intracellular potassium. Indeed, concentrations between 9 and 18 μg/ml for both ADBAC and DDAC inflicted almost maximum potassium leakage compared to concentrations of 4.5 μg/ml and the HEPES control. This observation is consistent with a relationship between potassium leakage and the onset of autolysis.

For ADBAC and DDAC at 35°C (Fig. 8C and D), statistical analyses were conducted to identify whether the biocide concentration was linked to the levels of autolysis recorded. For ADBAC and DDAC, a one-way analysis of variance was conducted to identify differences in the amounts of material that leaked at 4 h for each biocide concentration and the HEPES buffer control cells. Test analyses suggested that overall, a difference between biocide concentration existed for both ADBAC and DDAC, supported by the following outcomes: an F of 7.72 and a P value of 0.001 and an F of 6.19 and a P value of 0.002, respectively. Further analyses of the confidence interval plots and Tukey's pairwise comparison tests for ADBAC and DDAC suggested that two distinct response groups existed, HEPES buffer/4.5 μg/ml and the biocide range of 9 to 18 μg/ml, corresponding to no autolysis or induced autolysis, re-

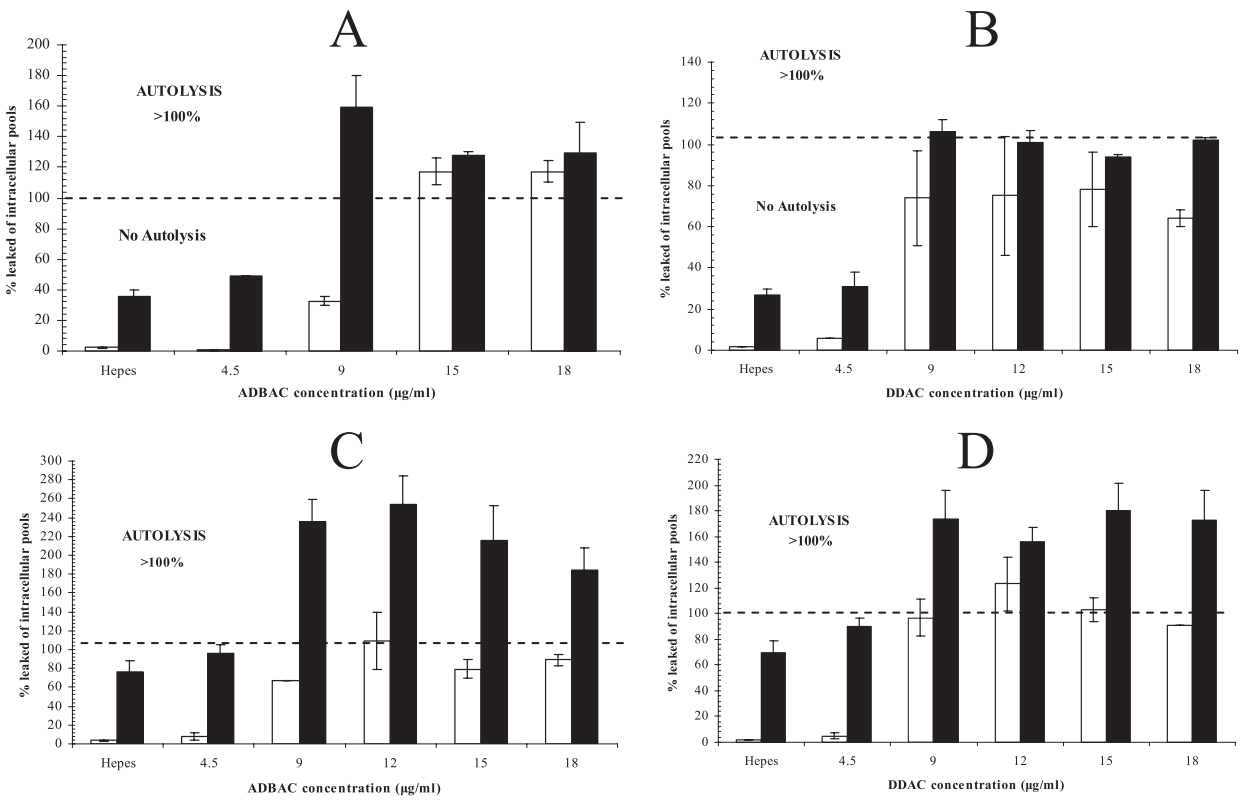

FIG. 8. Percent level of autolysis (black bars) induced by increasing ADBAC (A) and DDAC (B) concentrations after 240 min of contact at 25°C and their relationship to potassium (white bars) release (at 45 min). Autolysis is also presented for ADBAC (C) and DDAC (D) at 35°C. The means ± SEs for at least three observations are illustrated.

spectively, although within each group, there were no major differences in leakage performance.

Autolytic lethality. Control cells maintained in HEPES buffer after exposure to biocide decreased in viability by approximately 30% over 24 h (Fig. 9A). This suggests that HEPES buffer causes only limited stress on cells. A survivor count at 48 h for control cells decreased by approximately 90%, so data comparisons were made over 24 h only. Upon the removal of biocide, the cell numbers for both ADBAC- and DDAC-exposed cells decreased. Gilbert and Brown (17) previously reported that centrifugation procedures used in the standardization of bacterial suspensions killed a significant proportion of cells depending on the strains and also injured cells, increasing their susceptibility to bactericidal activity.

Over 24 h, ADBAC-exposed cells in HEPES buffer were reduced by just over 90%, and the effect of autolysis on DDAC-pretreated cells was 99% killing. It should be noted that over the first 6 h, the reduction in the cell number for DDAC-pretreated cells appeared to be quicker than that for cells treated with ADBAC. Between the 6-h and 18-h time points, both groups of cells were reduced by almost 90%. At 22 or 24 h, the decrease in cell number was minor and possibly suggested no further reduction. At the point where no further loss of cells was observed, cell death may be attributed to bactericidal action, with sublethally injured cells being unable to recover on the nutrient-rich medium and cells committed to death via autolysis. It is difficult to allocate a quantitative (CFU/ml) value to each of the previously named causes of cell

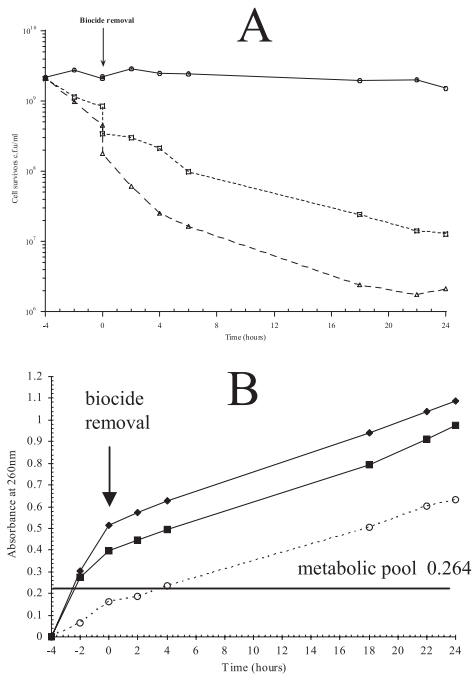

FIG. 9. (A) Cell survival in HEPES buffer (○) over 24 h after prior exposure to 9 μg/ml ADBAC (□) and DDAC (△) (n = 1). (B) Measurement of 260-nm-absorbing material over 24 h following neutralization of ADBAC (◆) and DDAC (■) (n = 1).

death. The remaining viable cells were able to survive exposure to a 9-μg/ml biocide dose. It was observed for ADBAC and DDAC that colonies at early time points varied in size, being either large (similar to HEPES control cells), which predominated, or small colonies. As the reaction time progressed, only large colonies were observed.

As shown in Fig. 9B, the HEPES control profile suggested that the breakdown of 260-nm-absorbing material was evident over 24 h, and this has been reported to occur in buffer environments (5). Leakage of 260-nm-absorbing material for cells exposed to ADBAC and DDAC exceeded that of the HEPES control cells, and this was largely due to the 4-h biocide contact period, prior to the removal of biocide. The leakage rates after biocide removal appeared to be the same irrespective of pretreatment conditions.

DISCUSSION

MIC properties of ADBAC and DDAC. The agar dilution (MIC) technique indicated that ADBAC and DDAC appeared to inhibit bacterial growth at 48 h when challenged with *S. aureus* densities ranging between 1×10^5 CFU/ml and 1×10^9 CFU/ml, with MICs between 0.4 and 1.8 μg/ml. These values were within the MIC range quoted for commercial QACs, 0.5 to 5.0 μg/ml (35). Acheampong (1) previously investigated the action of pure ADBAC homologues with carbon chain lengths of C_9 and C_{14} against *Escherichia coli*. Acheampong (1) suggested that when only small amounts of biocide agent were taken up by cells, increasing the cell concentration had little effect on the inhibition since sufficient free biocide was present in the original challenge dose. This observation was evident for ADBAC and DDAC over the concentration ranges of 1×10^5 CFU/ml to 1×10^7 CFU/ml, where the MICs were consistent for each agent. The increase in MICs at cell concentrations of $>1 \times 10^8$ CFU/ml could be attributed to an increasing reservoir of cellular negatively charged teichoic acids, which could interact with the cationic biocide molecules, reducing the available dose in solution.

Kill profiles for ADBAC and DDAC. Profiles of kill versus time at concentrations of 35, 45, and 55 μg/ml for ADBAC and DDAC at 25°C suggested an initial rapid interaction of biocide with cells, resulting in a 2- to 3-$\log_{10}$ reduction within 3 min. Such a quick reduction in cell number has been previously reported for QACs (30, 31). Other authors (8, 53) have commented on the shape of kill curves, referring to them as sigmoidal, terminating at a point of no further killing. This trend was generally more apparent at 25°C than at 35°C in this investigation.

In this study, regarding ADBAC, an increase in temperature from 25°C to 35°C produced faster killing and eradicated the initial delay in killing observed at 25°C. A similar trend was observed in a previous study of QACs by Gélinas et al. (16), where the activity between 4°C and 50°C increased with rising temperature and longer contact times. Ahlström et al. (3) previously observed that amphiphilic betaine esters (QACs) at 0°C were less active than at 30°C even though binding affinities remained unchanged. The reduction in killing was believed to be due to the reduced fluidity of the outer membrane of *Salmonella enterica* serovar Typhimurium. A reduction in temperature and pH resulted in a more tightly packed lipopolysaccharide, thus reducing activity. Gilbert et al. (18) previously reported that as membranes pass through the transition from high to low temperatures, they become more crystalline. In this investigation, DDAC exhibited the greatest biocidal activity and appeared not to be influenced by temperature (47). Temperature trends could be described by Q_{10} values, approximately 1 to 2 for DDAC. For ADBAC, the Q_{10} values suggested that approximately three to four times the activity occurred at 35°C compared to that at 25°C. Gélinas et al. (16) previously reported a twofold increase in activity for ADBAC against *S. aureus* between 20°C and 37°C. Sundheim et al. (45) previously reported that increased activity was observed when the temperature of a QAC disinfectant used in the food industry was raised to between 30 and 40°C.

During a disinfection treatment procedure, the concentration exponent may be subject to minor fluctuations. Hugo and Denyer (23) presented η values for phenol, which showed that η decreased with higher endpoint values.

Upon increasing the temperature from 25°C to 35°C, the η values for DDAC decreased (1.6 and 1.8 for ADBAC and 1.6 and 1.2 for DDAC at 25 and 35°C, respectively [r^2 values of 0.05]). Tilley (49) previously determined η values for some biocides and reported that upon increasing the temperature across the range of 10°C to 40°C, η values decreased. As temperature was increased, the kill time to reach specific endpoints decreased. This could possibly suggest that upon increasing the temperature, a difference in the cell/biocide interaction occurred that assisted killing, resulting in lower concentration exponents. The concentration exponent values for ADBAC marginally increased with temperature.

QAC uptake and membrane damage. Uptake isotherm studies for ADBAC and DDAC obeyed the L4 and H3/4 patterns, respectively. DDAC possessed a very strong binding affinity for *S. aureus* cells, and low residual levels in the supernatant liquid (equilibrium concentration) were recorded. This could explain the greater levels of killing experienced using the DDAC agent, and this could perhaps also be responsible for the significant slowing of killing after 5 min. The uptake isotherms and concentrations used in this study were also reported previously by Takasaki et al. (47, 48), who reported that DDAC was taken up rapidly, independently of the treatment temperature, and that the cytoplasmic membrane was the first uptake site, as the majority of the agent was distributed in the membrane and cytoplasmic fractions. The fact that temperature did not influence uptake might be inferred from the kill-survivor curves at 25°C and 35°C obtained in this study.

ADBAC at low concentrations exhibited moderate binding with the challenge inoculum, but at high concentrations, greater levels of biocide remained unbound. From the ADBAC uptake isotherm profile, there appeared to be a limit to the amount of ADBAC that could be adsorbed by cells, possibly due to the exhaustion of binding sites. This observation may be linked to the ADBAC kill curves, where a plateau effect was observed, suggesting no further killing of cells. Unbound ADBAC levels in the supernatant liquid could possibly be dominated by one single homologue chain length. Acheampong (1) previously observed that for ADBAC homologues, the C_{14} had a higher affinity than the C_9 against *E. coli* under identical conditions.

The inflection points observed in the isotherms were not marked but could disclose a concentration level where addi-

tional target sites become exposed and are made available to ADBAC and DDAC due to critical membrane damage (11, 26). Diphasic isotherms with both primary and secondary saturation plateaus have been classified for S, L, and H isotherms (2, 41). This was true for ADBAC and DDAC, whose primary plateau uptakes (marked A and B) were between 6 and 7 $\mu g/2 \times 10^9$ cells and 9 and 14 $\mu g/2 \times 10^9$ cells, respectively, approximating the threshold concentrations required for bactericidal activity where the leakage of intracellular materials in excess of control levels became apparent.

Leakage for both ADBAC and DDAC occurred at bactericidal concentrations (>9 μg/ml) rather than at concentrations that appeared to inhibit bacterial growth (<4.5 μg/ml). Greater levels of leakage at 9 μg/ml were induced by DDAC (0.0276 mM) than by ADBAC (0.0278 mM), and this concentration potentially marked the boundary between a cell releasing internal pool material to completion or resisting biocide-induced membrane changes. Potassium leaked rapidly from cells compared to 260-nm-absorbing material at any given biocide concentration. In general, DDAC inflicted quicker leakage, and at low concentrations, greater amounts of material leaked than did ADBAC. This was evident when concentrations that are considered to achieve membrane permeabilization (ADBAC, >9 μg/ml [>0.0278 mM]; DDAC, >4.5 μg/ml [>0.0138 mM]) and catastrophic membrane collapse (ADBAC, 15 μg/ml [0.0463 mM]; DDAC, 12 μg/ml [0.0368 mM]) were achieved. It was also evident that cells exposed to bactericidal concentrations of ADBAC and DDAC immediately released leakage markers, albeit in different quantities; internal pool material was released to completion within short exposure times, exhibiting actions similar to those of chlorhexidine (24). Concentrations that induced potassium leakage only were not evident, even at concentrations that appeared to inhibit bacterial growth for ADBAC and DDAC.

Evidence of autolysis, associated factors, and contributions to lethality. Autolysis was evident for cells exposed to biocide when leakage of 260-nm-absorbing material into the exogenous environment exceeded the metabolic pool. This was thought to be due to the breakdown of RNA material, including rRNA, by activated RNases. Measurements of 260-nm-absorbing material released at 35°C confirmed that both ADBAC and DDAC initiated autolysis, with autolytic levels increasing with longer contact times (42).

A greater amount of 260-nm-absorbing material leaked at 35°C than at 25°C, suggesting a temperature-dependent autolytic process and evidence of an enzymatic reaction (42). Hugo and Bloomfield (22) previously reported that experiments at 4°C inhibited autolytic enzyme activity, with only metabolic pool leakage being recorded. For both ADBAC and DDAC, the concentration appeared to dictate whether autolysis would be initiated in cells. Autolysis was observed at concentrations of 9 μg/ml and above, and this coincided with an intracellular potassium depletion from cells of more than 30% (26). The HEPES control and a concentration of 4.5 μg/ml failed to initiate autolysis in the experimental time scale, with a corresponding low level of potassium leakage. Sykes (46) and Lamikanra and Allwood (28) previously reported the importance of potassium for maintaining ribosome stability and the internal ionic environment.

At 35°C, levels of autolysis were greater for ADBAC than for DDAC. However, for ADBAC, autolysis peaked at 12 μg/ml and then appeared to decrease with higher biocide concentrations, which is suggestive of biocide retarding the action of RNase enzymes or possibly impeding the further release of material. For DDAC, the level of autolysis appeared constant, but lower, over the concentration range studied. Salton (42) previously observed a concentration relationship between cetyltrimethylammonium bromide (CTAB) and 260-nm release at several contact time points. Although Salton (42) examined concentrations greater (up to 900 ppm) than those used in this study, it was observed that a reduction in 260-nm release occurred above 270 ppm. This observation appeared in the region of maximum CTAB uptake by the cells. Salton (42) observed that 260-nm-absorbing material on the CTAB-saturated cells partly accounted for the fall in 260-nm detection. Takasaki et al. (48) previously reported that intracellular macromolecules such as phospholipids, proteins, and nucleic acids leaked from *S. aureus* cells and could be precipitated with cationic surfactants. In this study, the ADBAC uptake isotherm revealed that unbound ADBAC at challenge concentrations of 12 μg/ml and above would have been present in the reaction vessel and may have interacted with free 260-nm-absorbing material that leaked from cells. DDAC possessed an H (high) uptake isotherm, and only very low levels of residual biocide would be free to interact with 260-nm-absorbing material; hence, no concentration effects were observed. Hugo and Longworth (24) previously found that secondary leakage from cells was reduced due to the inhibition of autolytic enzymes by chlorhexidine. It was also believed that the cell surface or the cytoplasmic membrane congealed, preventing leakage.

Cell survivors monitored over 24 h after the removal of ADBAC and DDAC suggested that autolysis makes a limited contribution to bactericidal activity. During exposure of cells to 9 μg/ml ADBAC or DDAC for 4 h, a proportion of cells would have been killed due to bactericidal activity. Other cells would have received a sublethal dose, although it would have been sufficient to induce autolysis. Some cells would have avoided bactericidal or autolytic inducing activities and would therefore have been able to survive, provided that a suitable recovery medium was employed. Upon the removal of the biocide, cells that experienced autolysis or that were too badly damaged to recover would not be able to replicate and were committed to death over the 24-h detection period, and those cells unaffected by biocide would survive. The surviving cells were possibly responsible for the stabilized survivor counts observed at around 18 to 24 h.

Cells preexposed to 9 μg/ml ADBAC or DDAC indicated an accelerated release of 260-nm-absorbing material above the metabolic pool, in comparison to cells maintained in HEPES buffer. This suggested that ADBAC and DDAC induce autolysis, although upon the removal of biocide, the leakage rate appeared to decrease. Over 24 h, irrespective of cell pretreatment, autolysis was observed and appeared to be an irreversible event, as observed previously by Hugo and Bloomfield (22).

We conclude that both ADBAC and DDAC are membrane-active agents that interacted with the cytoplasmic membrane in *S. aureus*, inducing the immediate leakage of intracellular constituents, confirming the primary lesion for both. Over the

range of concentrations studied for leakage, MIC, and bactericidal investigations, DDAC was marginally more potent than ADBAC, but no outstanding differences in their properties were observed.

ADBAC and DDAC were both initiators of autolysis at low biocide concentrations (9 to 18 μg/ml), which, together with bactericidal activity, contributed to cell death. DDAC was less sensitive to temperature changes from 25°C to 35°C than ADBAC, and this was reflected in the Q_{10} values.

The protocols employed and data observations reported in this study together with the calculated numerical descriptors of biocide action (Q_{10} values and concentration exponents) will allow formulators and microbiologists using antiseptics and disinfectants to make a judicious selection of quaternary ammonium compound to achieve broad-spectrum activity and curb the threat of resistant outbreaks, especially in hospital settings. This will lead to improved antiseptic and disinfectant design while also considering the ultimate treatment of abiotic and biotic surfaces. These numerical efficacy parameters can also be used to better understand quaternary ammonium compound performance under a given set of conditions and advise on appropriate application whether for use as preservatives in pharmaceutical products or for general use in cleaning clinical or domestic and food environments.

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