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 ΔclpK deletion mutant and ΔclpK 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 (~1.5 × 10⁷ 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 C132-98ΔclpK 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 cf.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 (Mastropierro 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 (Zühlsdorf 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 ΔclpK mutant, and the C132-98 ΔclpK 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).


