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SHORT COMMUNICATION

Rapid inactivation of *Cronobacter sakazakii* on copper alloys following periods of desiccation stress

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Abstract *Cronobacter* spp. have been identified as the causative agent in meningitis and necrotizing enterocolitis in premature infants which can be linked to the bacterium's desiccation resistance and persistence in powdered infant formula. In this study we examined the efficacy of copper cast alloys in contact killing of *Cronobacter sakazakii* following periods of desiccation stress. *Cronobacter sakazakii* cells suspended in Tryptic Soy Broth (TSB) were killed within 10 min while kept moist on 99.9% copper alloys and within 1 min of drying on 99.9% copper alloys. Survival times were unchanged after cells suspended in TSB were desiccated for 33 days. *Cronobacter sakazakii* cells suspended in infant formula were killed within 30 min under moist conditions and within 3 min of drying on 99.9% copper alloys. However, when desiccated in infant formula for 45 days, survival times decreased to 10 and 1 min in moist and dry conditions, respectively. In contrast, no decrease in viable cells was noted on stainless steel surfaces under the experimental conditions employed in this study. *Cronobacter sakazakii* was rapidly killed on copper alloys under all testing conditions of this study indicating that desiccation and copper ion resistance do not prolong survival. These results could have important implications for the utilization of copper in the production and storage of powdered infant formula.

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Introduction

Neonatal meningitis and necrotizing enterocolitis are complications in newborns with mortality as high as 80% in low birth weight infants. Outbreaks in neonatal intensive care units (NICUs) over the past couple of decades have been associated with *Cronobacter* spp., formerly *Enterobacter sakazakii* (Iversen et al. 2008), isolated from infant formulas utilized in infant feedings (Simmons et al. 1989; Gurtler et al. 2005; Bowen and Braden 2006). Worldwide testing of infant formulas has confirmed that *Cronobacter* survive in powdered infant formula, powdered follow up formula, and other infant foods (Chap et al. 2009). Kucerova et al. (2010) sequenced the genome of *C. sakazakii* ATCC BAA-894 isolated from powdered formula used in a NICU disease outbreak. The genome revealed genes associated with invasion of brain microvascular endothelial cells, such as the *cusCFBA* and *cusR,* genes which are identified in *Escherichia coli* as encoding an RND-type copper efflux system (Rensing and Grass 2003; Kim et al. 2011). These genes were present in several *C. sakazakii* strains isolated from cases of neonatal infections (Kucerova et al. 2010). The reported persistence of *C. sakazakii* in milk based powdered infant formula is presumably due to increased resistance to desiccation which may be variable between strains (Caubilla-Barron and Forsythe 2007; Osaili and Forsythe 2009). Studies have shown that *C. sakazakii* reconstitution after desiccation results in rapid growth in nutrient medium (Osaili and Forsythe 2009). *Cronobacter sakazakii* has also been isolated from biofilms growing in infant feeding tubes

(Hurrell et al. 2009a). Hurrell et al. (2009b) investigated *C. sakazakii* biofilm development on materials utilized in feeding tubes and did not find significant differences in biofilm formation between polyvinyl chloride, polyurethane, and silver-impregnated flexilene. A study by Al-Holy et al. (2010) revealed a 1 to 2 logfold decrease in survival of *E. sakazakii* when 50–100 μg/ml copper sulfate was added to prepared infant formula. The combination of 0.2% lactic acid and 100 μg/ml copper sulfate completely inhibited growth of *E. sakazakii* after 2 h with the testing methods reported. Copper alloys have been studied extensively for their antimicrobial efficacy against antibiotic- and copper ion-resistant bacteria (Noyce et al. 2006; Elguindi et al. 2011), and mechanisms of killing have been identified (Grass et al. 2011; Espirito Santo et al. 2011). The ability to form capsules for increased desiccation resistance, increased resistance mechanisms to copper and silver, and the ability to survive in higher levels of copper, for example in brain microvascular epithelial tissue and macrophages (Townsend et al. 2007, 2008), may enhance survival of *Cronobacter* in dry milk based foods and consequently lead to more disease outbreaks. Thus, the main objectives of this study were to demonstrate the efficacy of copper alloys in contact killing of copper ion-resistant *Cronobacter* and to introduce the concept of utilizing copper materials in the production and storage of powdered infant formula.

Materials and methods

Bacterial strains

Cronobacter sakazakii BAA-894 isolates *E. sakazakii* MMCC# MZ0685 (Strain 20011001) and *E. sakazakii* MMCC# MZ0686 (Strain 20011012) were obtained as a gift from Michael McClelland of The Vaccine Research Institute in San Diego, California. The strains were grown in Tryptic Soy Broth (TSB) and stored in 10% glycerol at −80°C.

Copper alloys

The one inch-square copper alloys used in this study were a gift from the International Copper Association with copper contents of 99.9% Cu (Cu11000), 88.6% Cu, 10% Ni, 1.4% Fe (Cu70600) and 70% Cu, 30% Zn (Cu26000). Stainless steel (74% Fe, 8% Ni, 18% P) was used as the control surface. Prior to use the alloys were immersed for 30 s in a 3% NaOH solution heated to 70°C, rinsed in DH_2O, and followed by a 3–5 s immersion in 10% H_2SO_4 and rinsed with deionized H_2O. After a final immersion in 95% ethanol the alloys were air-dried and kept in a sterile closed container until use (Espirito Santo et al. 2008).

Media used

TSB and powdered infant formula (Similac Advance®, Abbott Nutrition 2011) were utilized as growth media for overnight cultures (ONC) and suspension media for inoculation on copper alloys. Tryptic Soy Agar (TSA) was used for minimal inhibitory concentration (MIC) of $CuCl_2$ in solid medium and enumeration of colony forming units (CFUs). The media were prepared according to manufacturer's instructions with de-ionized distilled water and autoclaved. Sterile preparation of Similac Advance® powdered formula had a copper sulfate concentration of 1.7 mg/l (Abbott Nutrition 2011), which is within the allowable limits of copper concentrations in drinking water. Phosphate buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and adjusted to pH 7.4 was used for recovery of bacterial cells from copper surfaces, preparation of dilution series, and plating on TSA.

MIC determination

Copper resistance in *C. sakazakii* was evaluated using the agar dilution method for determination of the lowest copper concentration required to inhibit the visible growth of the bacteria on TSA (Wiegand et al. 2008). Concentrations of $CuCl_2$ were added ranging from 0 to 17 mM. Cultured cells in TSB were streaked out on the plates, incubated at 37°C for 20–24 h, and growth assessed.

Bacterial survival on copper alloys

Overnight incubation of *C. sakazakii* strains in TSB or formula was followed by placing 100 μl of the cultures in 3 ml of the respective liquid growth medium and incubation on a rotating shaker at 150 rpm and 37°C to mid log phase at OD_{600} 0.5–0.7 to an average cell concentration of 5×10^8 per ml. Prepared formula and TSB without the inocula were plated on TSA in order to assess for the presence of competing microorganisms. Twenty five microlitre of cell culture was applied on each alloy to be tested, spread with a sterile glass rod over the entire surface, and kept moist in a sterile closed container (Wilks et al. 2005). For fast drying on surfaces, 1 ml of culture in mid-log growth phase was pelleted at rcf 18,000×*g* for 2 min, pellets were re-suspended in 80 μl of suspension medium, and 2 μl were spread over the testing surface drying in open air within 1 min. After defined incubation times at room temperature (25°C) the alloys were transferred into 50 ml centrifugation tubes which contained 10 ml sterilized PBS and 20–25 sterilized 2 mm diameter glass beads. After thorough mixing on a vortex mixer for 30–60 s serial dilutions in PBS were prepared, plated on TSA, and incubated at 37°C for 24 h.

The experiments were performed in triplicates and the mean and standard deviation calculated.

Desiccation of bacterial cells

Cronobacter sakazakii were cultured in TSB or formula as described above, and before testing on copper alloys 100 µl aliquots of the culture were placed in Petri dishes for desiccation. The samples were dried in a 40°C incubator for 2 h followed by room temperature for the remaining days of the desiccation period (Shaker et al. 2008). For testing, samples were re-constituted with the appropriate medium and viable cells were recovered in either liquid or solid culture medium. Capsule formation was assessed before and after desiccation.

Capsule stain

Cronobacter sakazakii capsule formation was determined by the appearance of yellow moist colonies on TSA and light microscopy utilizing Anthony's capsule stain (Smith and Hughes 2007).

Results

The MIC for *C. sakazakii* MZ0685 and MZ0686 was 10 mM in TSA containing $CuCl_2$ from 0 to 17 mM. Incubation of *C. sakazakii* strains on copper alloys resulted in rapid killing on contact in all experimental conditions tested. Cell cultures suspended in TSB survived <10 min on 99.9% copper alloys when incubated under moist conditions (Fig. 1a). Survival times were decreased to 1 min when the suspension was spread and air-dried on 99.9% copper alloys (Fig. 1b). Survival times of *C. sakazakii* on alloys with 88.6 and 70% copper content were not different from the survival times on 99.9% copper under these environmental conditions (data not shown). Additionally, desiccation of *C. sakazakii* in TSB for 33 days did not have any influence on survival times in moist or dry conditions (Fig. 1a, b). No decrease in in viability was noted on stainless steel surfaces under these testing conditions. A second set of experiments was conducted with *C. sakazakii* suspended in prepared powdered infant formula containing trace amounts of copper and zinc. As shown in Fig. 2b survival times of *C. sakazakii* were prolonged for up to 30 min when suspended in formula, spread over 99.9% copper surfaces, and kept moist during the incubation period. Moreover, *C. sakazakii* survival times also increased in dry conditions when suspended in formula. No countable CFUs remained after 3 min on 99.9% copper alloys (Fig. 2b) as compared to 1 min when suspended in TSB. However, desiccation in formula for 45 days decreased

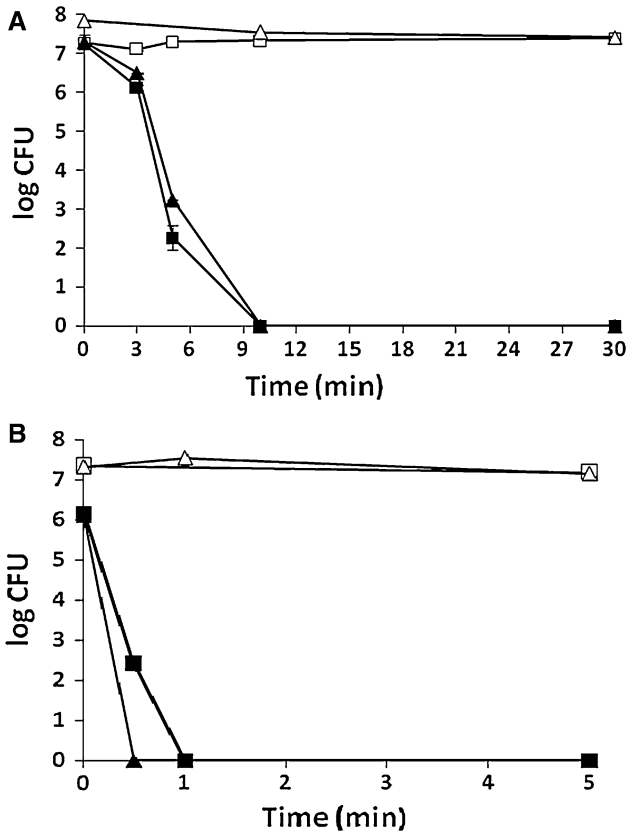

Fig. 1 Survival of *C. sakazakii* BAA-894 *E. sakazakii* MZ0685 suspended in TSB on 99.9% copper (*filled square*) and stainless steel (*open square*) in moist environmental conditions (**a**) and quick air drying (**b**). Survival rates are also shown after 33 days desiccation in TSB, on 99.9% copper (*filled triangle*) and stainless steel (*open triangle*)

survival times to <10 min in moist conditions and to <1 min in dry conditions on 99.9% copper (Fig. 2a, b). The experimental results obtained were similar for both strains tested, however, only data for MZ0685 are shown here. The results of two strains tested in this study confirmed that *C. sakazakii* could be killed effectively upon contact with copper alloys, particularly after suspension and desiccation in infant formula. The findings presented may indicate a possible application for copper surfaces in the production and packaging for storage of powdered milk based foods.

Discussion

The principal mechanisms for the antimicrobial properties of copper alloys are thought to be the release of copper ions from the surface, followed by increased copper influx into the cell as a result of changes in membrane permeability, and subsequent damage to iron-sulfur proteins (Macomber and Imlay 2009; Grass et al. 2011). In rich medium copper ions are likely to bind to the chelators present, thus

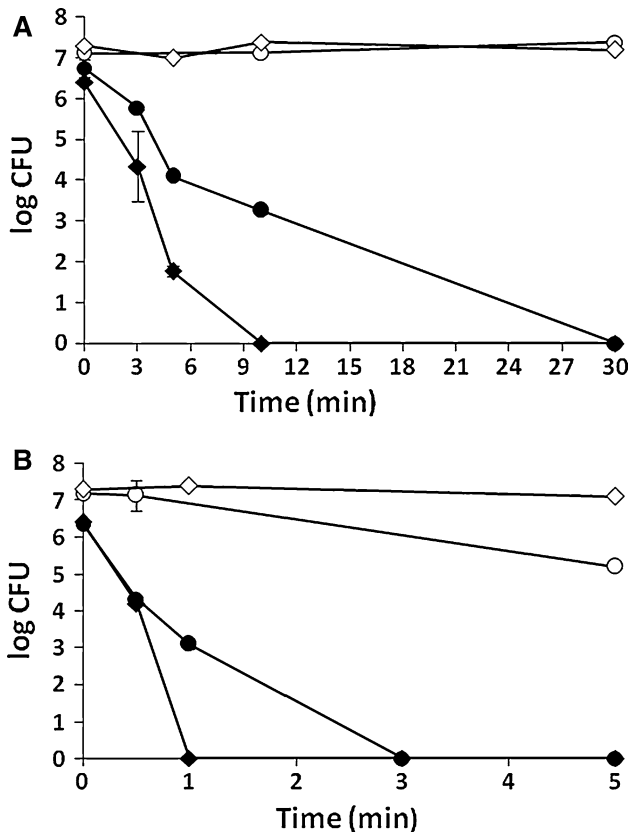

Fig. 2 Survival of *C. sakazakii* BAA-894 *E. sakazakii* MMCC# MZ0685 suspended in infant formula on 99.9% copper (*filled circle*) and stainless steel (*open circle*) in moist environmental conditions (**a**) and quick air drying (**b**). Survival rates are also shown after 45 days desiccation in infant formula, on 99.9% copper (*filled diamond*) and stainless steel (*open diamond*)

reducing bioavailability and slowing down copper ion influx, which could result in prolonged survival of *C. sakazakii* when suspended in formula. The formula used in this study (Similac Advance®), when prepared according to manufacturer's instructions, contains trace amounts of copper and zinc. However, no decrease of countable CFUs was noted when formula was the growth and incubation medium for *C. sakazakii* on stainless steel (Fig. 2a, b). After 45 days of desiccation in formula *C. sakazakii* showed increased sensitivity to copper resulting in decreased survival times on copper alloys. One explanation for this phenomenon could be a change in gene expression during the desiccation period. It had previously been shown that disruptions of genes involved in copper homeostasis in *Pseudomonas aeruginosa* and *Enterococcus hirae* resulted in decreased survival rates on copper alloys (Elguindi et al. 2009, Molteni et al. 2010). *Cronobacter sakazakii* suspended in TSB did not show a significant difference in survival after 33 days of desiccation, but differences may be too small to be detected by the experimental conditions employed in this study. More studies would be needed in order to determine possible genetic influences on the survival rates of *C. sakazakii* on copper alloys. In addition, rapid contact-killing on copper surfaces decreases the possibility for horizontal gene transfer (HGT) in *Cronobacter*. Evidence for past HGT was visible in the genome of *C. sakazakii* BAA-894 and could be very likely of prophage origin (Kucerova et al. 2010). The results of this study are encouraging as they provided evidence that increased copper and desiccation resistance in *C. sakazakii* did not enhance its survival on copper alloys. The findings may be useful for the application of copper materials in the production and storage of milk based foods. Copper would be most effectively utilized in the production of powdered formula, e. g. during the drying process, in order to eliminate live *Cronobacter* before packaging and subsequent storage. Copper could be further explored as an addition to enteral feeding tube material since there was no evidence that silver impregnation of flexilene decreased biofilm formation (Hurrell et al. 2009b).

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