Adhesion of the ulcerative pathogen *Mycobacterium ulcerans* to DACC-coated dressings

• **Objective:** *Mycobacterium ulcerans* is the causative agent of Buruli ulcer disease, the third most common mycobacteriosis after tuberculosis and leprosy and an emerging public health threat in sub-Saharan Africa. The bacteria produce a diffusible cytotoxin called mycolactone, which triggers the formation of necrotic lesions in cutaneous and subcutaneous tissues. The principal aim of this study was to characterise the cell surface hydrophobicity of *Mycobacterium ulcerans* and determine if bacteria bind to dialkyl carbamoyl chloride (DACC)-coated dressings through hydrophobic interactions *in vitro*. Since mycolactone displays hydrophobic groups, a secondary aim was to compare mycolactone binding to hydrophobic and standard dressings.

• **Method:** We used hydrophobic interaction chromatography to evaluate the cell surface hydrophobicity of *Mycobacterium ulcerans*, compared to that of other microorganisms colonising wounds. The binding of *Mycobacterium ulcerans* bacteria to DACC-coated and control dressings was then assessed quantitatively by measurement of microbial adenosine triphosphate (ATP), while that of mycolactone was evaluated by fluorescence spectroscopy.

• **Results:** Compared to *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, *Mycobacterium ulcerans* displayed the highest cell surface hydrophobicity, irrespective of the bacterial production of mycolactone. *Mycobacterium ulcerans* bacteria bound DACC-coated dressings were better than untreated controls. Mycolactone did not bind stably to hydrophobic, nor standard dressings, in the conditions tested.

• **Conclusion:** Retention of *Mycobacterium ulcerans* and other wound pathogens to DACC-coated dressings may help reduce the bacterial load in Buruli ulcers and thereby improve healing. Dressings efficiently capturing mycolactone may bring an additional clinical benefit, by accelerating the elimination of the toxin during the course of antibiotic treatment.

• **Declaration of interest:** The authors have no conflict of interest to declare.

wound healing; dressing; hydrophobic interaction; *Mycobacterium ulcerans*; mycolactone.

Mycobacterium ulcerans is a toxin-producing *Mycobacterium* belonging to the same family as *Mycobacterium tuberculosis* and *Mycobacterium leprae*,[1] which causes Buruli ulcer disease. It is an environmental pathogen that has been isolated from biofilms and aquatic animals of stagnant waters.[2-4] How *Mycobacterium ulcerans* is transmitted to humans nevertheless remains unclear.[5] A short stay in an endemic area is sufficient for contracting the disease.[6,7] However, human-to-human transmission is extremely rare. Based on epidemiological studies, polymerase chain reaction (PCR)-based detection of *Mycobacterium ulcerans* DNA and experimental data, transmission hypotheses involve biting insects and/or direct contact with an environmental source.[8] Today, Buruli ulcer is reported in over 30 tropical and subtropical countries, and constitutes a major public health concern in sub-Saharan Africa.[9] Over 1000 cases of Buruli ulcer are reported annually in Ghana.[10] If not diagnosed or treated appropriately, they can result in irreversible deformity, functional disability and life-threatening secondary infections.

Buruli ulcer, also known as Bairnsdale ulcer, Daintree ulcer, Mossman ulcer and Searl ulcer, is a chronic, necrotising disease of the skin and soft tissue.[11,12] Following an incubation period of 1–9 months,[13] lesions begin as a dermal papule, subcutaneous nodule or plaque (Fig 1). Ulceration occurs within 1–2 months, leading to massive tissue loss and destruction of nerves and blood vessels. Characteristic lesions have undermined edges and a sloughing necrotic base. They are generally painless unless complicated by secondary infections. *Mycobacterium ulcerans* multiplies in cutaneous and subcutaneous tissues within biofilms serving as a reservoir for mycolactone,[14] a diffusible cytotoxin with ulcerative and immunosuppressive properties.[1,15-17] Elimination of *Mycobacterium ulcerans* from infected

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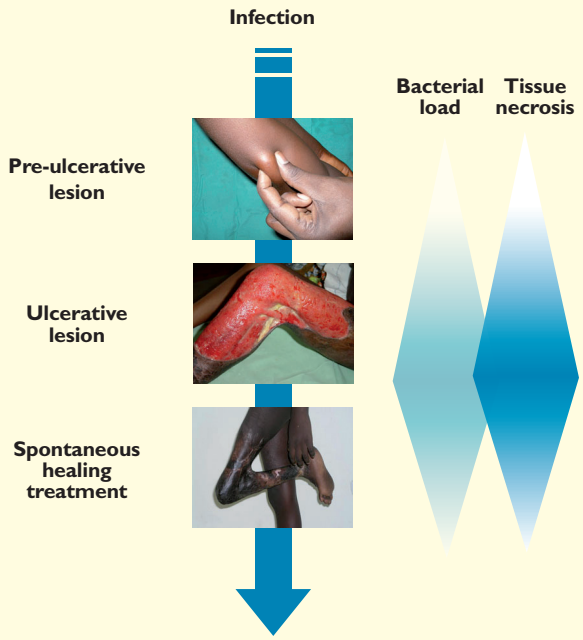
Fig. 1. Buruli ulcer disease progression. The typical presentation of Buruli ulcers, from the pre-ulcerative to the ulcerative and healing stages is shown.[10] The width of the pale and dark blue shapes denotes the extent of bacterial load and tissue necrosis, respectively



wounds requires systemic antibiotics daily for eight weeks.[18-20]. While this treatment is efficient at killing bacteria, mycolactone persists in ulcer exudates several weeks after completion of antibiotic therapy. [21] The mean time to Buruli ulcer healing is 20 weeks,[22] however, some patients develop paradoxical reactions during antibiotic therapy leading to clinical deterioration slowing down the healing process.[23] Since more than 60% of Buruli ulcer lesions are infected with *Staphylococcus aureus* and *Pseudomonas aeruginosa*,[24] secondary infections may also impair wound resolution. Finally, the recent observation that intra-lesional concentrations of mycolactone predict the time to healing of small nodules and ulcers strongly suggests that toxin persistence delays the resolution of Buruli ulcers.[25]

Staphylococcus aureus and *Pseudomonas aeruginosa* have been shown previously to bind to dialkyl carbamoyl chloride (DACC)-coated dressings (Sorbact, Abigo Medical AB).[26] These dressings use the basic physicochemical principle of hydrophobic interaction with the bacterial cell surface, a property that is expected to remove microbes expressing cell surface hydrophobicity from wounds. Since all members of the *Mycobacterium* genus display a lipid-rich, hydrophobic outer membrane—which provide bacteria with enhanced adhesive properties and resistance to unfavourable environmental conditions[27] — we postulated that *Mycobacterium ulcerans* may bind such hydrophobic dressings. In line with this hypothesis, previous studies have reported the hydrophobicity of the *Mycobacterium ulcerans* cell surface, as measured by bacterial adherence to hexadecane.[28] The aim of the present work was to further characterise the cell surface hydrophobicity of *Mycobacterium ulcerans* and determine if it confers bacteria with the capacity to bind DACC-coated dressings better than untreated controls. Since mycolactone is structured into a lactone core to which hydrophobic fatty acyl side-chains are appended,[29] we also examined the differential binding of this toxin to DACC-coated and control dressings.

Methods

Mycobacterium ulcerans (MU1615 strain, ATCC 35840), originally isolated from a Malaysian patient, was obtained from the Trudeau Collection.[15] An avirulent mycolactone-negative mutant with transposon insertion in the fabH gene (MU mut) was kindly provided by Prof. Pamela Small (University of Tennessee, Knoxville, USA). *Staphylococcus aureus* (HG001 strain), *Escherichia coli* (HB101 and BL21 strains) and *Pseudomonas aeruginosa* (PAO1 strain) were included as comparatives. Bacteria recovered from exponentially growing cultures were pelleted and re-suspended in phosphate-buffered saline (PBS), before cell dissociation on a gentleMAC apparatus (Miltenyi Biotec). Bacterial concentrations were estimated by absorbance measurement at 600nm using an Ultrospec 10 cell Density Meter (GE Healthcare Life Science), and cell viability assessed a posteriori by titration on agar plates and determination of colony-forming units.

Mycolactone preparation and binding assay

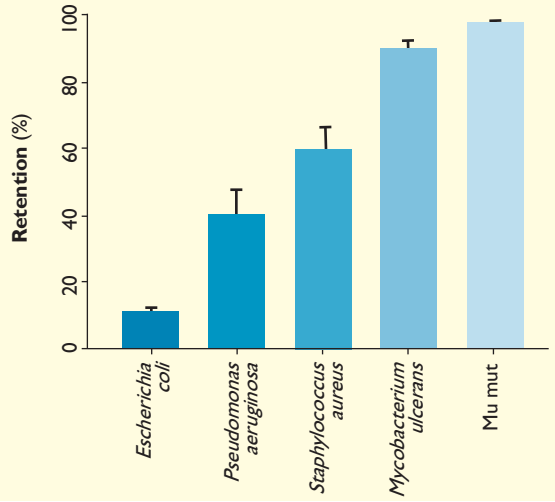
Mycolactone was purified from *in vitro* grown cultures of *Mycobacterium ulcerans*, a process described elsewhere.[15,30] Stock solutions were prepared in ethanol. We took advantage of the natural fluorescence of mycolactone to monitor its capacity to bind dressings. [30] Briefly, aliquots of purified mycolactone were pipetted onto pieces of dressings placed onto a glass slide, left to evaporate then washed with PBS. The fluorescent signature of mycolactone (Excitation 365nm/Emission 520nm) was then measured with ImageQuant LAS 4000 mini (GE Healthcare Life Science), and quantified with the ImageJ software (National Institutes of Health, Bethesda).

Hydrophobic interaction chromatography

Chromatography columns were prepared by adding 1ml Octyl-Sepharose beads (Sigma-Aldrich) to Poly-Prep chromatography columns (BioRad Laboratories). Before use, the columns were washed twice with 5ml of phosphate buffered saline (PBS). Dissociated bacterial cell suspensions (0.1ml, 10^9 bacteria/ml)

Fig 2. Cell surface hydrophobicity of *Mycobacterium ulcerans*, compared to that of other bacteria colonising wounds.

The percentage of bacteria remaining on Octyl-Sepharose column after PBS wash is shown for various strains, as a measure of bacterial cell surface hydrophobicity. Mu mut=mycolactone-deficient mutant.



were added to the column and eluted with PBS (3ml). The fraction of eluted bacteria was determined spectrophotometrically. Data are expressed as mean percentages +/- standard deviation (SD) of bacteria retained on the column, measured on triplicates.

Adenosine triphosphate assay

The amount of bacteria adhering to control or DACC-coated dressings was determined by measurement of microbial adenosine triphosphate (ATP), using the ATP Biomass Kit HS (BioThema). Briefly, fresh suspensions of *Mycobacterium ulcerans* or *Staphylococcus aureus* bacteria were prepared in PBS and kept on ice for no longer than 1 hour before the assay was performed. Aliquots of bacterial cell suspensions (10^8 bacteria/ml, 200µl) were incubated with 0.2cm² punched samples of dressings for 20 hours at 32°C. Dressings were then rinsed three times by quick vortexing in 1ml PBS then placed into 40µl PBS. Following addition of the kit extractant buffer (40µl), bacterial lysates were transferred into a white 96-well F-Bottom plate (Greiner Bio-One). Light emission was measured with a luminometer FLUOStar OPTIMA (BGM Labtech). Data are expressed as mean concentrations of ATP +/-SD, measured on over 10 replicates.

Results

The cell surface hydrophobicity of *Mycobacterium ulcerans* was investigated by hydrophobic interaction chromatography, a separation technique based on the interaction of cell wall components with hydrophobic ligands bound to a chromatography matrix. In the present study, we used octyl groups coupled to sepharose beads. Dispersed suspensions of bacteria were prepared in PBS and then loaded onto the chromatography columns. The proportion of bacteria remaining attached to the beads after washing of the column with PBS was estimated by

Fig 3. Enhanced binding of *Mycobacterium ulcerans* to dialkyl carbamoyl chloride (DACC) dressings.

The adhesion of *Mycobacterium ulcerans* and *Staphylococcus aureus* to DACC-coated or control dressings is shown, as determined by quantitative measurement of bacterial ATP. Data are presented as Box and Whiskers, with outlier cut-off determined by Tukey's test, and comparison with the non-parametric Wilcoxon ranksum (Mann-Whitney), *p<0.01, **p<0.005.

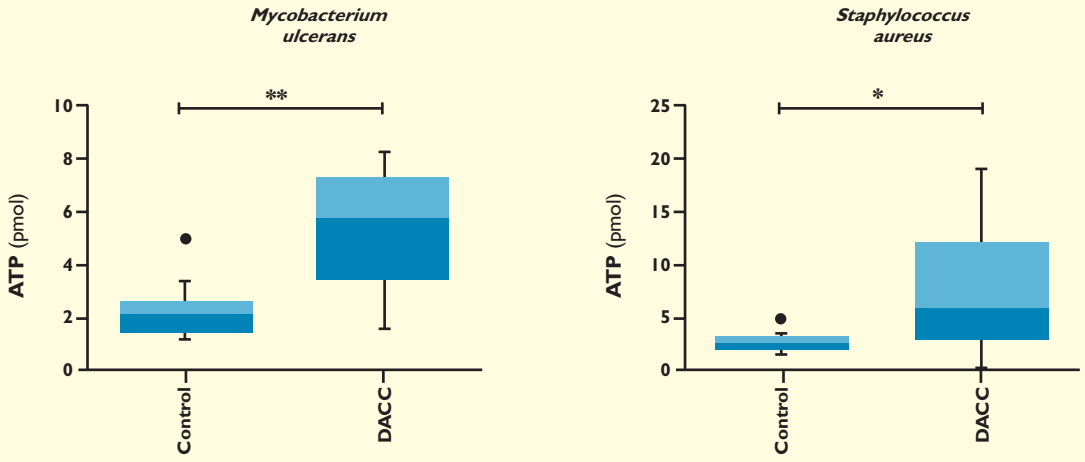
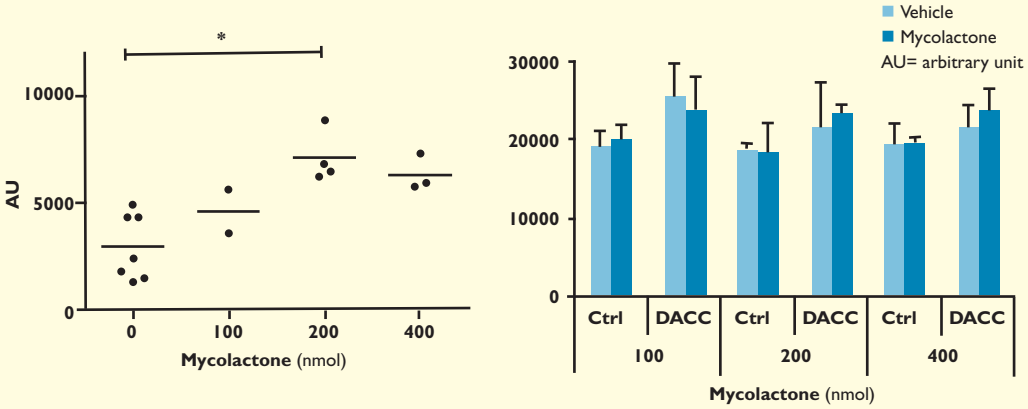


Fig 4. Mycolactone binding to DACC-coated and control dressings. Left: Differential detection of mycolactone on DACC-coated vs control dressings, following loading of ethanolic solutions and evaporation. Data were compared by ANOVA, followed by Dunn's multiple comparison test (p<0.05*). Right: Fluorescent signal of mycolactone or ethanol vehicle, following loading of ethanolic solutions onto control (Ctrl) or DACC-coated dressings, evaporation and three cycles of PBS wash.



quantifying the bacterial concentration in the flow-through. Consistent with previous reports,[26] *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* displayed significant cell surface hydrophobicity, evidenced by retention of 10%, 40% and 60% bacteria respectively. Compared with these strains, *Mycobacterium ulcerans* showed the highest cell surface hydrophobicity, with over 88% of bacteria remaining on the columns (Fig 2). Of note, the cell surface hydrophobicity of a mycolactone-deficient mutant (MU mut) was comparable to that of the wild-type strain, suggesting that mycolactone expression does not confer additional hydrophobicity to the bacteria.

Having shown that *Mycobacterium ulcerans* display a high cell surface hydrophobicity, we examined its capacity to adhere to hydrophobic dressings. Dispersed suspensions of *Mycobacterium ulcerans*, or *Staphylococcus aureus* as a control, were incubated with punched samples of Sorbact or control dressing for 20 hours at 32°C. These conditions were chosen to mimic the contact between bacteria and dressings in clinical setting. Dressings were then subjected to three cycles of PBS wash, to remove loosely adherent bacteria. Then, the amount of bacteria firmly attached to the dressings was determined by quantitative measurement of ATP in cell lysates, as previously described.[31] Similarly to *Staphylococcus aureus*, *Mycobacterium ulcerans* bound DACC-coated dressings significantly better than untreated controls (Fig 3).

Since mycolactone is key to Buruli ulcer pathogenesis,[12,15,17] we also investigated the possible binding of this cytotoxin to DACC-coated dressings. Mycolactone was purified from *Mycobacterium ulcerans* bacteria by means of organic solvent extraction followed by thin layer chromatography as described,[15] then dissolved in ethanol. Serial dilutions of this solution were then added to punched samples of DACC-coated or control dressings, then left to evaporate. In both types of dressings, a dose-dependent signal corresponding to the fluorescent signature of mycolactone[30] could be detected in the 0-400nmol range (unshown data). Notably, mycolactone bound DACC-coated dressings comparably or better than controls (Fig 4, left), suggesting that the hydrophobic coating of dressings may improve their capacity to bind mycolactone in these conditions. However, when submitted to subsequent cycles of PBS wash, mycolactone binding to the dressings (both DACC-coated and controls) was lost (Fig 4, right), indicative of weak interactions.

Discussion

The principle of DACC-coated dressings is to use hydrophobic interactions to bind and remove bacteria from wounds.[26] The present study was performed to determine if this original antimicrobial action may be of benefit in Buruli ulcer management. We found that the surface of *Mycobacterium ulcerans* is highly hydrophobic compared to that of other bacteria, and that *Mycobacterium ulcerans* binds to DACC-coated dressings better than untreated controls. Secondary infections of Buruli ulcer lesions have recently been shown to be common with organisms such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* before treatment, *Pseudomonas aeruginosa* during treatment and *Pseudomonas aeruginosa* or *Proteus miriabilis* after treatment.[24] Notably, 78% of patients with Buruli ulcers in Ghana and Benin use antibiotics other than the standard combination streptomycin and

rifampicin,[32] and *Staphylococcus aureus* in Buruli ulcer lesions showed a high frequency of resistance to the first-line drugs used in Ghana as well as the presence of MRSA. *Staphylococcus aureus* binds DACC-coated dressings regardless of the antibiotic resistance properties of the strain (Ronner et al, In Press). Our observation that *Mycobacterium ulcerans* and *Staphylococcus aureus* bind Sorbact thus suggests that these pathogens would be efficiently removed by such hydrophobic dressings.

Mycolactone binding to DACC-coated dressings did not resist PBS wash, indicative of weak interactions. Mycolactone was recently detected in mouse and human tissue infected with *Mycobacterium ulcerans* by assays of cytotoxicity and mass spectrometry. [25,33,34] Although current methods of investigation do not allow the accurate quantification of mycolactone in biological samples, mycolactone was shown to persist in ulcers several weeks after the end of antibiotic treatment. Given the pivotal role wwplayed by mycolactone in the pathogenesis of Buruli ulcers, dressings that efficiently capture mycolactone may help to improve its elimination from the wounds and shorten the current eight-week daily treatment with streptomycin and rifampicin.[22,35]

Conclusions

Topical agents used in the past with the intention to kill *Mycobacterium ulcerans* included nitric oxide-releasing creams,[36] application of French clay minerals,[37] or localised application of heat. Attempts to stimulate fibrogenesis with phenytoin were also reported.[38] To our knowledge, none of these strategies are yet to be translated into clinical practice. Currently, Buruli ulcer treatment clinics use vaseline, saline and iodine-based dressings. Buruli ulcer lesions less than 15cm in maximum diameter can take 52 weeks to heal,[22] illustrating the need to improve wound management. A suitable wound dressing in resource-poor rural settings should be cheap, easy to apply, and not cumber-

References

1. Demangel, C., Stinear, T.P., Cole, S.T. Buruli ulcer: reductive evolution enhances pathogenicity of *Mycobacterium ulcerans*. Nat Rev Microbiol 2009; 7: 1, 50-60.
2. Eddyani, M., Ofori-Adjei, D., Teugels, G. et al. Potential role for fish in transmission of Mycobactetium *ulcerans* disease (Buruli ulcer): an environmental study. Appl Environ Microb 2004; 70: 9, 5679-5681.
3. Marsollier, L., Severin, T., Aubry, J. et al. Aquatic snails, passive hosts of *Mycobacterium ulcerans*. Appl Environ Microbiol 2004; 70: 10, 6296-6298.
4. Marsollier, L., Stinear, T., Aubry, . et al. Aquatic plants stimulate the growth of and biofilm formation by *Mycobacterium ulcerans* in axenic culture and harbor these bacteria in the environment. Appl Environ Microbiol 2004; 70: 2, 1097-1103.
5. Jacobsen, K.H., Padgett, J.J. Risk factors for *Mycobacterium ulcerans* infection. Int J Infect Dis 2010; 14: 8, e677-681.
6. Lavender, C.J., Fyfe, J.A., Azuolas, J. et al. Risk of Buruli ulcer and detection of *Mycobacterium ulcerans* in mosquitoes in southeastern Australia. PLoS Negl Trop Dis 2011; 5: 9, e1305.
7. McGann, H., Stragier, P., Portaels, F. et al. Buruli ulcer in United Kingdom tourist returning from Latin America. Emerg Infect Dis 2009; 15: 11, 1827-1829.
8. Merritt, R.W., Walker, E.D., Small, P.L. et al. Ecology and transmission of Buruli ulcer disease: a systematic review. PLoS Negl Trop Dis 2010; 4: 12, e911.
9. Asiedu, K., Wansbrough-Jones, M. *Mycobacterium Mycobacterium ulcerans* infection (Buruli or Bairnsdale ulcer): challenges in developing management strategies. Med J Aust 2007; 186: 2, 55-56.
10. World Health Organization (2014) Buruli Ulcer. WHO: http://www.who.int/buruli/en/ [accessed 14 July 2014]
11. Chany, A.C., Tresse, C., Casarotto, V., Blanchard, N. History, biology and chemistry of *Mycobacterium ulcerans* infections (Buruli ulcer disease). Nat Prod Rep 2013; 30: 12, 1527-1567.
12. Guenin-Mace, L., Oldenburg, R., Chretien, F., Demangel, C. Pathogenesis of skin ulcers: lessons from the *Mycobacterium ulcerans* and Leishmania spp. pathogens. Cell Mol Life Sci 2014; 71: 13, 2443-2450.
13. Trubiano, J.A., Lavender, C.J., Fyfe, J.A., Bittmann, S., Johnson, P.D. The incubation period of Buruli ulcer (*Mycobacterium ulcerans* infection). PLoS Negl Trop Dis 2013; 7: 10, e2463.
14. Marsollier, L., Brodin, P., Jackson, M. et al. Impact of *Mycobacterium ulcerans* biofilm on transmissibility to ecological niches and Buruli ulcer pathogenesis. PLoS Pathog 2007; 3: 5, e62.
15. George, K.M., Chatterjee, D., Gunawardana, G. et al. Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science 1999; 283: 5403, 854-857.
16. Guenin-Mace, L., Simeone, R., Demangel, C. Lipids of Pathogenic Mycobacteria: Contributions to Virulence and Host Immune Suppression. Transbound Emerg Dis 2009; 56: 6-7, 255-268.
17. Guenin-Mace, L., Veyron-Churlet, R., Thoulouze, M.I., et al. Mycolactone activation of Wiskott-Aldrich syndrome proteins underpins Buruli ulcer formation. J Clin Invest 2013; 123: 4,1501-1512.
18. Etuaful, S., Carbonnelle, B., Grosset, J. et al. Efficacy of the combination rifampin-streptomycin in preventing growth of *Mycobacterium ulcerans* in early lesions of Buruli ulcer in humans. Antimicrob Agents Chemother 2005; 49: 8, 3182-3186.
19. Ji, B.H., Lefrancois, S., Robert, J., Chauffour, A., Truffot, C., Jarlier, V. *In vitro* and *in vivo* activities of rifampin, streptomycin, amikacin, moxifloxacin, R207910, linezolid, and PA-824 against *Mycobacterium ulcerans*. Antimicrob Agents Ch 2006; 50: 6, 1921-1926.
20. Johnson, P.D. Should antibiotics be given for Buruli ulcer? Lancet 2010; 375: 9715, 618-619.
21. Sarfo, F.S., Le Chevalier, F., Aka, N. et al. Mycolactone diffuses into the peripheral blood of buruli ulcer patients - implications for diagnosis and disease monitoring. PLoS Negl Trop Dis 2011; 5: 7, e1237.
22. Phillips, R.O., Sarfo, F.S., Abass, M.K. et al. Clinical and Bacteriological Efficacy of Rifampin-Streptomycin Combination for Two Weeks followed by Rifampin and Clarithromycin for Six Weeks for Treatment of *Mycobacterium ulcerans* Disease. Antimicrob Agents Chemother 2014; 58: 2, 1161-1166.
23. Nienhuis, W.A., Stienstra, Y., Abass, K.M. et al. Paradoxical responses after start of antimicrobial treatment in *Mycobacterium ulcerans* infection. Clin Infect Dis 2012; 54: 4, 519-526.
24. Yeboah-Manu, D., Kpeli, G.S., Ruf, M.T. et al. Secondary bacterial infections of buruli ulcer lesions before and after chemotherapy with streptomycin and rifampicin. PLoS Negl Trop Dis 2013; 7: 5, e2191.
25. Sarfo, F.S., Phillips, R.O., Zhang J. et al. Kinetics of mycolactone in human subcutaneous tissue during antibiotic therapy for *Mycobacterium ulcerans* disease. Bmc Infect Dis 2014; 14: 202.
26. Ljungh, A., Yanagisawa, N., Wadstrom, T. Using the principle of hydrophobic interaction to bind and remove wound bacteria. J Wound Care 2006; 15: 4, 175-180.
27. Brennan, P.J., Nikaido, H.. The Envelope of Mycobacteria. Annual Review of Biochemistry 1995; 64: 29-63.
28. Deshayes, C., Angala, S.K., Marion, E. et al. Regulation of mycolactone, the *Mycobacterium ulcerans* toxin, depends on nutrient source. PLoS Negl Trop Dis 2013; 7: 11, e2502.
29. Hong, H., Demangel, C., Pidot,

some. Capillary dressings may have a place, particularly in the management of exuding Buruli ulcers.[39] The present study suggests that hydrophobic dressings may have a positive impact on the healing process by reducing the wound bioburden. Both capillary and hydrophobic dressings are more expensive than standard compresses. Clinical studies will be required to determine if they confer clinical improvement. Should a clinical benefit be seen, cost-effectiveness may not only derive from a decreased healing time, but also from decreased need for antibiotics, higher skin graft success rate, less demand for and/or incidence of extensive debridement or limb amputations. In sub-Saharan Africa, the socioeconomic costs associated with Buruli ulcers are extensive.[40-43] In addition to the high cost of treatment (up to 89% of a work-year per patient in Ghana in 2001), the fear of disfigurement and amputation often prevent people from seeking surgical treatment. Long hospital stays also represent a huge loss in productivity for adult patients and family caregivers, and loss of educational opportunities for children.[10] All of these parameters should also be taken into account in future cost–benefit analyses. ■

S.J., Leadlay, P.F., Stinear, T. Mycolactones: immunosuppressive and cytotoxic polyketides produced by aquatic mycobacteria. Nat Prod Rep 2008: 25: 3, 447-454.

30. Spangenberg, T., Kishi, Y. Highly sensitive, operationally simple, cost/time effective detection of the mycolactones from the human pathogen *Mycobacterium ulcerans*. Chem Commun 2010; 46: 9, 1410-1412.

31. Hoffner, S., Jimenez-Misas, C., Lundin, A. Improved extraction and assay of mycobacterial ATP for rapid drug susceptibility testing. Luminescence 1999; 14: 5, 255-261.

32. Barogui, Y.T., Klis, S., Bankole, H.S. et al. Towards rational use of antibiotics for suspected secondary infections in Buruli ulcer patients. PLoS Negl Trop Dis 2013; 7:1, e2010.

33. Sarfo, F.S., Phillips, R.O., Rangers, B. et al. Detection of Mycolactone A/B in *Mycobacterium ulcerans*-Infected Human Tissue. Plos Neglect Trop D 2010; 4: 1: e577

34. Converse, P.J., Xing, Y., Kim, K.H. et al. Accelerated Detection of Mycolactone Production and Response to Antibiotic Treatment in a Mouse Model of *Mycobacterium ulcerans* Disease. PLoS Negl Trop Dis 2014; 8: 1, e2618.

35. Sarfo, F.S., Phillips, R., Asiedu, K. et al. Clinical Efficacy of Combination of Rifampin and Streptomycin for Treatment of *Mycobacterium ulcerans* Disease. Antimicrob Agents Ch 2010; 54: 9, 3678-3685.

36. Phillips, R., Adjei. O., Lucas, S., Benjamin, N., Wansbrough-Jones, M. *Mycobacterium* Pilot randomized double-blind trial of treatment of *Mycobacterium ulcerans* disease (Buruli ulcer) with topical nitrogen oxides. Antimicrob Agents Chemother 2004; 48: 8, 2866-2870.

37. Williams, L.B., Metge, D.W., Eberl, D.D. et al. What makes a natural clay antibacterial? Environ Sci Technol 2011; 45(8): 3768-3773.

38. Adjei, O., Evans, M.R., Asiedu, A. Phenytoin in the treatment of Buruli ulcer. Trans R Soc Trop Med Hyg 1998; 92: 1, 108-109.

39. Reynolds, T., Russell, L., Deeth, M. et al. Randomised controlled trial comparing Drawtex with standard dressings for exuding wounds. J Wound Care 2004; 13: 2, 71-74.

40. Amoakoh, H.B., Aikins, M. *Mycobacterium* Household cost of out-patient treatment of Buruli ulcer in Ghana: a case study of Obom in Ga South Municipality. BMC health services research 2013; 13: 507.

41. Asiedu, K. Etuaful Staphylococcus Socioeconomic implications of Buruli ulcer in Ghana: a three-year review. Am J Trop Med Hyg 1998; 59: 6, 1015-1022.

42. Grietens, K.P., Boock, A.U., Peeters, H. et al. *Mycobacterium* "It is me who endures but my family that suffers": social isolation as a consequence of the household cost burden of Buruli ulcer free of charge hospital treatment. PLoS Negl Trop Dis 2008; 2: 10, e321.

43. Aujoulat, I., Johnson, C., Zinsou, C. et al. Psychosocial aspects of health seeking behaviours of patients with Buruli ulcer in southern Benin. Trop Med Int Health 2003; 8: 8, 750-759.