The newly discovered ClpK protein strongly promotes survival of *Klebsiella pneumoniae* biofilm subjected to heat shock

Klebsiella pneumoniae is an important nosocomial pathogen causing urinary tract, respiratory and blood infections (Podschun & Ullmann, 1998). Immunocompromised individuals such as intensive care unit (ICU) patients and people suffering from severe underlying diseases are frequently susceptible to infection (Sahly *et al.*, 2000). Thus, it is estimated that *K. pneumoniae* is second only to *Escherichia coli* as a cause of Gram-negative nosocomial sepsis (Sahly & Podschun, 1997), and regarding bacteraemic cases within ICUs *K. pneumoniae* has been identified as the most common Gram-negative agent in recent studies (Ben Jaballah *et al.*, 2007; Couto *et al.*, 2007; Michalopoulos *et al.*, 2011). As a ubiquitous organism, *K. pneumoniae* is a normal resident of the human gastrointestinal tract and nosocomial transmission from this reservoir is considered to occur mainly by personal contact and through inappropriately decontaminated hospital equipment (Montgomerie, 1979).

In hospitals, and in ICUs in particular, diagnostic and medical treatment procedures involving endoscopes require strict infection control measures since these devices are subject to repeated contact with mucosal surfaces of the patients. Reusable flexible endoscopes cannot be steam sterilized due to their material composition, and are generally reprocessed by cleaning, high-level disinfection and drying. High-level disinfection is often accomplished by a thermochemical treatment below 60 °C in washer-disinfectors. Reports of transmission and outbreaks of infectious agents being linked to endoscopes are numerous; e.g. *K. pneumoniae* outbreak due to an infected bronchoscope (Cetre *et al.*, 2003), outbreak of multidrug-resistant (extended-spectrum β -lactamase) *K. pneumoniae* linked to a duodenal scope used for endoscopic retrograde cholangiopancreatography (Aumeran

et al., 2010) and endoscope-mediated transmission of a carbapenem-resistant isolate belonging to the internationally successful *K. pneumoniae* clone ST258 (Naas *et al.*, 2010).

Bacterial biofilms are typically quite resistant to adverse environmental conditions imposing chemical and physical stresses (Costerton *et al.*, 1987). Studies have shown that patient-ready endoscopes may be persistently contaminated despite adherence to recommended decontamination procedures (Bisset *et al.*, 2006) and confirm the presence of bacterial biofilm on endoscopes by scanning electron microscopy (Pajkos *et al.*, 2004), suggesting biofilm formation as a contributor to failure of pathogen elimination from scopes. We recently reported on an endemic *K. pneumoniae* strain (C132-98) persisting at an ICU which was eliminated concomitantly with an increased focus on endoscope reprocessing procedures. A pronounced heat resistance phenotype of planktonic cultures of C132-98, which was suggested to facilitate its nosocomial persistence, was shown to be mediated by a plasmid-encoded heat resistance locus encompassing a gene encoding a novel ClpK protein (Bojer *et al.*, 2010). Approximately 1/3 of a collection of nosocomial *K. pneumoniae* isolates tested positive for the *clpK* gene and a correlation to a heat resistance phenotype of these isolates was established (Bojer *et al.*, 2010). Since it is known that *K. pneumoniae* biofilm cells can be more resistant to ethanol disinfection (Chambers *et al.*, 2006) as well as heat inactivation and some disinfectants (Faille *et al.*, 2003; LeChevallier *et al.*, 1988), we sought to assess a possible clinical significance of this heat resistance locus by determining whether ClpK confers thermoprotection of *K. pneumoniae* biofilm-stage cells.

Determination of viability pre- and post-heat shock was performed and compared

in biofilm cells of wild-type (WT) *K. pneumoniae* C132-98 and its isogenic $\Delta clpK$ deletion mutant and $\Delta clpK$ mutant cells complemented by *clpK* (pClpK) or the complete heat resistance locus encompassing *clpK* (pMB58-sub) constructed previously (Bojer *et al.*, 2010). In essence, individual biofilms were allowed to develop on Lab-Tek Permanox Chamber Slides (Nunc) for 48 h by 1/100 dilution of overnight cultures in M9 medium (Sigma) supplemented with 0.02 M glucose/0.002 M MgSO₄ and incubation at 37 °C and 150 r.p.m. Planktonic and loosely adherent cells were removed by washing cover slides three times in PBS after which cover slides were placed in 50 ml PBS in plastic tubes. Tubes for heat shock were pre-heated and maintained at 58 °C for 5 min followed by cooling in an ice-water bath. Untreated and heat-shocked biofilms were displaced from cover slides by addition of 0.1 % Triton X-100 and applying two rounds of 10 min in a Branson ultrasonic cleaner (B-2200) separated by vigorous vortexing, and quantified as c.f.u. cm⁻² by plating. Survival rates based on three independent experiments were analysed (1-way ANOVA; GraphPad Prism) following log-transformation of data to normalize the variance. Biofilm removal from cover slides was assessed qualitatively by traditional crystal violet staining.

Quantification of bacterial biofilms showed that *K. pneumoniae* C132-98 produces an extensive biofilm ($\sim 1.5 \times 10^7$ c.f.u. cm⁻²). Also, the *clpK* deletion mutant and its complementation derivatives showed comparable amounts of biofilm (Fig. 1b). However, the survival rate following heat shock was strongly influenced by ClpK. While WT C132-98 biofilm remained practically unaffected (~ 70 % survival) by heat shock at 58 °C for 5 min, the C132-98 $\Delta clpK$ mutant biofilm was significantly impaired (~ 0.02 % survival) (Fig. 1c). The observed >1000 -fold reduction in heat resistance of mutant

cells was restored by complementation. Full complementation necessitates complementation with adjacently located genes (pMB58-sub) suggesting a polar effect from *clpK* deletion as indicated for planktonically grown cells also (Bojer *et al.*, 2010). The relevance of the *clpK* gene alone in the heat resistance of *K. pneumoniae* biofilm is, however, evident from the significant protection (>100-fold improved survival) provided by pClpK (Fig. 1c).

Since biofilms are considered to constitute a significant proportion of the bacterial biomass clinically as well as environmentally, assessment of bacterial physiological responses should include biofilm-embedded cells. The sessile cells of bacterial biofilms are by definition physically different in organization to their planktonic counterparts and are also considered to be physiologically distinct (Donlan & Costerton, 2002). Indeed, we observed that *K. pneumoniae* C132-98

biofilm cells remained viable in greater proportions than planktonic cells; i.e. the c.f.u. of planktonic cultures were generally reduced to approximately 20% following heat shock at 58 °C for 5 min (compared to the ~70% survival of biofilm-embedded cells reported herein). Even at 60 °C heat shock, biofilm cells were only reduced approximately twofold in viability after 5 min whereas planktonic cells were practically eliminated. Thus, any effect by the ClpK heat resistance determinant might be masked by an intrinsic resistance of *K. pneumoniae* biofilm. This report does, however, show that ClpK significantly contributes to the survival of heat-shocked biofilm. The repeated reprocessing of medical devices may lead to build-up of resistant biofilms (Alfa & Howie, 2009) and endoscopic procedures can be independent risk factors for acquisition of nosocomial pneumonia and bloodstream infections (Mastropiero *et al.*, 2009; von Baum *et al.*, 2005). Also, reprocessing temperatures in endoscope

washer–disinfectors have been shown to be critical for minimizing the microbial load (Zühlendorf *et al.*, 2003). Altogether, the strong thermoprotection provided by ClpK may play a role in nosocomial persistence of certain *K. pneumoniae* strains. Elimination of *K. pneumoniae* C132-98 did coincide with increased focus on reprocessing procedures supporting this hypothesis. The possible clinical significance of this notion is underlined by a recent report on a multi-hospital outbreak of KPC-2-producing ST258 *K. pneumoniae* partially explained by endoscope transmission (Carbonne *et al.*, 2010) as well as the aforementioned cases of nosocomial infection and transmission being linked to contaminated endoscopes.

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Fig. 1. (a) Plasmid-encoded heat resistance locus in *K. pneumoniae* C132-98 and subcloned (pACYC184) fragments covered by pClpK and pMB58-sub. (b) Amount of biofilm formed by WT C132-98, its isogenic Δ clpK mutant, and the C132-98 Δ clpK mutant complemented by either pClpK or pMB58-sub. (c) Survival of biofilm cells following 5 min of heat shock at 58 °C (NS, no significant difference; * P <0.05; *** P <0.001).

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- Alfa, M. J. & Howie, R. (2009).** Modeling microbial survival in buildup biofilm for complex medical devices. *BMC Infect Dis* **9**, 56.
- Aumeran, C., Poincloux, L., Souweine, B., Robin, F., Laurichesse, H., Baud, O., Bommelaer, G. & Traoré, O. (2010).** Multidrug-resistant *Klebsiella pneumoniae* outbreak after endoscopic retrograde cholangiopancreatography. *Endoscopy* **42**, 895–899.
- Ben Jaballah, N., Bouziri, A., Mnif, K., Hamdi, A., Khaldi, A. & Kchaou, W. (2007).** Epidemiology of hospital-acquired bloodstream infections in a Tunisian pediatric intensive care unit: a 2-year prospective study. *Am J Infect Control* **35**, 613–618.
- Bisset, L., Cossart, Y. E., Selby, W., West, R., Catterson, D., O'Hara, K. & Vickery, K. (2006).** A prospective study of the efficacy of routine decontamination for gastrointestinal endoscopes and the risk factors for failure. *Am J Infect Control* **34**, 274–280.
- Bojer, M. S., Struve, C., Ingmer, H., Hansen, D. S. & Krogfelt, K. A. (2010).** Heat resistance mediated by a new plasmid encoded Clp ATPase, ClpK, as a possible novel mechanism for nosocomial persistence of *Klebsiella pneumoniae*. *PLoS ONE* **5**, e15467.
- Carbonne, A., Thiolet, J., Fournier, S., Fortineau, N., Kassis-Chikhani, N., Boytchev, I., Aggoune, M., Segulier, J., Senechal, H. & other authors (2010).** Control of a multi-hospital outbreak of KPC-producing *Klebsiella pneumoniae* type 2 in France, September to October 2009. *Euro Surveill* **15**, pii:19734.
- Cetre, J. C., Salord, H. & Vanhems, P. (2003).** Outbreaks of infection associated with bronchoscopes. *N Engl J Med* **348**, 2039–2040.
- Chambers, S. T., Peddie, B. & Pithie, A. (2006).** Ethanol disinfection of plastic-adherent microorganisms. *J Hosp Infect* **63**, 193–196.
- Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M. & Marrie, T. J. (1987).** Bacterial biofilms in nature and disease. *Annu Rev Microbiol* **41**, 435–464.
- Couto, R. C., Carvalho, E. A., Pedrosa, T. M., Pedroso, E. R., Neto, M. C. & Biscione, F. M. (2007).** A 10-year prospective surveillance of nosocomial infections in neonatal intensive care units. *Am J Infect Control* **35**, 183–189.
- Donlan, R. M. & Costerton, J. W. (2002).** Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **15**, 167–193.
- Faile, C., Fontaine, F., Lelièvre, C. & Bénézech, T. (2003).** Adhesion of *Escherichia coli*, *Citrobacter freundii* and *Klebsiella pneumoniae* isolated from milk: consequence on the efficiency of sanitation procedures. *Water Sci Technol* **47**, 225–231.
- LeChevallier, M. W., Cawthon, C. D. & Lee, R. G. (1988).** Factors promoting survival of bacteria in chlorinated water supplies. *Appl Environ Microbiol* **54**, 649–654.
- Mastropiero, R., Bettinzoli, M., Bordonali, T., Patroni, A., Barni, C. & Manzato, A. (2009).** Pneumonia in a cardiothoracic intensive care unit: incidence and risk factors. *J Cardiothorac Vasc Anesth* **23**, 780–788.
- Michalopoulos, A., Falagas, M. E., Karatza, D. C., Alexandropoulou, P., Papadakis, E., Gregorakos, L., Chalevelakis, G. & Pappas, G. (2011).** Epidemiologic, clinical characteristics, and risk factors for adverse outcome in multiresistant gram-negative primary bacteremia of critically ill patients. *Am J Infect Control* **39**, 396–400.
- Montgomerie, J. Z. (1979).** Epidemiology of *Klebsiella* and hospital-associated infections. *Rev Infect Dis* **1**, 736–753.
- Naas, T., Cuzon, G., Babics, A., Fortineau, N., Boytchev, I., Gayral, F. & Nordmann, P. (2010).** Endoscopy-associated transmission of carbapenem-resistant *Klebsiella pneumoniae* producing KPC-2 beta-lactamase. *J Antimicrob Chemother* **65**, 1305–1306.
- Pajkos, A., Vickery, K. & Cossart, Y. (2004).** Is biofilm accumulation on endoscope tubing a contributor to the failure of cleaning and decontamination? *J Hosp Infect* **58**, 224–229.
- Podschun, R. & Ullmann, U. (1998).** *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* **11**, 589–603.
- Sahly, H. & Podschun, R. (1997).** Clinical, bacteriological, and serological aspects of *Klebsiella* infections and their spondylarthropathic sequelae. *Clin Diagn Lab Immunol* **4**, 393–399.
- Sahly, H., Podschun, R. & Ullmann, U. (2000).** *Klebsiella* infections in the immunocompromised host. *Adv Exp Med Biol* **479**, 237–249.
- von Baum, H., Ober, J. F., Wendt, C., Wenzel, R. P. & Edmond, M. B. (2005).** Antibiotic-resistant bloodstream infections in hospitalized patients: specific risk factors in a high-risk population? *Infection* **33**, 320–326.
- Zühlsdorf, B., Winkler, A., Dietze, B., Floss, H. & Martiny, H. (2003).** Gastroscope processing in washer-disinfectors at three different temperatures. *J Hosp Infect* **55**, 276–282.