Low concentrations of commercial alcohol hand rubs facilitate growth of and secretion of extracellular proteins by multidrug-resistant strains of *Acinetobacter baumannii*

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Acinetobacter baumannii is increasingly recognized as an important multidrug-resistant nosocomial pathogen. Recent work has highlighted enhanced growth and heightened virulence in the presence of ethyl alcohols. As alcohol-based hand rubs (ABHRs) are extensively used in health care settings, the authors set out to determine whether the hand rubs could also influence the growth of prevalent multidrug-resistant strains circulating in UK hospitals. A significant increase in growth was observed when minimal media were supplemented with concentrations of 1 % and lower of four commercially available hand rubs. In addition, growth in ABHR-supplemented media resulted in secretion of proteins into the culture supernatant. One of these was identified as *OmpA*, which is recognized as having emulsifying activity, which could potentially confer enhanced pathogenicity to *A. baumannii*.

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INTRODUCTION

Acinetobacter baumannii is a non-fermentative Gram-negative rod of the family *Moraxellaceae*. The organism is an opportunistic pathogen and has emerged in recent years as an important cause of ventilator-associated pneumonia, bacteraemia and sepsis in patients with burns, immunosuppression and critical illness (Joly-Guillou, 2005). Many strains also exhibit formidable antimicrobial resistance, leading to serious concerns over our ability to treat and control infection with this organism. Most of the multidrug-resistant strains circulating in UK hospitals belong to three distinct clonal groups with common PFGE profiles, namely OXA-23 clone-1, OXA-23 clone-2 and the South-East clone (Coelho *et al.*, 2006).

A. baumannii demonstrates a remarkable ability to persist in the hospital environment, with bed linen, bedside cabinets, telephone receivers, computer keyboards, sink taps, patient charts and ventilator housings all identified as environmental reservoirs (Bergogne-Berezin & Towner, 1996). The main mechanism for transmission, however, remains spread via the hands of hospital staff. Adequate hand hygiene is therefore thought to be crucial in

preventing the nosocomial transmission of *A. baumannii* (D'Agata *et al.*, 2000). In many hospitals, alcohol-based hand rubs (ABHRs), consisting of varying concentrations of ethyl or isopropyl alcohol often in combination with chlorhexidine and organic emollients, have found widespread acceptance as an alternative to handwashing (Kampf & Kramer, 2004). Recent work has shown that *A. baumannii* is able to readily metabolize low concentrations of ethyl alcohols, and, furthermore, that this leads to both enhanced growth and pathogenicity via the secretion of an unidentified substance (Smith *et al.*, 2004). This observation is intriguing, as *A. baumannii* is likely to be frequently exposed to alcohol in hospital environments where a 'background level' of alcohol may exist due to the extensive use of ABHRs. Eradication of *A. baumannii* once established has been shown to be particularly difficult. In view of the versatile metabolic capacity of *Acinetobacter* species it is possible that traces of alcohol or other organic compounds in the environment could facilitate their growth and survival, perhaps even heightening their pathogenicity to humans. We therefore set out to determine whether low concentrations of commercially available ABHRs could affect the growth of prevalent strains of multidrug-resistant *A. baumannii* (MDRAB) and also to identify any secreted proteins which could represent virulence factors.

Abbreviations: ABHRs, alcohol-based hand rubs; MDRAB, multidrug-resistant *A. baumannii*.

METHODS

Bacterial strains and media. Three multidrug-resistant strains of *A. baumannii* belonging to the South East, OXA-23 clone-1 and OXA-23 clone-2 PFGE groups were used throughout this study. All three isolates were resistant to aminoglycosides and third-generation cephalosporins (MDRAB) and both of the OXA-23 strains were also resistant to carbapenems (MDRAB-C). Strains were grown in either the enriched medium YPAD containing 1 % yeast extract, 2 % Bacto peptone, 2 % glucose and 300 μ M adenine or in M9 Minimal Media (MM) consisting of 1 $\times$ M9 salts (0.6 % NaHPO₄, 0.3 % KH₂PO₄, 0.05 % NaCl, 0.01 % NH₄Cl) supplemented with 0.1 mM CaCl₂, 0.002 M MgSO₄ and 0.4 % glucose.

Growth analyses. Stationary phase cultures were made by incubation of each strain overnight at 37 °C in 5 ml YPAD. One hundred microlitres of this culture was used to inoculate 10 ml of both YPAD and MM with the addition of 0.1–10 % (v/v) of either 100 % ethanol or the commercially available ABHRs Skinman, Softalind, Spirigel and Purell. The cultures were reincubated and growth was quantified spectrophotometrically by recording the OD₆₀₀ at set time intervals. All readings were blanked versus uninoculated media to account for any turbidity resulting from the addition of ethanol or ABHR to the media. Measurements were taken approximately every hour for 6–8 h in experiments conducted using YPAD with final measurements taken overnight. In experiments performed using MM, measurements were taken twice daily for 48 h. Incubation of each strain was also performed, at the same concentrations, following supplementation of the media with the non-alcohol-based hand rub Clinell.

Viable counts. To confirm that any observed increase in OD₆₀₀ was due to an increase in the number of viable bacteria, the number of c.f.u. ml⁻¹ was determined as follows. For each time point, serial dilutions of 100 μ l of culture were made down to a concentration of 10⁻⁹. Counts were performed following overnight incubation of 100 μ l aliquots of each dilution spread onto LB agar plates.

Analysis of extracellular protein secretion. Proteins secreted following growth in MM with and without ABHR supplementation were prepared as follows. Cells from 1.5 ml of stationary phase culture were pelleted by centrifugation at 16 000 g for 4 min. Supernatant (1 ml) was transferred to a fresh tube and proteins were precipitated by addition of 350 μ l 50 % (w/v) trichloroacetic acid. After incubation on ice for 30 min, precipitated protein was collected by centrifugation at 16 000 g for 10 min. Pellets were washed with 1 ml acetone and resuspended in 15 μ l NuPAGE lithium dodecyl sulphate sample buffer. SDS-PAGE analysis was carried out using XCell SureLock Mini-Cell (Invitrogen) apparatus. Samples were heated at 70 °C for 10 min before loading onto NuPAGE Novex 4–12 % (v/v) Pre-Cast Bis-Tris gels and electrophoresed at 200 V for 35–50 min in NuPAGE MOPS SDS Running Buffer. Gels were removed from the apparatus, washed three times with 100 ml deionized H₂O and then stained for 1 h with 20 ml SimplyBlue SafeStain (Invitrogen). Clear backgrounds for photography were obtained by further washing with deionized H₂O for 1–3 h. Molecular mass was determined by comparison with prestained standards (New England Biolabs).

Proteomics analysis. Protein bands labelled 1 and 2 (Fig. 4) were excised from the gels, destained, reduced and alkylated prior to in-gel digestion with trypsin. Briefly, the gel pieces were destained with 50 mM ammonium bicarbonate/50 % acetonitrile, reduced with dithiothreitol (10 mM in 100 mM ammonium bicarbonate for 30 min) and alkylated with iodoacetamide (55 mM in 100 mM ammonium bicarbonate for 20 min). Following a wash step with 100 mM ammonium bicarbonate and dehydration with acetonitrile,

the gel pieces were digested with 25 μ l 6 ng trypsin μ l⁻¹ in 50 mM ammonium bicarbonate for 5 h at 40 °C. The tryptic peptide fragments were extracted twice: firstly with 2 % acetonitrile/1 % formic acid for 30 min and then with 50 % acetonitrile/50 % aqueous 1 % formic acid for another 30 min. All treatments were performed robotically using a MassPrep Workstation (Micromass). All extracts from a given gel piece were combined, reduced to 1–2 μ l in a Speed-Vac concentrator and reconstituted in 0.1 % formic acid for mass spectral analysis by LC-MS/MS using the CapLC/Q-ToF2 (Micromass). Samples were transferred to the autosampler of a CapLC and eluted through a reverse-phase capillary column directly into the nano-electrospray ion source of a Q-ToF2 mass spectrometer. The instrument was operated in survey scanmode, with the range *m/z* 400–1800. All fragment ion (MS/MS) spectra from the sample were searched using the MASCOT search tool (Matrix Science) using the MS protein sequence database to obtain protein identification. MASCOT scores only for tryptic peptide fragments that were above the significance level indicating identity or extensive homology were reported.

RESULTS

Effect of ethanol and ABHR supplementation on the growth of MDRAB

All three strains of *A. baumannii* were able to grow in YPAD supplemented with ethanol at 1 % or less. Growth in YPAD supplemented with ethanol at 10 % was not supported. There was no significant difference in growth rate between supplemented and unsupplemented YPAD media. Supplementation at 0.1 % or less did, however, result in growth to a higher final OD₆₀₀ for all strains (Fig. 1).

When strains were grown in MM supplemented with 1 % ethanol or less, both a significantly faster initial rate of growth and growth to a higher final OD₆₀₀ were observed, with 1 % ethanol being the optimal concentration. All strains failed to grow in MM + 10 % ethanol (Fig. 2).

Fig. 1. Growth of MDRAB in enriched media following supplementation with 0.01–10 % ethanol.

Fig. 2. Growth of MDRAB in minimal media following supplementation with 0.01–10 % ethanol.

Growth of all strains in YPAD media supplemented with 0.1 % (v/v) of both Softalind and Skinman ABHRs resulted in a small but insignificant rise in OD₆₀₀. When MM was used as the growth medium, growth enhancement was observed for all strains following supplementation with 1 %, 0.1 % and 0.01 % of each ABHR. Optimal growth occurred at 1 % supplementation although growth was initially faster at 0.1 %. Strains were unable to grow at 10 % supplementation or when supplemented with Clinell. The same pattern was seen in all strains when incubated with all four ABHRs (Fig. 3). Increases in OD₆₀₀ were also found to correlate with an increase in the number of viable bacteria, as measured by colony counts, regardless of the growth media used or supplementation with ethanol or ABHRs.

Effect of ABHRs on secretion of extracellular proteins

Proteins present in culture supernatants of all three strains of MDRAB grown in MM supplemented with 1 % of each ABHR were examined by SDS-PAGE. A protein of between 17 and 20 kDa was detected in experiments performed

Fig. 4. Secretion of extracellular proteins by MDRAB following growth in MM supplemented with ABHRs. Lanes: 1, MM alone; 2, MM + 1 % Softalind; 3, MM alone; 4, MM + 1 % Skinman. Protein bands labelled 1 and 2 were excised from the gel and examined by Q-ToF MS/MS analysis.

using OXA-23 clone-1 and OXA-23 clone 2 whilst a protein of approximately 33 kDa was produced by the South-East clone (Fig. 4)

Identification of secreted proteins by MS

Bands labelled 1 and 2 (Fig. 4), containing the proteins, were cut from the gel and subjected to proteomics analysis for protein identification. Tryptic peptide fragments were analysed by a Q-TOF2 mass spectrometer and the mass spectral data were used to search protein databases by the

Fig. 3. Growth of MDRAB in minimal media supplemented with 1 % ABHR. All three strains of MDRAB grew to a higher OD₆₀₀ after 48 h following supplementation with 1 % Softalind, Skinman, Purell or Spirigel ABHRs ($P < 0.05$, paired Student's *t*-test).

Table 1. Identification of secreted proteins from MDRAB following growth in minimal media supplemented with 1 % ABHR

Protein no.	Identification	MASCOT score	Peptides matched/ protein coverage	Theoretical molecular mass	Actual molecular mass
1	Hypothetical protein from <i>Acinetobacter</i> ADP-1	117	1/7 %	26.1 kDa	22 kDa
2	Outer-membrane protein A – <i>A. baumannii</i>	922	14/44 %	38.4 kDa	34 kDa

MASCOT search tool. Based on a high probability MOWSE score, the protein secreted by the South-East clone was identified as outer-membrane protein A (OmpA) from *A. baumannii* and the proteins secreted by OXA-23 clones 1 and 2 were identified as a hypothetical protein from *A. baumannii* ADP-1. The number of tryptic peptide fragments matched, together with protein coverage and putative MOWSE scores by MASCOT, are summarized in Table 1. There was good correlation between the theoretical and actual masses of the proteins identified. Although the MOWSE score for band 1 was low, this reflects poor quality data rather than an incorrect match. The high score for band 2 indicates a highly significant hit for the protein.

DISCUSSION

Previous work has demonstrated that low concentrations of ethanol enhance both the growth and virulence of *Acinetobacter* spp. The results of our study indicate that the growth of multidrug-resistant strains of *A. baumannii* is also enhanced when exposed not only to ethanol but also to ABHRs. This phenomenon is most apparent when MDRAB is incubated in a minimal growth medium as, presumably, the addition of ethanol or ABHR provides an additional carbon source which is readily utilized. All four ABHRs tested, Skinman, Softalind, Spirigel and Purell, were able to significantly enhance MDRAB growth at 1 % or less. In NHS hospitals, these rubs are either wall-mounted or placed next to a patient's bedside. The solutions are usually pump-dispensed directly onto the hands, which may result in considerable spillage and contamination of the surrounding environment. In contrast to the ABHRs tested, the non-alcohol-based product Clinell, which relies on the biocidal activity of two cationic surfactants, did not support the growth of MDRAB at all. If low levels of ABHRs do indeed exist in clinical areas where they are intensively used, this could affect the survival and persistence of MDRAB.

Our results also indicate that there are distinct differences in the extracellular protein profiles when MDRAB is grown in MM or MM supplemented with ABHRs. Exposure of the OXA-23 clones 1 and 2 to ABHRs resulted in the secretion of a protein whose function is yet to be identified; however, the identification of OmpA as the protein in the supernatant of the South-East clone is particularly interesting. OmpA possesses emulsifying activity, which

could be useful in scavenging carbon for growth from complex hydrocarbon energy sources. The secretion of OmpA by *Acinetobacter* following exposure to 0.5 % ethanol in minimal media has also been described by Walzer *et al.* (2006), suggesting that it may be an important response to growth and survival under low-nutrient conditions. As well as conveying a growth advantage, bioemulsifiers may also play an important role in bacterial adhesion, quorum sensing and the development of biofilms (Ron & Rosenberg, 2001). Direct evidence for a role of *A. baumannii* outer-membrane proteins in pathogenesis comes from work by Choi *et al.* (2005), whereby purified outer-membrane protein 38 (Omp38) was able to induce apoptosis in respiratory epithelial cells. The highly clonal, hospital-acquired, multidrug-resistant strains of *A. baumannii* circulating in the UK appear to be less virulent than drug-sensitive community strains (Leung *et al.*, 2006). Therefore, any enhanced effect on virulence due to ABHR exposure could have major clinical implications.

This study presents some interesting results which could have significant implications for infection control practices in those hospitals experiencing outbreaks of MDRAB. Concerns over the use of ABHRs have already been expressed with respect to their lack of activity against the spores that potentiate *Clostridium difficile* infection. In light of this study, the widespread use of ABHRs, in areas where *A. baumannii* infection is seen as a particular concern, may also need to be reassessed. Further epidemiological studies are needed to assess the impact of widespread use of ABHRs for the control of MDRAB.

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