Biometals (2015) 28:1087–1092
DOI 10.1007/s10534-015-9891-3

Synergistic effect of copper and low temperature over *Listeria monocytogenes*

Mauricio Latorre · Ana María Quesille-Villalobos · Felipe Maza · Angel Parra · Angélica Reyes-Jara

Received: 16 October 2015 / Accepted: 26 October 2015 / Published online: 29 October 2015

Abstract The capacity to grow at low temperatures has allowed *Listeria monocytogenes* to become one of the primary food pathogens to date, representing a major public health problem worldwide. Several works have described the homeostatic response of *L. monocytogenes* under different copper (Cu) treatments growing at mild temperature (30 °C). The aims of this report were to evaluate if changes in the external concentration of Cu affected viability and Cu homeostasis of *L. monocytogenes* growing at low temperature. Ours results showed that *L. monocytogenes* growing at 8 °C had a reduced viability relative to 30 °C when exposed to Cu treatments. This decrease was correlated with an increase in the internal concentration of Cu, probably linked to the transcriptional down-regulation of mechanisms involved in Cu homeostasis. This combined effect of Cu and low temperature showed a synergistic impact over the viability and homeostasis of *L. monocytogenes*, where low temperature exacerbated the toxic effect of Cu. These results can be useful in terms of the use of Cu as an antibacterial agent.

Keywords *Listeria monocytogenes* · Copper · Low temperature · Synergistic effect

Abbreviations

Cu	Copper
TSBYE	Tripticase soy broth yeast extract media
TSAYE	Tripticase soy agar yeast extract media
OD	Optical density
MBC	Minimum bactericidal concentration
TXRF	Total reflection X-ray fluorescence
csoR	Transcription factor
ctpA	ATPase Cu efflux
copZ	Cu chaperone
cutC	Cu homeostasis

M. Latorre
Laboratorio de Bioinformática y Expresión Génica, INTA, Universidad de Chile, El Líbano 5524, Macul, Santiago, Chile

M. Latorre
Center of Genome Regulation (Fondap 15090007), Universidad de Chile, Blanco Encalada 2085, Santiago, Chile

M. Latorre
Mathomics, Center for Mathematical Modeling, Universidad de Chile, Beauchef 851, 6th Floor, Santiago, Chile

M. Latorre
Center of Mathematical Modeling, Universidad de Chile, Beauchef 851, Santiago, Chile

A. M. Quesille-Villalobos · F. Maza · A. Parra · A. Reyes-Jara (✉)
Laboratorio de Microbiología y Probióticos, INTA, Universidad de Chile, El Líbano 5524, Macul, Santiago, Chile
e-mail: areyes@inta.uchile.cl

Background

Listeria monocytogenes is a Gram positive, non-sporulating and ubiquitous microorganism. In humans, this bacterium causes listeriosis via consumption of contaminated food, producing a wide range of manifestations that range from febrile gastroenteritis to more severe, invasive disease (Franciosa et al. 2005).

Unlike other foodborne pathogens, the strategy of applying low temperature to avoid bacteria proliferation is not applicable for *L. monocytogenes*, as it has the ability to proliferate at refrigeration temperature (Chan and Wiedmann 2009). This ability has positioned this bacterium as one of the principal drivers of foodborne diseases, representing a major public health problem that involves large global economic losses.

Several attempts have been made to improve effectiveness of various antimicrobial surfaces in order to control this bacterial pathogen. Recently, the use of copper (Cu) has achieved significant attention (Bleichert et al. 2014; De Muynck et al. 2010; Giao et al. 2015). In relation to *L. monocytogenes*, survival of this bacterium was greatly reduced on Cu alloys compared to other metal surfaces (Wilks et al. 2006). Thus, a Cu surface appears to be an excellent alternative to control *L. monocytogenes* in the food industry.

Cu is an essential micronutrient widely required for several metabolic processes. While, several works have been described the adaptation of *L. monocytogenes* to different metal concentration growing at mild temperature (Corbett et al. 2011; Chang et al. 2014; Francis and Thomas 1997; Hantke 2001; Lechowicz and Krawczyk-Balska 2015), its Cu homeostatic response at low temperature is still unknown. In this context, the aims of this work were to determinate if the capacity of *L. monocytogenes* to growth at low temperature can be altered by changes in the external Cu concentration and, if this fluctuation impacts Cu homeostasis of the bacterium.

Methods

Bacterial growth curves

L. monocytogenes strain List2-2 isolated from seafood was stored in skimmed milk at −80 °C. The strain was recovered in Oxford selective agar (Oxoid, Basingstoke, UK). Growth curves were conducted in Tripticase Soy Broth (BBL, Becton–Dickinson, USA) containing 0.6 % yeast extract (Oxoid, Basingstoke, UK) (TSBYE). For bacterial growth curves at low temperature, a single colony of *L. monocytogenes* was cultured overnight in TSBYE at 30 °C (160 rpm). The next day, 100 µL of culture were transferred to fresh medium to be incubated at 8 °C during 72 h (low temperature adaptation stage). After this, 30 mL of TSBYE media was adjusted at optical density at 600 nm (OD_{600nm}): 0.1 and grown at 8 °C (160 rpm). Growth curves at mild temperature (30 °C), 100 µL from an overnight culture in TSBYE at 30 °C were inoculated to the same fresh media with a starting OD_{600nm} of 0.1 and grown at 30 °C (160 rpm). The effect of Cu on the growth of *L. monocytogenes* was determined by the addition of different concentrations of Cu (control = no metal added, 0.5, 1.0, 2.0 and 3.0 mM of $CuSO_4{\cdot}5H_2O$). Bacterial growth was monitored reading OD_{600nm}. All growth curve experiments were carried out in triplicate.

Minimum bactericide concentration of copper (MBC-Cu) assay

Antimicrobial copper effectiveness was determined using the broth dilution method which conformed to the recommended standards of the National Committee for Clinical Laboratory Standards (NCCLS) as described below (NCCLS 1999). Briefly, a fresh TSBYE broth (pH 6.0) supplemented with different concentrations of copper ($CuSO_4{\cdot}5H_2O$: 1–16 mM) were inoculated with 1×10^5 CFU/mL (for assay at low temperature, bacterial strains were previously adapted at 8 °C). The bacteria were cultured (a) during 5 days (or evident visual turbid of the control) at 8 °C with stirring 120 rpm and (b) during 18 h for experiments at 30 °C, also using stirring. To determine the MBC-Cu, an aliquot of 100 µL from each culture with copper was not growth as observed, was taken. The suspension was inoculated in Tripticase Soy agar containing 0.6 % yeast extract (TSAYE) and incubated overnight at 30 °C. The next day the lowest copper concentration at which bacteria was killed (no colony detected), was identified. MBC-Cu assay was performed in triplicate.

Intracellular Cu content

Bacterial cultures of *L. monocytogenes* growing at early exponential phase (OD_{600nm} of 0.3) at 30 and 8 °C

(adapted previously) were exposed to 0.5 mM of $CuSO_4 \cdot 5H_2O$ during one hour under shaker. Next, bacterial were adjusted at a concentration close to 1×10^8 CFU/mL. The cells were washed to remove external Cu as previously described (Reyes-Jara et al. 2010). Cells were then suspended in 200 µL of HNO_3 (Merck) and digested during 24 h at 65 °C. After the acid lysis, total cell Cu content was determinate by Total Reflection X-ray Fluorescence (TXRF) (Gonzalez et al. 1999). Values were normalized to the CFU. Statistical differences were assessed by Student's *t* test $p < 0.05$ (GraphPad Prism 4 software).

Results and discussion

Effects of Cu exposure on *L. monocytogenes* grown at low temperature

Figure 1 describes the effect of fluctuation in the external concentration of Cu over the viability of *L. monocytogenes* growing at two different temperatures. In the control situation (without Cu supplementation), the bacterial cultures growing at 8 °C showed a decrease in velocity in relation to 30 °C, this reduction was increased in the Cu treatments. The addition of 0.5 mM generated a reduction in the viability of *L. monocytogenes* at a low temperature, a reduction not observed in the mild temperature.

This combined effect between Cu and low temperature suggested an additive effect over the viability of *L. monocytogenes*. Cultures of *Rhodopseudomonas palustris* exposed simultaneity to Cu and iron showed a higher reduction in cellular viability in relation to the addition of the single metals, a combined effect called synergistic toxicity (Bird et al. 2013).

To evaluate the toxicity of Cu as an antibacterial agent at low temperature we performed a minimum bactericidal concentration assay (Cu-MBC). According to the bacterial growth curves, at 8 °C a lower concentration of Cu is required to kill *L. monocytogenes* relative to 30 °C.

As the external concentration of Cu was the same in both temperature conditions, it is possible to propose that the internal content of this metal can be affected by low temperature, explaining the synergistic effect over *L. monocytogenes*.

Intracellular Cu content of *L. monocytogenes* at low temperature

Cu homeostasis can be defined as the correct control of the internal concentration of this metal to avoid the total cellular death (Kim et al. 2008). According to the growth curves, we exposed *L. monocytogenes* to 0.5 mM of Cu in order to maintain Cu homeostasis.

Figure 2 describes Cu content of *L. monocytogenes* growing at 8 and 30 °C after one hour of exposure to a non-toxic external concentration of the metal. Under a control growth condition (no metal added), non-significant changes were observed between temperatures. On the other hand, after the Cu treatment, cultures of *L. monocytogenes* growing at mild temperature triplicated its Cu content, a similar behavior

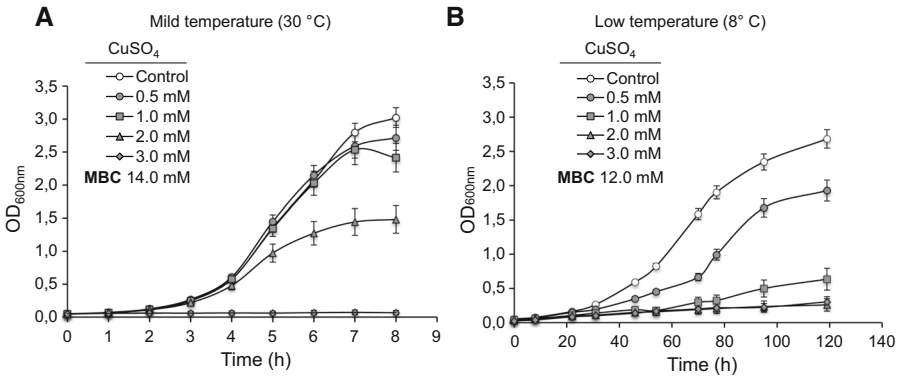

Fig. 1 Bacterial growth curves of *L. monocytogenes*. Bacterial cultures growing at 8 °C (**a**) and 30 °C (**b**). Control curve indicates cultures without supplementation of Cu. All values represent the average of three measurement of absorbance of three independent biological replicates (*error bars* denotes standard deviation). *MBC* minimum bactericidal concentration

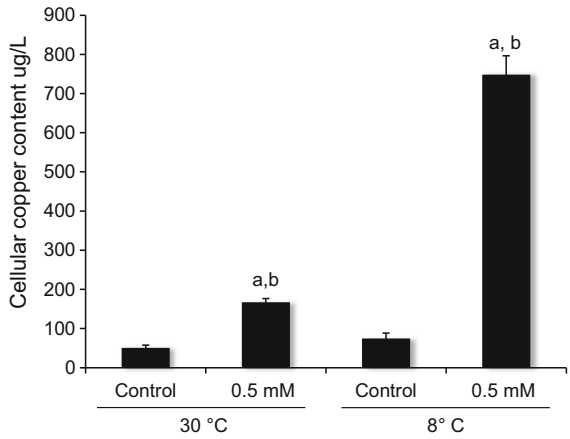

Fig. 2 Intracellular Cu content of *L. monocytogenes* at mild and cold temperature. All values are expressed as the direct Cu content normalized by the colony-forming unit (1×10^8). Letters indicate significant differences (student test $p < 0.05$, three independent biological samples), *a* between the control and the Cu at the same temperature, *b* between Cu treatments at 8 and 30 °C

observed in other pathogen bacterial species (Grass and Rensing 2001; Reyes-Jara et al. 2010).

Interestingly, the *L. monocytogenes* growing at low temperature increased more than ten times their internal content of Cu after the metal exposure, phenotype previously described in this bacterium. Corbett et al. (2011) showed that mutant of *L. monocytogenes* for the Cu ATPase (CtpA) treated with a non-toxic concentration of 20 μM of $CuSO_4$, increases its Cu content more than 40 times in relation with the wild type strains growing at 37 °C by 22 h (Corbett et al. 2011). The same phenotype was observed when mechanisms involved in Cu homeostasis were removed from *Enterococcus faecalis* (Latorre et al. 2014, 2011). Null mutant strains in this bacterium for the *cop* operon (CopY: transcription factor; CopA: ATPase Cu efflux and CopZ: Cu chaperone) and the Cu homeostasis protein CutC, increase its internal Cu content more than ten times in relation to the wild-type strain after the metal treatment, without affecting bacterial viability.

According to this data, it is plausible to hypothesize that the significant increase in the Cu content observed at low temperature in *L. monocytogenes*, could be generated by transcriptional down-regulation of Cu homeostasis mechanisms, principally by the reduction of the Cu efflux system. Durak et al. in 2013 published a complete set of microarrays describing the global transcriptional response of *L. monocytogenes* to low temperature (4° C compared to 30 °C) (Durack et al. 2013). As expected, all the Cu genes of *L. monocytogenes* (*csoR*: transcription factor (lmo1854); *ctpA*: ATPase Cu efflux (lmo1852); *copZ*: Cu chaperone (lmo1853) and *cutC*: copper homeostasis (lmo0026, lmo1018) were down regulated by the low temperature in the microarray assay, supporting the idea that the Cu internal increment observed during the low temperature condition can be explained by the repression of mechanisms involved in Cu homeostasis. In addition, genes activated by cold temperature which encode for components related to the correct folding of nascent proteins (cyclophilin family) contain Cu ions in their structure (Witkowska et al. 2012). Interestingly, genes involved in maintaining the integrity of DNA (like *topA, parE* and nusG) induced by cold in *L. monocytogenes* have responded to Cu treatments in *Pseudomonas aeruginosa* (Teitzel et al. 2006), suggesting the presence of common stress factors able to induce the expression of genes involved in cellular protection.

Conclusions

In this work, we studied the effect of changes in the external concentration of Cu over viability and Cu homeostasis of *L. monocytogenes* growing at a low temperature. *L. monocytogenes* growing at a low temperature reduced its cellular viability during the Cu treatments and increased Cu content. Considering that Cu can generate free radicals toxic for the cell, this metal increment at a low temperature could be generating a toxic internal condition, affecting the viability as showed in the bacterial curves.

The effects of Cu combined with other stressor agents have been widely documented (Vijver et al. 2011). Regarding our experiments, it is possible to propose that Cu, in combination with low temperature, are producing a synergistic effect over *L. monocytogenes*, a phenotype previously described in multicellular organisms (Ozoh and Jones 1990). In this scenario, the bacterium reaches a higher internal concentration of Cu in relation to the bacterium growing at mild temperature. This increment can be associated with the transcriptional down regulation of components involved in the Cu efflux (CtpA ATPase).

The capacity of *L. monocytogenes* to survive and proliferate at low temperatures opens an interesting field in terms of bacterial Cu homeostasis. The information generated not only provides important data for understanding how Cu homeostasis can be affected by low temperature, but also provides potential insights in terms of pathogens control regarding the use of Cu as an antibacterial agent.

Acknowledgments The authors thank Prof. Guillermo Figueroa for providing the *L. monocytogenes* strain List2-2. This work was supported by Grant CONICYT, Nos. 791100002 and FONDECYT 11121449 and Fondo Nacional de Desarrollo de Areas Prioritarias, FONDAP, Project Number 15090007, Center for Genome Regulation (CGR).

Authors' contributions ML and AR-J designed the research, conducted the research, analyzed data and wrote the paper. AMQ-V, FM and AP performed all the experiments. All authors read and approved the final content.

Compliance with Ethical Standards

Conflict of interest All the authors of this work declare that they have no conflict of interest.

References

Bird LJ, Coleman ML, Newman DK (2013) Iron and copper act synergistically to delay anaerobic growth of bacteria. Appl Environ Microbiol 79:3619–3627. doi:10.1128/AEM.03944-12

Bleichert P, Espirito Santo C, Hanczaruk M, Meyer H, Grass G (2014) Inactivation of bacterial and viral biothreat agents on metallic copper surfaces. Biometals 27:1179–1189 doi:10.1007/s10534-014-9781-0

Chan YC, Wiedmann M (2009) Physiology and genetics of *Listeria monocytogenes* survival and growth at cold temperatures. Crit Rev Food Sci Nutr 49:237–253. doi:10.1080/10408390701856272

Chang FM et al (2014) Cu(I)-mediated allosteric switching in a copper-sensing operon repressor (CsoR). J Biol Chem 289:19204–19217. doi:10.1074/jbc.M114.556704

Corbett D, Schuler S, Glenn S, Andrew PW, Cavet JS, Roberts IS (2011) The combined actions of the copper-responsive repressor CsoR and copper-metallochaperone CopZ modulate CopA-mediated copper efflux in the intracellular pathogen *Listeria monocytogenes*. Mol Microbiol 81:457–472. doi:10.1111/j.1365-2958.2011.07705.x

De Muynck W, De Belie N, Verstraete W (2010) Antimicrobial mortar surfaces for the improvement of hygienic conditions. J Appl Microbiol 108:62–72. doi:10.1111/j.1365-2672.2009.04395.x

Durack J, Ross T, Bowman JP (2013) Characterisation of the transcriptomes of genetically diverse *Listeria monocytogenes* exposed to hyperosmotic and low temperature conditions reveal global stress-adaptation mechanisms. PloS One 8:e73603. doi:10.1371/journal.pone.0073603

Franciosa G, Maugliani A, Floridi F, Aureli P (2005) Molecular and experimental virulence of *Listeria monocytogenes* strains isolated from cases with invasive listeriosis and febrile gastroenteritis. FEMS Immunol Med Microbiol 43:431–439. doi:10.1016/j.femsim.2004.11.005

Francis MS, Thomas CJ (1997) The *Listeria monocytogenes* gene ctpA encodes a putative P-type ATPase involved in copper transport. Mol Gen Genet 253:484–491

Giao MS, Wilks SA, Keevil CW (2015) Influence of copper surfaces on biofilm formation by *Legionella pneumophila* in potable water. Biometals 28:329–339. doi:10.1007/s10534-015-9835-y

Gonzalez M, Tapia L, Alvarado M, D. Tornero J, Fernandez R (1999) Intracellular determination of elements in mammalian cultured cells by total reflection X-ray fluorescence spectrometry. J Anal Atomic Spectrom 14:885–888. doi:10.1039/A808748B

Grass G, Rensing C (2001) Genes involved in copper homeostasis in *Escherichia coli*. J Bacteriol 183:2145–2147. doi:10.1128/JB.183.6.2145-2147.2001

Hantke K (2001) Bacterial zinc transporters and regulators. Biometals 14:239–249

Kim BE, Nevitt T, Thiele DJ (2008) Mechanisms for copper acquisition, distribution and regulation. Nat Chem Biol 4:176–185. doi:10.1038/nchembio.72

Latorre M, Olivares F, Reyes-Jara A, Lopez G, Gonzalez M (2011) CutC is induced late during copper exposure and can modify intracellular copper content in *Enterococcus faecalis*. Biochem Biophys Res Commun 406:633–637. doi:10.1016/j.bbrc.2011.02.109

Latorre M et al (2014) Enterococcus faecalis reconfigures its transcriptional regulatory network activation at different copper levels. Metallomics 6:572–581. doi:10.1039/c3mt00288h

Lechowicz J, Krawczyk-Balska A (2015) An update on the transport and metabolism of iron in *Listeria monocytogenes*: the role of proteins involved in pathogenicity. Biometals 28:587–603 doi:10.1007/s10534-015-9849-5

NCCLS (1999) Clinical and Laboratory Standards Institute. Methods for determining bactericidal activity of antimicrobial agents (NCCLS document M26-A), 1st edn. Wayne, Pennsylvania, USA

Ozoh PT, Jones NV (1990) The effects of salinity and temperature on the toxicity of copper to 1-day and 7-day-old larvae of *Hediste* (Nereis) *diversicolor* (O. F. Muller). Ecotoxicol Environ Saf 19:24–32

Reyes-Jara A, Latorre M, Lopez G, Bourgogne A, Murray BE, Cambiazo V, Gonzalez M (2010) Genome-wide transcriptome analysis of the adaptive response of *Enterococcus faecalis* to copper exposure. Biometals 23:1105–1112. doi:10.1007/s10534-010-9356-7

Teitzel GM, Geddie A, De Long SK, Kirisits MJ, Whiteley M, Parsek MR (2006) Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. J Bacteriol 188:7242–7256. doi:10.1128/JB.00837-06

Vijver MG, Elliott EG, Peijnenburg WJ, de Snoo GR (2011) Response predictions for organisms water-exposed to

metal mixtures: a meta-analysis. Environ Toxicol Chem 30:1482–1487. doi:10.1002/etc.499

Wilks SA, Michels HT, Keevil CW (2006) Survival of *Listeria monocytogenes* Scott A on metal surfaces: implications for cross-contamination. Int J Food Microbiol 111:93–98. doi:10.1016/j.ijfoodmicro.2006.04.037

Witkowska D, Valensin D, Rowinska-Zyrek M, Karafova A, Kamysz W, Kozlowski H (2012) Coordination of Ni^{2+} and Cu^{2+} to metal ion binding domains of *E. coli* SlyD protein. J Inorg Biochem 107:73–81. doi:10.1016/j.jinorgbio.2011.11.012