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Effects of *ortho*-phthalaldehyde, glutaraldehyde and chlorhexidine diacetate on *Mycobacterium chelonae* and *Mycobacterium abscessus* strains with modified permeability

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The mechanisms of the mycobactericidal action of *ortho*-phthalaldehyde (OPA), glutaraldehyde (GTA) and chlorhexidine diacetate (CHA) were investigated using mycobacterial spheroplasts of two reference strains, *Mycobacterium chelonae* NCTC 946, *Mycobacterium abscessus* NCTC 10882 and two GTA-resistant strains, *M. chelonae* Epping and *M. chelonae* Harefield. Transmission electron microscopy of the spheroplasts revealed an altered cell wall structure compared with the parent cells. Structural alterations resulting from the spheroplasting process were in part correlated to a loss of lipid content. Low concentrations of CHA induced protein coagulation in *M. chelonae* NCTC 946 spheroplasts, which also exhibited the highest loss of free non-polar lipids. Higher concentrations of CHA were required to produce similar results to the other spheroplasts investigated in which there was a less substantial decrease in lipid content. OPA (0.5% w/v) readily penetrated the residual cell wall and cytoplasmic membrane, producing significant protein coagulation in *M. chelonae* NCTC 946. GTA (0.5% v/v) induced a similar effect but to a lesser extent. Pre-treatment of the spheroplasts with OPA and GTA and their subsequent suspension in water demonstrated that GTA was a more potent cross-linking agent. This protective effect of GTA results from extensive cross-linking of amino and/or sulphydryl side-chain groups of proteins. The rapid mycobactericidal effect of OPA probably arises from its more efficient penetration across biological membranes. Mycobacterial spheroplasts represented a useful cellular model with an altered cell wall permeability. This study also showed the importance of the mycobacterial cell wall in conferring intrinsic resistance to CHA.

Keywords: mechanism of action, cell wall, permeability

Introduction

Non-tuberculous mycobacteria (NTM) are defined as those mycobacteria that are not part of the *Mycobacterium tuberculosis* complex. Many NTM are free-living saprophytes that are widely distributed in a variety of environments such as soil, water, dust and aerosols.[1,2] The rapidly growing NTM such as *Mycobacterium chelonae*, *Mycobacterium abscessus* and *Mycobacterium fortuitum* also comprise a major cause of opportunistic hospital-acquired infections in immunocompromised patients.[3,4] In medical care units, the spread of these atypical mycobacteria can be reduced by adequate disinfection of equipment, the local environment and the staff.

Mycobacteria as a group are highly impermeable to hydrophilic molecules, including nutrient molecules such as glucose and glycerol, and antibiotics such as β-lactams.[5,6] It is well established that the complex lipid-rich mycobacterial cell wall, which constitutes an efficient impermeability barrier, plays a major role in the intrinsic resistance of mycobacteria to antibiotics, antiseptics and disinfectants.[7–11]

In this study, we have focused our attention on the mechanisms of action of some biocides that are widely used in

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hospital environments and particularly on some aldehydes used for high-level disinfection in endoscopy units. The bis-biguanide chlorhexidine, used as the diacetate or gluconate, is a clinically important antiseptic (hand-washing and oral products), disinfectant and preservative.[12] Mycobacteria are generally highly resistant to chlorhexidine diacetate (CHA), but the MICs for some mycobacterial strains are of the order of those for CHA-sensitive Gram-positive cocci.[10,12] The mechanism by which CHA is mycobacteriostatic rather than mycobactericidal remains unknown, although the cell wall permeability barrier seems to play an important role.[13] Glutaraldehyde (GTA) remains a popular high-level disinfectant for flexible endoscope disinfection[14] despite its slow mycobactericidal activity and the increasing emergence of 2% v/v GTA-resistant *M. chelonae* strains isolated from endoscope washer disinfectors.[15] More recently, *ortho*-phthalaldehyde (OPA), an aromatic dialdehyde, has been proposed as a possible alternative to GTA for high-level disinfection of endoscopes.[16] A previous study demonstrated the rapid efficacy of 0.5% w/v OPA against a range of NTM and more importantly against GTA-resistant mycobacterial strains.[17]

Bacterial spheroplasts exhibit a residual altered cell wall structure and composition, and we have used such models to obtain information about: (i) the nature and role of the mycobacterial cell wall in conferring resistance to CHA; (ii) the role of the permeability barrier represented by the mycobacterial cell wall towards OPA and GTA; and (iii) the possible protective effect, associated with cross-linking properties, of OPA and GTA in preventing spheroplast lysis in water.

Materials and methods

Bacterial strains and growth conditions

The organisms studied were *M. chelonae* NCTC 946 (ATCC 35752) and *M. abscessus* NCTC 10882 (National Type Culture Collection, London, UK), and two GTA-resistant washer disinfector isolates, *M. chelonae* Epping and *M. chelonae* Harefield, obtained from Dr P. A. Griffiths (City Hospital NHS Trust, Birmingham, UK). The growth medium consisted of 90 mL of Middlebrook 7H9 broth supplemented with 0.2% v/v glycerol and 10% v/v Middlebrook ADC enrichment (albumin, dextrose, catalase; Difco Laboratories, Cowley, Oxford, UK). Cells were grown to their mid-log growth phase at 30°C (except for *M. abscessus* NCTC 10882, which was grown at 37°C) with gentle shaking (100 rpm, Gallenkamp water-bath; Fisher, Loughborough, UK).

Chemicals and biocide solutions

Solutions of OPA (ASP Biocides, Irvine, CA, USA), GTA (Sigma–Aldrich, Gillingham, Dorset, UK) and CHA (Sigma–Aldrich) were freshly prepared in 30 mM Tris–HCl buffer (pH 8.0) containing 20% w/v sucrose. All other chemicals were from Sigma–Aldrich.

Preparation of spheroplasts

Mycobacterial spheroplasts were prepared as described previously.[18] Briefly, mid-log growth phase cultures of the different mycobacterial strains were treated with a mixture containing 60 mg/L D-cycloserine, 1.4% w/v glycine, 200 mg/L lysozyme, 2000 mg/L EDTA and 1000 mg/L LiCl for 18 h (*M. chelonae* NCTC 946 and *M. abscessus*) or 36 h (*M. chelonae* Epping and *M. chelonae* Harefield). Pre-spheroplasts or cell wall-deficient forms were washed with 30 mM Tris–HCl buffer (pH 8.0) containing 20% sucrose and incubated with 10 mM potassium-EDTA and 0.5 mg/mL lysozyme on a shaker (100 rpm) at 37°C. Spheroplasts were formed after 4 h incubation. To remove intact cells and aggregated wall ghosts, the suspension was centrifuged at 300**g** for 7 min.[19]

Transmission electron microscopy (TEM) studies

Both spheroplast and bacillary forms (parental forms) of the four mycobacterial strains were examined. Cells were fixed for 24 h in 2.5% v/v GTA in 0.1 M sodium cacodylate buffer, pH 7.2. Cells were washed twice with cacodylate buffer, post-fixed for 1 h at room temperature using 1% w/v osmium tetroxide (OsO_4) in cacodylate buffer, and then washed again with cacodylate buffer. Samples were dehydrated through a graded ethanol series of 50, 70, 80 and 95% v/v for 5 min each and then washed three times for 10 min each in 100% ethanol. The cell pellets were resin-infiltrated for 48 h at 60°C using a mixture of araldite cy212 (5 g), dodecenyl succinic anhydride (5 g), dibutyl phthalate (0.5 g) and *N*-benzyldimethylamine (0.38 g) at 1:1 v/v with propylene oxide. Blocks were thin sectioned on a Reïchert Ultracut microtome (Ultracut, Reïchert-Jung, Vienna, Austria) with a glass knife and mounted on to polioform-coated copper grids. Sections were counterstained with 2% w/v uranyl acetate for 10 min in the dark, washed twice with water and stained with Reynolds' lead citrate for 5 min. Thin sections were examined and photographed using a Philips EM208 TEM operating at 60 kV under standard conditions with a liquid-nitrogen cold trap in place.

Extraction of mycobacterial free lipids

Non-polar lipids of whole mycobacterial cells and spheroplasts were extracted using the method of Dobson *et al.*[20] Lipids were extracted from dry cells for 15 min with a biphasic mixture of methanolic saline (methanol–0.3% aqueous NaCl; 100:10 v/v) and petroleum ether at room temperature with continuous stirring. The upper layer was removed and retained, and the lower layer was re-extracted with petroleum ether for a further 15 min. The upper layer was removed and

pooled with the upper layer retained from the first extraction, and evaporated to dryness at room temperature to yield the non-polar lipids.

Experiments on spheroplasts

Effect of CHA, OPA and GTA. Spheroplasts were resuspended in 1 mL of 30 mM Tris–HCl buffer (pH 8.0) containing 20% sucrose to give an optical density at 640 nm (OD_{640}) of approximately 5. A 100 µL aliquot was then mixed with 700 µL of CHA, OPA or GTA solution in sucrose buffer to give appropriate final biocide concentrations of 25–500 mg/L (CHA) or 0.01–0.5% (OPA and GTA). Aliquots of 100 µL were also subjected to deionized water or sucrose buffer for the determination of maximum lysis and the assessment of auto/spontaneous lysis, respectively. Changes in OD_{640} were measured over 60 min using a Helios α UV–visible spectrophotometer (Spectronic Unicam, Cambridge, UK). The sucrose buffer was used as the blank and the reduction in absorbance was calculated by subtracting the absorbance taken at 2–60 min from the absorbance taken at time $t = 0$.

Effect of OPA or GTA pre-treated spheroplasts on induced lysis by deionized water. Spheroplasts were resuspended in 5 mL of 0.1% OPA or GTA to give an OD_{640} of 1. Concurrently, one sample was resuspended in 5 mL of 30 mM Tris–HCl buffer (pH 8.0) containing 20% sucrose and was used as a control without pre-treatment. After 30 min exposure at 20°C, samples were centrifuged at 16 000**g** for 45 min and resuspended in 1 mL of deionized water. An aliquot of 100 µL was immediately mixed with 700 µL of deionized water and changes in OD_{640} measured as described above. All experiments on spheroplasts were carried out in triplicate.

Results

Ultrastructure and envelope profiles of mycobacterial spheroplasts

TEM data obtained by double staining bacteria with OsO_4 and uranyl acetate plus lead citrate are illustrated in Figure 1. Figure 1(a) shows an untreated control preparation of *M. chelonae* NCTC 946; the cells are intact, typically rod-shaped, with a ribosome-rich cytoplasm, a DNA-rich nucleoplasm, a distinct cytoplasmic membrane (CM), a peptidoglycan layer (PG) and an equally thick electron-transparent layer (ETL) that separates the PG from the thin outer wall layer (OL). The three other untreated strains presented the same morphological appearance as the reference strain (data not shown). When compared with the ultrastructure of intact untreated *M. chelonae* NCTC 946 (Figure 1a), spheroplasts appeared as spherical structures that lacked a distinct, compact and dense cell wall as well as a densely packed cytoplasm (Figure 1b–e). The high speed centrifugations required during spheroplast preparation were probably responsible for the leakage of intra-cytoplasmic constituents. High magnification envelope profile micrographs of *M. chelonae* NCTC 946 showed details of the cell wall ultrastructure of untreated cells (Figure 2a) and spheroplasts (Figure 2b). The envelope of untreated *M. chelonae* NCTC 946 consisted of a well defined CM presenting a thickness of ~2.5–3.5 nm (innermost electron-dense layer), a PG layer of 5.5–7.5 nm in width (second innermost heavily stained layer, creating the periplasmic space with the CM) that underlies the ETL corresponding to the hydrophobic domain of the cell wall, which mainly consists of the mycolic acids covalently bound to arabinogalactan (AG).[7] Finally, a poorly discernible OL was identified (Figure 2a). The total wall thickness (comprising PG, ETL and OL) in control cells was ~20 nm. In contrast, there were striking differences in the cell envelope profiles between spheroplasts and parent strains. *M. chelonae* NCTC 946 spheroplasts showed a disorganized cell wall structure (Figure 2b). The CM was distinguishable, and the thickness and intensity of the PG layer was decreased up to 50% of that of the intact *M. chelonae* NCTC 946. Interestingly, it also appeared that the CM and PG layers were detached from one another when compared with the multilayered cell envelope of the control cells in which the CM was tightly associated with the PG layer forming an evenly opposed periplasmic space. No OL was visible with any of the spheroplast residual envelopes.

Effect of spheroplasting process on mycobacterial free non-polar lipid content

Preliminary mixing of whole cells or spheroplasts with a biphasic mixture of methanolic saline and petroleum spirit yielded the non-polar lipids in the petroleum spirit layer. A further chloroform–methanol extraction of the lower aqueous methanol layer and the partially extracted cells yields the free cell wall polar lipids as well as some plasma membrane polar phospholipids.[21] Because this study focused on the role of the cell wall as a permeability barrier to biocides, a second extraction of plasma membrane lipids was not investigated. The non-polar lipid content in whole mycobacterial cells and spheroplasts is outlined in Table 1. For each strain, a significant reduction (*t*-test, $P < 0.001$) was observed in the non-polar lipid content of the spheroplasts in comparison with the corresponding untreated strain. However, this decrease in free lipids was not of the same magnitude for all the strains and was significantly different between the GTA-resistant and reference strains. A smaller loss of free lipids (43% and 37% for *M. chelonae* Harefield and *M. chelonae* Epping, respectively) occurred with the GTA-resistant strains than the reference strains (*M. chelonae* NCTC 946 and *M. abscessus* NCTC 10882), which showed a considerable lipidic loss (79% and 61%, respectively). There was no structural evidence from the TEM study of any differences in lipid content between

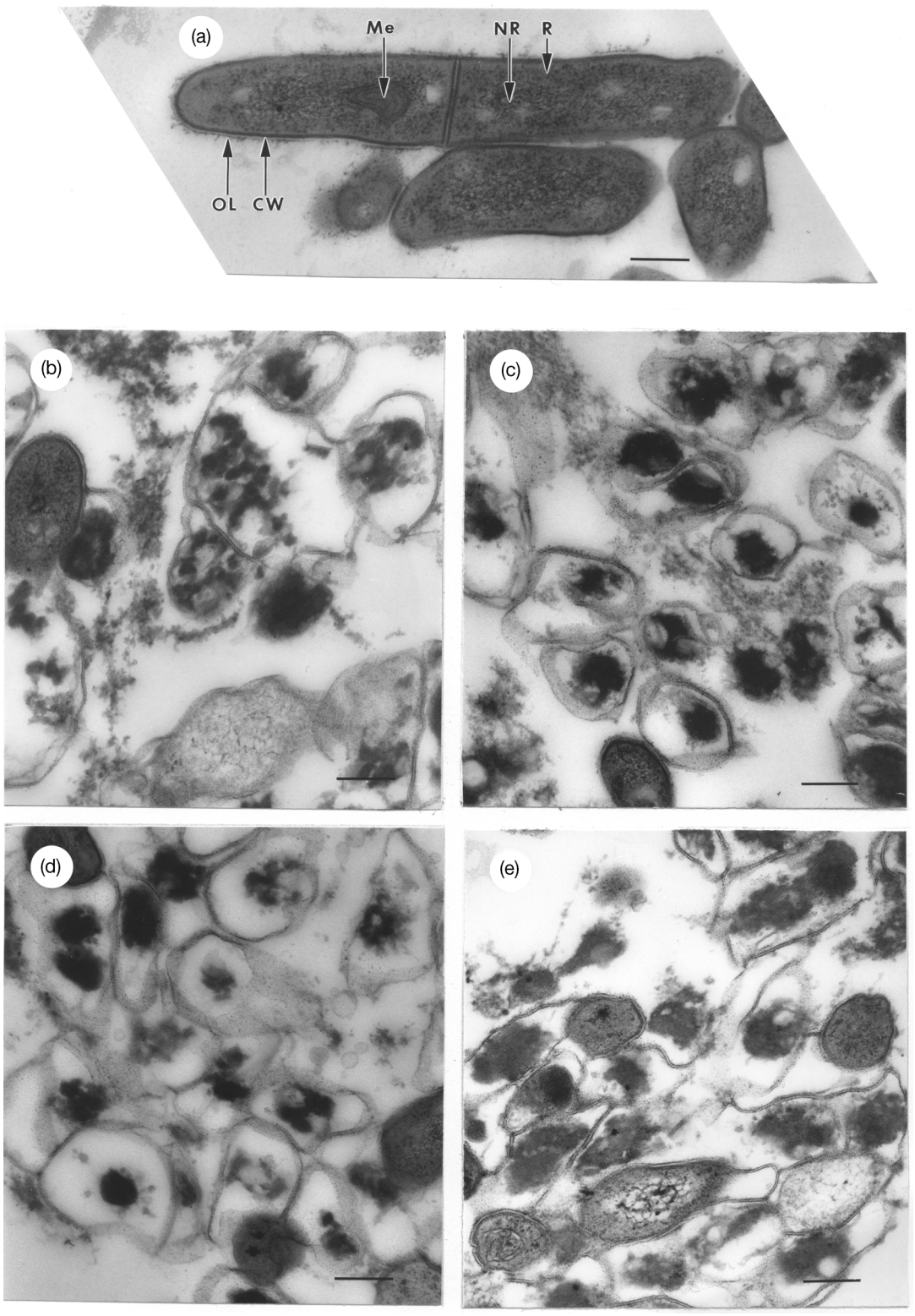

Figure 1. Transmission electron micrograph of thin sections of (a) untreated control bacilli *M. chelonae* NCTC 946, characterized as rod-shaped bacteria, surrounded by a well-defined cell wall, (b) *M. chelonae* NCTC 946 spheroplasts, (c) *M. abscessus* NCTC 10882 spheroplasts, (d) *M. chelonae* Harefield spheroplasts and (e) *M. chelonae* Epping spheroplasts. Spheroplasts are characterized as spherical forms lacking a distinct and compact wall structure. The micrographs shown are typical of the population as a whole, as judged by viewing many independent fields. OL, outer layer; CW, cell wall; Me, mesosome; NR, nuclear region; R, ribosomes. Magnification in all cases, ×25 000. Bars, 300 nm.

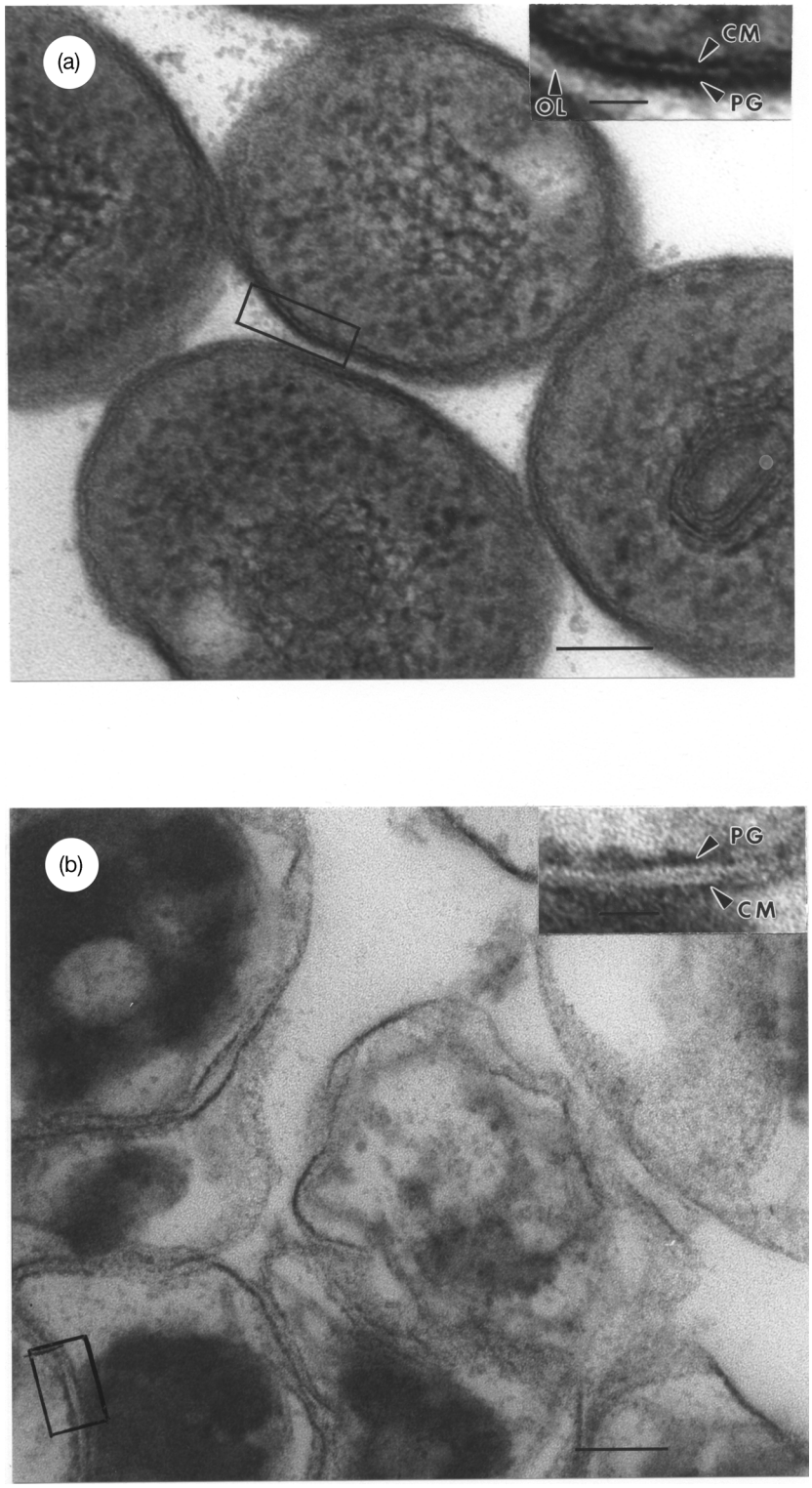

Figure 2. High-magnification (×100 000) transmission electron micrograph of thin section of (a) untreated control *M. chelonae* NCTC 946 stained by lead citrate and uranyl acetate present an intact mycobacterial cell wall structure with a dense cytoplasm. Cells show a thin cytoplasmic membrane (CM), and a thick electron-dense layer corresponding to the cell wall. A thin and poorly stained outer layer (OL) is also visible. (b) Stained by the same method as above, *M. chelonae* NCTC 946 spheroplasts show a diffuse residual cell wall. The CM and peptidoglycan (PG) are distinguishable but less electron-dense and compact than the parental form. Bars, 100 nm; inset, bars indicate 25 nm.

Table 1. Free non-polar lipid content of whole cells and spheroplasts of mycobacteria

Species	Non-polar lipid content in mg/g dry weight (mean ± S.D.)[a]		Loss of lipids (%)
	whole cells	spheroplasts	
M. chelonae NCTC 946	61.26 ± 5.83	12.76 ± 4.82[b]	79
M. abscessus NCTC 10882	66.71 ± 2.32	26.00 ± 2.38[b]	61
M. chelonae Harefield	54.41 ± 2.08	31.15 ± 2.17[b]	43
M. chelonae Epping	61.21 ± 4.69	38.80 ± 0.23[b]	37

[a]Values are averages from two independent cultures measured in duplicate.
[b]Significant difference ($P < 0.001$) between whole cells and spheroplasts.

the remaining envelope of spheroplasts of GTA-resistant and -sensitive strains, presumably because the water-soluble stains, such as the uranyl acetate used in this study, failed to penetrate substantially into the extremely hydrophobic hydrocarbon-rich regions.

Stability of spheroplasts

In all experiments, the spheroplasts were diluted either in deionized water to assess their resistance to osmotic shock (control for maximum lysis) or in sucrose buffer (stabilizing buffer) to evaluate their stability (control auto/spontaneous lysis) by measuring the decrease in OD_{640} (Δ_{OD}) (Figures 3–8). The rate of lysis d(1/OD)/dt in water was slow ($\Delta_{OD\ 60min}$ ranging from –0.18 to –0.37), but greater than the rate of lysis induced in sucrose buffer. Indeed, spheroplasts of all four strains were relatively stable when resuspended in hypertonic sucrose solution, achieving a $\Delta_{OD\ 60\ min}$ of approximately –0.1.

Effect of CHA on spheroplast suspensions

The OD of *M. chelonae* NCTC 946 spheroplast suspensions treated with CHA was higher than that of the control in stabilizing buffer (Figure 3). The increase in OD was greater as the concentration of CHA increased. Figure 4 depicts the effect of CHA on *M. chelonae* Harefield spheroplasts. The spheroplast suspensions were stabilized in solutions of 25–100 mg/L CHA and had similar time curves to the control in sucrose buffer. However, a slight increase in OD was observed when 500 mg/L CHA was used. Similar results were observed with *M. abscessus* NCTC 10882 and *M. chelonae* Epping (not shown).

Effect of OPA and GTA on spheroplast suspensions

Spheroplasts of the GTA-resistant strains agglutinated in the presence of OPA and GTA, leading to an erroneous reading of the fall in OD, although (i) agglutination of spheroplasts in OPA solutions was less extensive than that observed in GTA solutions, and (ii) *M. chelonae* Harefield showed less agglutination than *M. chelonae* Epping in both biocide solutions. Thus, the effects of OPA and GTA solutions were only investigated for spheroplasts of *M. chelonae* NCTC 946 and *M. abscessus* NCTC 10882. Low concentrations of OPA appeared to stabilize the spheroplast suspensions of *M. chelo-*

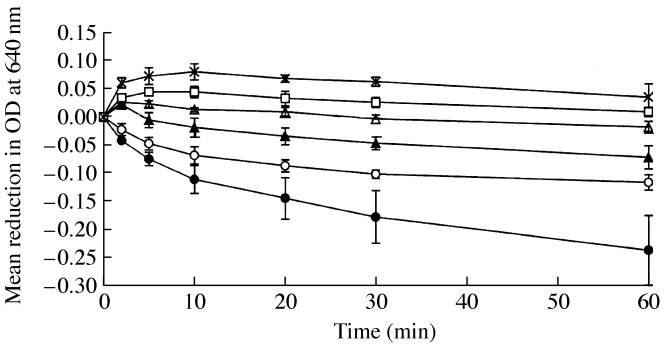

Figure 3. Effect of CHA on *M. chelonae* spheroplasts in stabilizing buffer. White circles, control buffer; black circles, water; concentration (mg/L) of CHA: black triangles, 25; white triangles, 50; squares, 100; crosses, 500. Each plot indicates the mean ± S.D. of three different independent experiments.

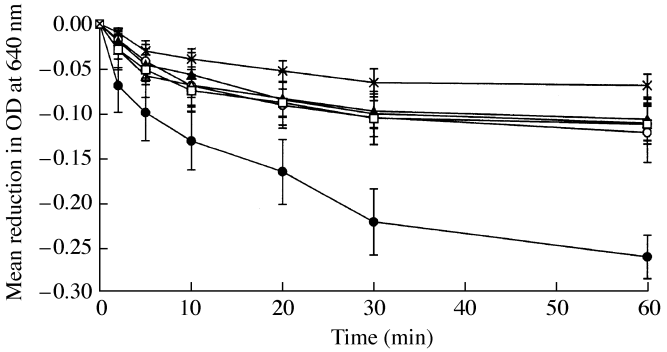

Figure 4. Effect of CHA on *M. chelonae* Harefield spheroplasts in stabilizing buffer. White circles, control buffer; black circles, water; concentration (mg/L) of CHA: black triangles, 25; white triangles, 50; squares, 100; crosses, 500. Each plot indicates the mean ± S.D. of three different independent experiments.

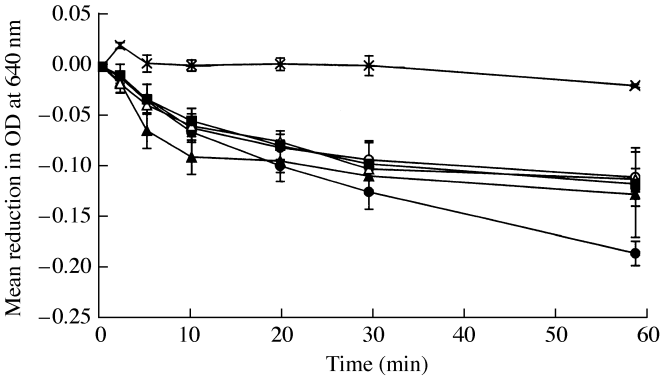

Figure 5. Effect of OPA on *M. chelonae* spheroplasts in stabilizing buffer. White circles, control buffer; black circles, water; concentration (mg/L) of OPA: black triangles, 0.01; white triangles, 0.05; squares, 0.1; crosses, 0.5. Each plot indicates the mean ± S.D. of three different independent experiments.

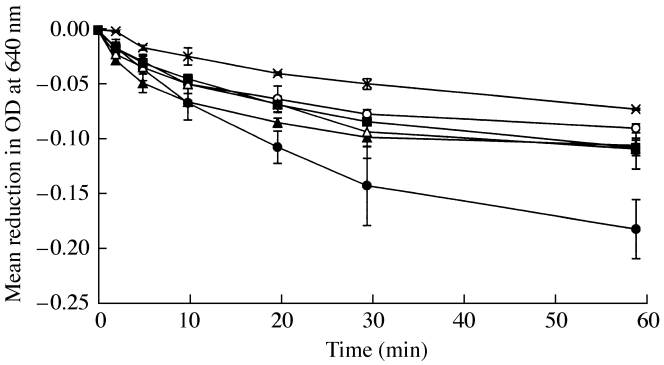

Figure 6. Effect of GTA on *M. chelonae* spheroplasts in stabilizing buffer. White circles, control buffer; black circles, water; concentration (mg/L) of GTA: black triangles, 0.01; white triangles, 0.05; squares, 0.1; crosses, 0.5. Each plot indicates the mean ± S.D. of three different independent experiments.

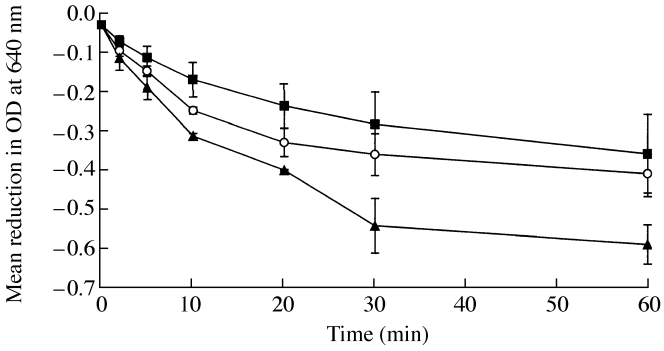

Figure 7. Effect of pre-treatment of *M. chelonae* spheroplasts for 30 min at 22°C with OPA (0.1%) or GTA (0.1%) before dilution in water. Circles, untreated (control); triangles, pre-treated with OPA; squares, pre-treated with GTA. Each plot indicates the mean ± S.D. of three different independent experiments.

nae NCTC 946, whereas at a higher concentration (0.5%), OPA induced a significant increase in OD in comparison with the rate of lysis of spheroplasts in the stabilizing buffer (Figure 5). Treatment of *M. chelonae* NCTC 946 spheroplasts with GTA solutions produced similar results to that of OPA, although when the highest concentration of GTA (0.5% v/v) was used, the increase in OD was less marked (Figure 6). Similar results were obtained with *M. abscessus* NCTC 10882 (data not shown).

Stability of OPA- and GTA-pre-treated spheroplasts

Pre-treatment of *M. chelonae* NCTC 946 spheroplasts with 0.1% GTA significantly inhibited lysis when cells were subsequently transferred to deionized water (Figure 7). Conversely, pre-treatment with OPA did not stabilize spheroplasts, but increased their susceptibility to lysis induced by water (Figure 7). The rate of lysis of untreated spheroplasts in water was greater in these experiments than in the previous ones. This sensitization could be due to the extra centrifugation stage needed after the 30 min treatment (in this case, suspension in sucrose buffer). GTA-treated spheroplasts of *M. chelonae* Harefield were stabilized by 0.1% GTA and exhibited a low rate of lysis in water compared with the untreated control (Figure 8). Again, pre-treatment with OPA did not protect spheroplasts from lysis once they were transferred to water. However, pre-treated spheroplasts showed a rate of lysis of the same magnitude as the untreated spheroplasts. Similar results to that of *M. chelonae* Harefield were obtained with *M. abscessus* NCTC 10882 (not shown). As a result of the subsequent agglutination following treatment with OPA or GTA, *M. chelonae* Epping spheroplasts could not be included in this set of experiments.

Discussion

Spheroplast formation and cell wall changes

Bacterial spheroplasts provide a useful tool for studying the mechanisms of action of biocides. The method of spheroplast preparation from mycobacteria described earlier by Rastogi & David[18] was effective for the four fast-growing strains investigated, the cell population consisting of >90% spherical forms as judged by TEM. The morphology of the spheroplasts was in agreement with earlier results.[22] Untreated bacilli had a well-defined cell envelope of ~20 nm thickness. Damage inflicted to the cell envelope was significant, but some cell wall components remained in modified cell forms, leading to their characterization as spheroplasts. Spheroplasts lacked an OL but the PG layer was still visible although this altered layer alone was not sufficient to maintain the rigidity of the cell wall.

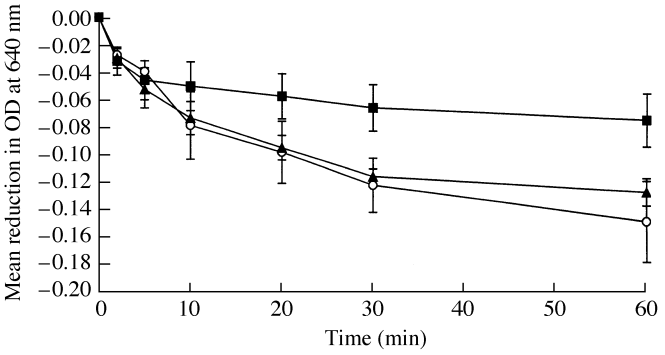

Figure 8. Effect of pre-treatment of *M. chelonae* Harefield spheroplasts for 30 min at 22°C with OPA (0.1%) or GTA (0.1%) before dilution in water. Circles, untreated (control); triangles, pre-treated with OPA; squares, pre-treated with GTA. Each plot indicates the mean ± S.D. of three different independent experiments.

Changes in cell wall structure could also be linked to a decrease in free-extractable lipids. Of the two reference strains, *M. chelonae* NCTC 946 was more sensitive to the spheroplasting process. Spheroplast formation was completed in 22 h and resulted in a decrease in free lipids of 79% and 61% for *M. chelonae* NCTC 946 and *M. abscessus* NCTC 10882, respectively. Conversely, the two GTA-resistant strains were less susceptible to the spheroplast formation treatment, requiring a period of 40 h. This resistance to the spheroplasting process was confirmed by the cellular content of free lipids, which was less dramatically reduced. In agreement with other studies, the loss of cell wall was associated with alterations in the lipid composition.[23,24] The mixture of lysozyme, D-cycloserine and glycine used for spheroplast preparation acts specifically at the level of bacterial PG biosynthesis and represents the foundation layer of the cell wall. Alterations to PG may lead to reduced linkages with mycobacterial AG, which may in turn cause the release of mycolic acid residues. Consequently, the release of mycolic acid loosely associated with the insoluble matrix esterified to a variety of carbohydrate-containing molecules, free lipids and the bacterial OL (which are otherwise non-covalently linked to the arabinogalactan-mycolate through lipid–lipid interactions in the cell wall) will also take place. The PG of *M. chelonae* Epping and *M. chelonae* Harefield may be more resistant to lysozyme or the PG target site less exposed, thus inducing a smaller loss of total cell wall lipids than with the reference strains. Nevertheless, the content of free lipids extracted in all four strains using this method was above expected theoretical values. According to a previous study,[25] the mycolic acid content of *Mycobacterium smegmatis* ATCC 607 is ~180 mg/g dry weight of cells. Major disorganizations and alterations of the cell envelope, especially in lipid composition, and the detachment of the PG layer from the cytoplasmic membrane increase the mycobacterial cell permeability to antimicrobial substances.[13,26–29]

Effects of CHA

In sensitive bacteria, CHA collapses the membrane potential and the membrane disruption plays a role in its lethal effect.[12] Damage to the CM is followed by leakage of intracellular constituents although higher concentrations of CHA cause coagulation of cytoplasmic protein, enzymes and nucleic acids.[30] Protein coagulation or precipitation, which changes the refractive index of the microbial surface and cell constituents, will increase the OD of cell suspensions.[31] Increases in OD were observed when *M. chelonae* NCTC 946 spheroplasts were suspended in CHA, with the highest concentration giving the maximum increase in OD. These results indicate that intracellular sealing occurred in CHA solutions ranging from 25 to 500 mg/L. The MICs of CHA for mycobacteria range from 0.5 to 1 mg/L, with 50 mg/L for a more resistant strain, *M. avium* 3906 (laboratory isolate). These low concentrations of CHA are mycobacteriostatic at concentrations of the same order as those that inhibit other bacteria (i.e. *Staphylococcus aureus*) but are not mycobactericidal,[32] with no reduction in colony forming units after 60 min exposure to 100 mg/L CHA using a quantitative suspension test.[33] In another study, 40 000 mg/L CHA produced no more than a 2 $\log_{10}$ reduction in viability against *M. tuberculosis* H37Rv.[34] Cytosol coagulation in bacteria entails irreversible cellular damage, consequently this effect should undoubtedly be correlated with cell death. Low, non-mycobactericidal concentrations of CHA (25–100 mg/L) induced protein precipitation in *M. chelonae* NCTC 946 spheroplasts but not in the other strains. In contrast, concentrations of CHA up to 150 mg/L caused lysis but no precipitation of the proteins and nucleic acids from *Escherichia coli*.[30] The significant cell wall alterations (highlighted by the EM analysis and the dramatic loss of lipids) observed in *M. chelonae* NCTC 946 spheroplasts seem to be responsible for the increased cellular permeability of this strain to CHA. However, a higher concentration (500 mg/L) was needed to produce a similar effect in *M. chelonae* strains Epping and Harefield, ostensibly because the permeability barrier had been altered to a lesser extent. These findings support the hypothesis that the low fluidity and the hydrophobic nature of the mycobacterial cell wall limit permeability of CHA to the cytoplasmic membrane and thereafter into the cell cytoplasm.[10,13]

Effects of GTA and OPA

A concentration (0.5% w/v, 37 mM) slightly below the hospital in-use concentration of OPA (0.55% w/v, 40.7 mM) produced significant protein coagulation in *M. chelonae* NCTC 946 spheroplasts. In comparison, 0.5% v/v GTA (50 mM) induced a similar but less marked effect on the latter organism. The lipophilic nature of the molecule will play a key role in diffusion through hydrophobic domains such as biological membranes.[6] OPA, with its lipophilic nature (log P_{oct} = +1.53,

determined by Crippen's fragmentation using CS ChemDraw Ultra software),[35] penetrated the permeability barriers more efficiently than GTA, which is fairly hydrophilic (log P_{oct} = –0.75), inducing more cytoplasm sealing. Concentrations <0.5% of both dialdehydes caused no protein coagulation. It is pertinent to note that the free form of chlorhexidine, which is much more hydrophobic (log P_{oct} = +6.18) than OPA, induced coagulation in *M. chelonae* NCTC 946 spheroplasts at much lower concentrations (25–500 mg/L, corresponding to 0.04–0.8 mM CHA). Such low concentrations of CHA do not induce protein coagulation in whole mycobacterial cells, otherwise this agent would be mycobactericidal.

Mycobacterial spheroplasts pre-treated with GTA, but not with OPA, showed a significant ability to withstand osmotic shock-induced cell lysis. The protective or stabilizing effect observed with GTA is a typical consequence of extensive cross-linking between the aldehyde groups of the biocide and ε-NH_2 and/or -SH side-chain groups of proteins, glycopeptides and certain lipids such as sulpholipids present in the residual mycobacterial envelope. The spheroplasts are strengthened and are thereby protected from lysis. Differences observed between osmotic shock-induced lysis between GTA-pre-treated resistant and sensitive strains might be explained by the residual components associated with the cell wall of spheroplasts of the resistant strain after the spheroplasting process, thus allowing an enhanced GTA cross-linking. Pre-treatment of cells with OPA and GTA on lysis induced by EDTA–Tris pH 9, sodium lauryl sulphate and lysostaphin on whole cells of *Pseudomonas aeruginosa*, *E. coli* and *S. aureus*, respectively, demonstrated that GTA was a better cross-linking agent than OPA, although the latter did protect against lysis to a certain extent.[36] GTA is able to join together two contiguous side-chains within the PG in the case where both tripeptides carry free ε-NH_2 groups (i.e. DL-α,ε-diaminopimelic acid in mycobacteria). The distance between contiguous peptides along a single polysaccharide backbone would, at maximum, be the length of the disaccharide unit, which is ~10.3 Å.[37] The cross-linking reaction with GTA would allow a cross-bridge considerably longer than 10 Å, due to the presence of ~85% of long α,β-unsaturated aldehyde polymers at 25°C and alkaline pH,[38] but the most simple cross-bridge, containing a five-carbon chain, would fit neatly the distance between adjacent peptide side-chains. From a consideration of bond angles and bond distances of the two aldehydic functions in OPA, the spatial disposition and proximal distance between the residues containing ε-NH_2 and/or -SH groups involved in the cross-linking must be ~3 Å.[39] Thus, cross-linking of PG by OPA would be physically impossible. The reason as to why OPA partially protects whole bacterial cells (other than mycobacteria) but not mycobacterial spheroplasts from lysis remains unclear. The residual mycobacterial wall possessed by spheroplasts contains much less protein or structures containing side-chains that can react with the aldehydes than those in organisms such as *P. aeruginosa* or *E. coli*. Thus, the PG may remain a main target for cross-linking in the spheroplasts.

Mycobactericidal action of OPA and GTA

It is conceivable that OPA and GTA achieve a mycobactericidal effect by different mechanisms. GTA is a more effective cross-linking agent and its own uptake may be decreased by virtue of its extensive cross-linking at the bacterial cell surface. GTA thus inactivates cells at a slower rate than OPA of the same percentage concentration.[17] The lipophilic OPA molecule may readily penetrate the lipid-rich mycobacterial cell wall, and because of the lack of extensive cross-linking in the outer layers or within the PG, the effective dialdehyde concentration will not be reduced. Thus, sufficient OPA molecules will be able to reach critical target sites in the periplasmic space, the cytoplasmic membrane and/or cytoplasm.

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