TITLE

Characterization of the binding capacity of mercurial species in *Lactobacillus* strains

RUNNING TITLE

Mercury binding by lactobacilli

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ABSTRACT

BACKGROUND:

Metal sequestration by bacteria has been proposed as a strategy to counteract metal contamination in foodstuffs. Lactobacilli can interact with metals, but studies with important foodborne metals such as inorganic [Hg(II)] or organic (CH_3Hg) mercury are lacking. Lactobacilli were evaluated for their potential to bind these contaminants and the nature of the interaction was assessed by the use of metal competitors, chemical and enzymatical treatments and mutants affected in the cell-wall structure.

RESULTS:

Lactobacillus strains efficiently bound Hg(II) and CH_3Hg. Mercury binding by *Lactobacillus casei* BL23 was independent of the cell viability. In BL23 both forms of mercury were cell-wall bound, their interaction was not inhibited by cations and it was resistant to chelating agents and protein digestion. *L. casei* mutants affected in genes involved in the modulation of the negative charge of the cell-wall anionic polymer lipoteichoic acid showed increased mercury biosorption. In these mutants, mercury toxicity was enhanced compared to wild-type bacteria. These data suggest that lipoteichoic acid itself or the physicochemical characteristics that it confers to the cell-wall play a major role in mercury complexation.

CONCLUSION

48 This is the first example of the biosorption of Hg(II) and CH_3Hg in lactobacilli
49 and it represents a first step towards their possible use as agents for
50 diminishing mercury bioaccessibility from food at the gastrointestinal tract.

51

52 **KEYWORDS:** mercury, methylmercury, *Lactobacillus*, biosorption, cell-wall

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INTRODUCTION

Metals form chemical compounds that are naturally present in the environment. Furthermore, human activities such as mining, industry and transport increase their levels in the environment. Therefore, they can also occur as residues in food and their accumulation in the body can lead to harmful effects over time.

Lactic acid bacteria (LAB) have a long history of safe use in a variety of food fermentation processes for which they have received a GRAS/QPS status. Furthermore, many LAB are also natural inhabitants of the gastrointestinal and urogenital tracts, and some strains are considered as probiotics. LAB have been assayed for a great number of possible health applications, among them, the binding of food contaminants such as toxins and metals, which would result in diminished entry of the toxics into the systemic circulation after ingestion.[1] The capacity of LAB to act as biosorbents of heavy metals has been studied and the use of strains with specific capacities to interact with metals has been proposed as a strategy to diminish the risk associated to the intake of metal-contaminated foods and drinks.[2, 3] This concept is also supported by the identification of the intestinal microbiota as a key player in the limitation of cadmium and lead accumulation after oral intake.[4]

Several mechanisms have been described for heavy metal interaction with bacteria, including specific uptake (bioaccumulation) and biosorption, a metabolically independent process based on surface adsorption, ionic

interactions, chelation or microprecipitation.[3] Studies on heavy metal(oid) sequestration by LAB have been mainly carried out with Cd(II) and Pb(II)[5-7] but also Cr(VI)[8], Cu(II)[9], Al(III)[10] and As(III)/As(V).[11, 12] However, despite the wide distribution of mercury and its importance as a major contaminant in food,[13] binding of mercurial compounds by LAB has received very little attention and, as far as we know, only one report is found in the literature where the capacity to sequester Hg(II) by LAB has been examined.[12] In addition, no data are available about the interaction of LAB with methylmercury (CH_3Hg), which is the main mercury form present in food.[14]

Besides the *in vitro* binding of heavy metals in aqueous solutions, proof of concept experiments have established the efficacy of lactobacilli in the removal of Cd *in situ* from contaminated foods such as fruit juices,[15] but also in *in vivo* animal trials. For example, *Lactobacillus plantarum* strains that possess high capacity of Pb, Cd and Cu binding have been assayed in mice.[5, 7, 16, 17] In this model, a reduction in metal tissue accumulation and increased metal fecal excretion has been observed in animals suffering metal chronic exposure through drinking water. Some of these studies have also evidenced that the beneficial effects of lactobacilli in alleviating the toxic effects of metals probably involve additional mechanisms beyond the sequestering of metals at the intestinal lumen. These include protection against oxidative stress and enhancement of the intestinal barrier function that lead to a decreased permeability to metals.[16, 17] Current knowledge of the capacity of LAB in protecting humans from metal toxicity is quite limited:

100 a single clinical trial has been carried out in children and pregnant women in
101 an at-risk population. Oral administration of a yogurt containing
102 *Lactobacillus rhamnosus* GR-1 resulted in a low but significant reduction in
103 mercury and arsenic amounts in blood, although the exposure level of the
104 studied group was low.[18] In summary, current available evidence suggests
105 that LAB have potential applications as protectants against metal toxicity.

106 With the goal of characterizing the potential for mercury sequestration by
107 lactobacilli and to examine the underlying mechanisms, the capacity of
108 several strains for binding inorganic [Hg(II)] and organic mercury
109 (methylmercury), and the binding characteristics of *Lactobacillus casei*
110 BL23 were analyzed, as a first step towards the possible application of LAB
111 as mercury detoxification agents.

EXPERIMENTAL

Bacterial strains and growth conditions

The bacterial strains utilized in this study are listed in Table 1. Cells were routinely grown in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories) at 37 ºC under static conditions. Agar plates were made by adding agar at 18 g L^{-1}.

Assays of mercury binding by lactobacilli

For each strain, 200 mL of MRS were inoculated at 5% (v/v) with an overnight culture and incubated at 37 ºC for 24 hours. The cells were harvested by centrifugation (5500x*g*, 5 min) and washed with a volume of phosphate-buffered saline (PBS, Sigma-Aldrich). The bacterial pellets were resuspended in PBS and 10 mL aliquots of cellular suspensions in PBS adjusted to different OD_{595} were prepared for subsequent assays. To these suspensions, 1 mg L^{-1} of mercury ($Hg(NO_3)_2$ 1000 mg L^{-1}, Merck, Spain) or methylmercury (CH_3HgCl, 1000 mg L^{-1}, Alfa Aesar, Spain) were added. After incubation at 37 ºC for different times ranging from 2 to 60 min, bacterial cells were pelleted by centrifugation (5500x*g*, 10 min) and the supernatant was collected. The pellets were washed with PBS and mercury contents were determined in bacterial cells, supernatant and washing fractions. Blank samples without bacteria and containing mercury were incubated at 37 ºC for similar periods. The amount of mercury determined in these samples was used to calculate the percentage of added mercury that

was retained by bacteria. Independent experiments were carried in duplicate or triplicate.

Equilibrium study of mercury sorption by *L. casei* BL23 cells

L. casei cells prepared as outlined above were suspended in 1 mL of PBS at an OD_{550nm} of 4 and allowed to bind Hg(II) or CH_3Hg at different concentrations (ranging from 0.25 to 15 mg L^{-1}) for 1 h at 37 ºC. The bacterial pellets were recovered by centrifugation (12000x*g*, 5 min) and mercury concentrations were determined in the supernatant and in the bacteria. Bacterial dry weight was determined after incubation of bacterial suspensions at 100 ºC for 24 h. A Langmuir isotherm model for solute absorption to surfaces described in the following equation was applied: $q_e = q_{max}[bC_f / (1+bC_f)]$. In this model the values of q_e (bound metal per unit of mass) and C_f (concentration of metal remaining in solution) are experimentally determined at the equilibrium. The q_{max} parameter represents the maximal binding capacity and the b coefficient is related to the affinity of the binding reaction. Fitting of the experimental data to the model was performed with the curve fit tool implemented in Graphpad Prism 4 (GraphPad Software Inc., USA).

Bacterial treatments

To assess the nature and the strength of mercury retention to bacterial cells, different treatments were applied.

(i) EDTA washing. *L. casei* BL23 bacterial pellets from the mercury binding experiments (OD_{550nm} of 4, 1 mg L^{-1} of mercurial species) were resuspended in an equal volume of PBS containing 10 mmol L^{-1} EDTA (Sigma-Aldrich) and the suspension was gently shaken for 15 min at room temperature. Subsequently, cells were recovered by centrifugation, washed with PBS as described above and the amounts of mercury retained in the cells and present in the supernatants were determined.

(ii) In order to test the effects of the presence of divalent cations in mercury binding, the binding assays (OD_{550nm} of 4, 1 mg L^{-1} of mercurial species) were performed in the presence of different concentrations (1 to 25 mmol L^{-1}) of $CaCl_2$, $MgCl_2$, $MnSO_4$ and $FeSO_4$ (all from Panreac, Spain).

(iii) Surface proteins were digested by proteinase K treatment. *L. casei* BL23 cells resuspended to an OD_{595} of 2 were treated with proteinase K (100 μg mL^{-1} final concentration, Roche) for 1h at 37 ºC. The bacteria were harvested by centrifugation, washed with PBS and used in mercury binding assays with 1 mg L^{-1} of mercurial species.

(iv) Non-covalently bound surface proteins were extracted by resuspending *L. casei* cells to an OD_{595} of 2 in 1.5 mol L^{-1} LiCl (Sigma-Adrich) in PBS and incubating for 1h at 4 ºC. Cells were subsequently recovered by centrifugation, washed with PBS and assayed for mercury binding (1 mg L^{-1} of mercurial species). Post-treatment experiments were also performed in which cells that had previously incorporated mercury were subjected to

proteinase K or LiCl treatments under the same conditions described above and mercury contents in the treated cells were determined. Controls were carried out in all experiments in which the bacteria were incubated and washed without the addition of proteinase K or LiCl.

(v) The capacity to retain mercury by heat-killed bacteria (OD_{550nm} of 4, 1 mg L^{-1} of mercurial species) was investigated by performing binding experiments with cells that had been pre-treated at 80 ºC for 30 min in PBS. This treatment reduced the viability by more than seven logarithmic units as determined by viable cell plate counting.

(vi) Bacterial cell-wall digestion. *L. casei* BL23 cells that had bound mercury (OD_{595} 0.7, 1 mg L^{-1} of mercurial species) were washed with PBS and resuspended in a solution containing 200 g L^{-1} polyethylene glycol 4000, 10 mmol L^{-1} Tris-HCl pH 8, 5 mmol L^{-1} sodium maleate, 5 mmol L^{-1} $MgCl_2$, 10 mg mL^{-1} lysozyme (Roche) and 10 U mL^{-1} mutanolysine (Sigma-Aldrich) and incubated for 1h at 37 ºC. The bacteria were pelleted by centrifugation and mercury was determined in the cells and supernatants. The absence of lysis in digested cells was checked by comparing lactate dehydrogenase activity (measured in 200 µl of 100 mmol L^{-1} triethanolamine buffer pH 6.8, 5 mmol L^{-1} fructose-1,6-bisphosphate, 5 mmol L^{-1} pyruvate and 0.15 mmol L^{-1} NADH) between supernatants of cells treated and non-treated with the hydrolytic enzymes.

Determination of bacterial growth

L. casei strains (Table 1) were grown in MEI medium [19] (tryptone, 5 g L^{-1}; yeast extract, 5 g L^{-1}; K_2HPO_4, 6 g L^{-1}; KH_2PO_4, 4 g L^{-1}; $MgSO_4.7H_2O$, 0.2 g L^{-1}; $MnSO_4$, 0.05 g L^{-1}; Tween 80, 1 mL L^{-1}) in 96-well plates (150 μl per well) with different concentrations of Hg(II) or CH_3Hg (0.25 to 1 mg L^{-1}) at 37 ºC. Growth was recorded as OD_{595nm} in a Polarstar plate reader (BMG Labtech GmbH, Germany).

Mercury determination

The samples were digested using a microwave accelerated reaction system (MARS, CEM, Vertex, Spain). The supernatants, washing fractions and the cell pellets were placed in Teflon reactors and 4 mL of 14 mol L^{-1} HNO_3 (Merck) and 1 mL of H_2O_2 (30% v/v, Prolabo, Spain) were added. The reactors were irradiated (180 °C, 15 min) and the digest obtained was allowed to rest for 12 h to eliminate nitrous vapour. Then it was made up to volume with 0.6 mol L^{-1} HCl and mercury was quantified by cold vapour atomic fluorescence spectrometry (CV-AFS, Millennium Merlin PSA 10.025, PS Analytical, UK). The analytical conditions employed were: reducing agent, 20 g L^{-1} $SnCl_2$ (Scharlab, Scharlau Chemie, Spain) in 1.8 mol L^{-1} HCl (Merck), 4.5 mL min^{-1}; reagent blank, 0.6 mol L^{-1} HCl, 9 mL min^{-1}; carrier gas, argon, 0.3 L min^{-1}; dryer gas, air, 2.5 L min^{-1}; delay time, 15 s; analysis time, 40 s; memory wash time, 60 s. Quality control for quantification by CV-AFS was performed by analysing a liquid reference material (QCI-049-1 Trace Metals AA Sample 1, LGC Standards, Spain) with a certified mercury concentration of 40.8 ± 1.19 μg L^{-1}.

Statistical analysis

Statistical analysis was performed by means of Student's t-test (Graphpad Prism 4). Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Lactobacillus cells bind inorganic mercury and methylmercury

The capacity of several strains of lactobacilli to remove Hg(II) and CH_3Hg from an aqueous solution was investigated. These strains were originally chosen on the basis of their previously determined capacity to synthesize polyphosphate, an inorganic phosphate polymer that has been related to the capacity of retaining mercury in microorganisms, owing to its chelating potential.[20] However, polyphosphate synthesis in these strains was not linked to their mercury-binding capacity (unpublished observations). As seen in Fig. 1, all strains possessed a high capacity for removing Hg(II) and CH_3Hg from buffered solutions with percentages of mercury sequestration ranging from 54 to 100%. In general, under our experimental conditions, CH_3Hg was retained to a greater extent by lactobacilli compared to Hg(II) (median of 69.2% for Hg(II) and 85.0% for CH_3Hg). These results confirm a previous work that showed a high potential of LAB for Hg(II) removal [12] and further extend the study on the capacity of mercury binding of lactobacilli to CH_3Hg, the main mercurial form present in food.[14] Kinoshita *et al.*[12] had already reported Hg(II) binding in eleven strains of LAB of food or intestinal origin belonging to the *Lactobacillus*, *Weissella*, *Enterococcus*, *Pediococcus* and *Streptococcus* genera. Removal of Hg(II) from solution by these strains was also in the 80-90% range and it was much higher than that observed for Cd(II), Pb(II) or the anionic metalloid As(III) under the same conditions.

Mercury binding in *L. casei* BL23 cells

Further characterization of mercury binding was performed in *L. casei* BL23. This strain was selected due to its wide use in physiology and genetic research in LAB and its genetic amenability,[21] which allows molecular approaches aimed at disclosing mercury complexation mechanisms. Mercury binding was dependent on the amount of cells used in the assay, reaching a plateau at OD 1.25 for Hg(II) and 10 for CH_3Hg (Fig. 2A). Binding kinetics was also a fast process, and maximum mercury accumulation was already achieved after 2 min incubation (Fig. 2B). Characterization of the mercury retention capacity using the Langmuir model gave a maximum binding capacity (q_{max}) of 898,8 ± 138,9 ng per mg bacterial dry weight for Hg(II) and 204,8 ± 13,03 ng per mg bacterial dry weight for CH_3Hg (Fig. 3). The b coefficient of this model, which reflects the binding affinity, was 0,00029 ± 0,00012 for Hg(II) and 0,00272 ± 0,00057 for CH_3Hg, which denotes that although the maximum binding capacity of bacteria was higher for Hg(II), the binding affinity was superior in the case of CH_3Hg. This higher b coefficient suggests that *L. casei* BL23 would be specially suited to remove CH_3Hg at low concentrations, such as those that are expected in the gastrointestinal tract. [3] q_{max} values for both mercury forms were higher compared to q_{max} for toxic metals reported in other lactobacilli, [3] which, in line with the results obtained by Kinoshita et al.,[12] would follow the order Hg > Cd(II) > Pb(II) > As(III).

Mercury binding in *L. casei* BL23 is not inhibited by cations and does not require live cells.

We determined the effect of diverse cations (Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+}) as likely competitors in the process of mercury biosorption by lactobacilli. None of these cations interfered with mercury binding and no inhibition was observed under our experimental conditions (data not shown). This is in contrast with the results obtained with Pb(II) in *L. plantarum*, whose binding was inhibited in the presence of Fe^{2+}, Ca^{2+} or Mn^{2+}.[22] This result suggests that the nature of mercury binding might also involve non-ionic interactions. In line with these results, treatment of the bacterial cells with EDTA was not sufficient to remove bound mercury from the bacteria and as much as 99.8 % and 99.6 % of bound Hg(II) and CH_3Hg, respectively, still remained associated to cells after a 15 min washing treatment with 10 mM of the chelating agent. This differs from previous results with other metals, as a rapid release from the cells was observed for Cd(II) and Pb(II) bound to *Lactobacillus fermentum* ME3, *Bifidobacterium longum* 46, or *L. plantarum* CCFM8661 when the cells were treated with lower EDTA concentrations than those used here.[22, 23]

Mercury binding did not require viable cells, and heat-killed bacteria were able to bind the two forms of the metal with an increased efficacy (33.0 ± 1.0 % increase for Hg(II) binding and 25.0 ± 10.0 % increase for CH_3Hg).

293 This observation supports the idea that mercury binding is not a process
294 carried out by specific/unspecific binding proteins or transporters, similar to
295 Cd(II) and Pb(II) binding in other lactobacilli.[24] Mercury transport systems
296 are present in bacterial strains that possess mercury detoxification
297 mechanisms and they participate in the intracellular incorporation of
298 mercury, which is later reduced to produce the less toxic and volatile
299 metallic (Hg^0) form.[25] However, although some systems for metal
300 detoxification have been reported in lactobacilli,[26] no mercury-specific
301 detoxifying systems have been described for this microbial group. The most
302 plausible explanation for the increased binding capacity in heat-killed cells
303 would be that heat treatment produces an increased exposure of as yet
304 unidentified binding sites that become more accessible to mercury, as has
305 been described for other metals.[3]

306

307 **Mercury is mainly associated to the cell wall in *L. casei* BL23**

308 Mercury has been shown to be very reactive towards thiol groups, such as
309 those present in proteins containing Cys and proteinaceous substances
310 have been linked to metal biosorption processes in bacteria. In fact, binding
311 of other metals such as Cu(II), Cd(II) or Pb(II) has been reported for some
312 S-layer proteins from bacteria, including lactobacilli.[27, 28] Recently, a 14 KDa
313 Hg(II)-binding surface protein containing Cys-X-X-Cys motifs from the LAB
314 *Weissella viridescens* was identified.[12] In order to test whether

315 proteinaceous substances were involved in capturing mercury in BL23, cells
316 were either treated with proteinase K or extracted with LiCl prior binding
317 assays, which would proteolyze the bacterial surface or extract most of the
318 non-covalently bound surface proteins, respectively. These treatments had
319 minor effects on the retention capacity for both forms of mercury assayed:
320 post-treatment of bacteria that had incorporated Hg(II) and CH_3Hg with
321 proteinase K or LiCl, resulted in a release of 15% of bound Hg(II), whereas
322 22 and 33%, respectively, of CH_3Hg was removed from the bacteria after
323 these treatments. This would imply that complexation with proteins does not
324 appear to be the only mechanism of mercury binding in the assayed strain.

325 Other extracellular polymers, such as exopolysaccharides, have been
326 linked to metal complexation in LAB,[10, 29] although this possibility can be
327 ruled out in *L. casei* BL23, an exopolysaccharide-negative strain. Cell-wall
328 polymers are more likely targets for mercury complexation in bacteria.[30]
329 According to this, when BL23 cells that had previously incorporated mercury
330 were subjected to a treatment with cell-wall hydrolytic enzymes (lysozyme
331 and mutanolysin), about half of the retained Hg(II) (53.3 ± 4.4%) and 39.3 ±
332 1.3% of CH_3Hg were released from the bacteria, indicating that a
333 substantial proportion of mercury was associated to cell-wall structures. Cell
334 wall components also have a major contribution to Pb(II) binding in *L.*
335 *plantarum*.[22]

336

Mercury binding is affected in *L. casei* strains defective in cell surface components.

The identification of which constituent/s of the cell-wall is/are involved in mercury complexation requires special consideration. It has been postulated that heavy metal biosorption in bacteria can be mediated by the interactions of the metals with the negatively-charged bacterial surface. Gram-positive bacteria contain cell-wall polymers, such as lipoteichoic acid (LTA, a negatively-charged polymer of glycerol-phosphate), that can be involved in such interactions. We tested mercury binding in *L. casei* BL23 mutants affected in the composition of the cell surface.[31] The first mutant (*dltA*) was defective in the D-alanine-D-alanyl carrier protein ligase, an activity involved in the process of D-alanylation of LTA polymer, which has been postulated to modulate the net charge of the cell-wall or the structural arrangement of the cell wall (for a review see Revilla-Guarinos *et al.*[32]). A second mutant (*mprF*) was affected in the enzyme responsible for the lysinylation of the anionic phospholipids at the cell membrane, thus augmenting the cell membrane net negative charge. Finally, the ΔRR12 strain was defective in a transcriptional activator (encoded by LCABL_19600 in *L. casei* BL23) necessary for induction of *dltA* and *mprF* genes.[31] As can be seen in Fig. 4, the *dltA* and ΔRR12 mutations resulted in an increased capacity (19 to 28% increase) of Hg(II) binding, maybe as a result of an increased negative charge density at the cell surface. CH_3Hg also showed a trend towards increased retention in the *dltA* strain, although

statistical significance was not achieved (p=0.11). This would favour the hypothesis that negatively charged LTA interacts with mercury. However, other studies suggest that D-alanylation leads to structural modifications of the cell wall, making it more compact and less permeable.[32, 33] In this scenario, it may be speculated that low D-alanylation would expose additional binding sites in the cell wall.

Growth of the strains in media containing mercury also evidenced that these mutations enhanced the sensitivity to mercury of the bacteria. Despite the absence of an increase in mercury binding in the *mprF* mutant under our assay conditions, this strain also displayed higher mercury sensitivity. However, the highest impact was for the *dltA* and ΔRR12 mutations, as shown by their strongly decreased growth at mercury concentrations above 0.5 mg L^{-1} (Fig. 5). In several microorganisms and, specifically in *L. casei* BL23, mutants in *dltA* and *mprF* are more sensitive to cationic antimicrobial peptides (CAMPS).[31] The fact that *L. casei* mutants defective in these genes, especially those involved in LTA modification, were more sensitive to both forms of mercury, agrees with the concept that the modification of the cell envelope physico-chemical properties facilitates the surface interaction or increases the local concentration of mercury at the surface in a process analogous to that reported for CAMPS. In conclusion, although LTA appears as a likely target for mercury complexation, the involvement of other surface components such as peptidoglycan cannot be excluded. The

construction of LAB strains defective in LTA synthesis, which have been obtained in one LAB species,[34] will help to answer these questions.

The results presented here extend the research of metal binding by lactobacilli to a metal that constitutes an important health threat. Also, they open the doors to investigate to which extent the capacity of mercury binding by lactobacilli can be influenced by the gastrointestinal conditions and by the presence of mercury in a real food matrix, with the aim of designing strategies to reduce the risk associated to mercury exposure through food.

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Table 1. Strains used in this study

Designation	Strain	Origin/reference
BL299	*Lactobacillus plantarum* 299V (DSM[a] 9843)	Human intestine
BL36	*Lactobacillus brevis* ATCC[b] 14869	Human intestine
BL17	*Lactobacillus acidophilus* ATCC 4356	Human intestine
BL166	*Lactobacillus plantarum* WCFS1	Human intestine[35]
BL259	*Lactobacillus reuteri* BL[c] 259	Rat caecum
BL23	*Lactobacillus casei* BL23	Laboratory strain[21]
BL377	*Lactobacillus rhamnosus* GG (ATCC 53103)	Human intestine
BL5	*Lactobacillus johnsonii* ATCC 11506	Human intestine
BL260	*Lactobacillus intestinalis* BL260	Rat caecum
BL327	*Lactobacillus rhamnosus* ATCC 9595	Human oral cavity
DLT	*Lactobacillus casei* BL23 *dltA*::pRV300	[31]
MPRF	*Lactobacillus casei* BL23 *mprF*::pRV300	[31]
ΔRR12	*Lactobacillus casei* BL23 ΔLCABL_19600	[36]

[a] Deutsche Sammlung von Mikroorganismen und Zellkulturen
[b] American Type Strain Culture Collection
[c] Our laboratory collection

FIGURE LEGENDS

Figure 1. Binding of mercury by *Lactobacillus* strains. Different strains of lactobacilli were incubated at an OD_{550nm} of 4 with 1 mg L^{-1} of mercury (Hg(II) and CH_3Hg) for 1 h at 37 ºC in PBS and the percentages of mercury retained in the cells were determined after washing. Data are means and standard deviation from two experiments carried out with bacteria from independent cultures.

Figure 2. Binding of mercury by *L. casei* BL23 cells incubated in PBS buffer with mercury at a final concentration of 1 mg L^{-1}. (A) percentages of binding for Hg(II) (filled circles) and CH_3Hg (circles) in the presence of different amounts of *L. casei* cells (as units of optical density at 550nm (OD)). The solid lines represent mercury retained in the cells, whereas the dashed lines represent mercury in the supernatants of the binding reaction. (B) Binding of Hg(II) (filled circles) and CH_3Hg (circles) by *L. casei* as a function of time. The solid lines represent mercury in the cells, whereas the dashed lines represent mercury in the supernatants of the binding reaction. Data are means and standard deviation from two experiments carried out with bacteria from independent cultures.

Figure 3. Effect of mercury concentration on binding by *L. casei* cells. The Langmuir isotherm model was applied. q_e is the binding capacity as ng of mercury per mg of bacterial dry weight (dw), whereas C_f represents the

metal concentration in the solution at the equilibrium. The inner plots represent the linearized form of the Langmuir model.

Figure 4. Effect of different mutations affecting surface characteristics on mercury binding by *L. casei*. *dltA*, mutant in the D-alanine-D-alanyl carrier protein ligase enzyme; *mprF*, mutant affected in the synthesis of lysylphosphatidylglycerols; RR12, mutant with a deletion in the response regulator, encoded by LCABL_19600, which controls expression of *dltA* and *mprF*. Data are means and standard deviation of five replicates with bacteria from independent cultures. Asterisks indicate a statistically significant difference compared to the wild type strain (p<0.01). dw, dry weight.

Figure 5. Growth of different *L. casei* strains in the presence of Hg(II) or CH_3Hg. *dltA*, mutant in the D-alanine-D-alanyl carrier protein ligase enzyme; *mprF*, mutant affected in the synthesis of lysylphosphatidylglycerols; ΔRR12, mutant with a deletion in the response regulator, encoded by LCABL_19600, which controls expression of *dltA* and *mprF*. The graphs represent the means of three bacterial growth curves.

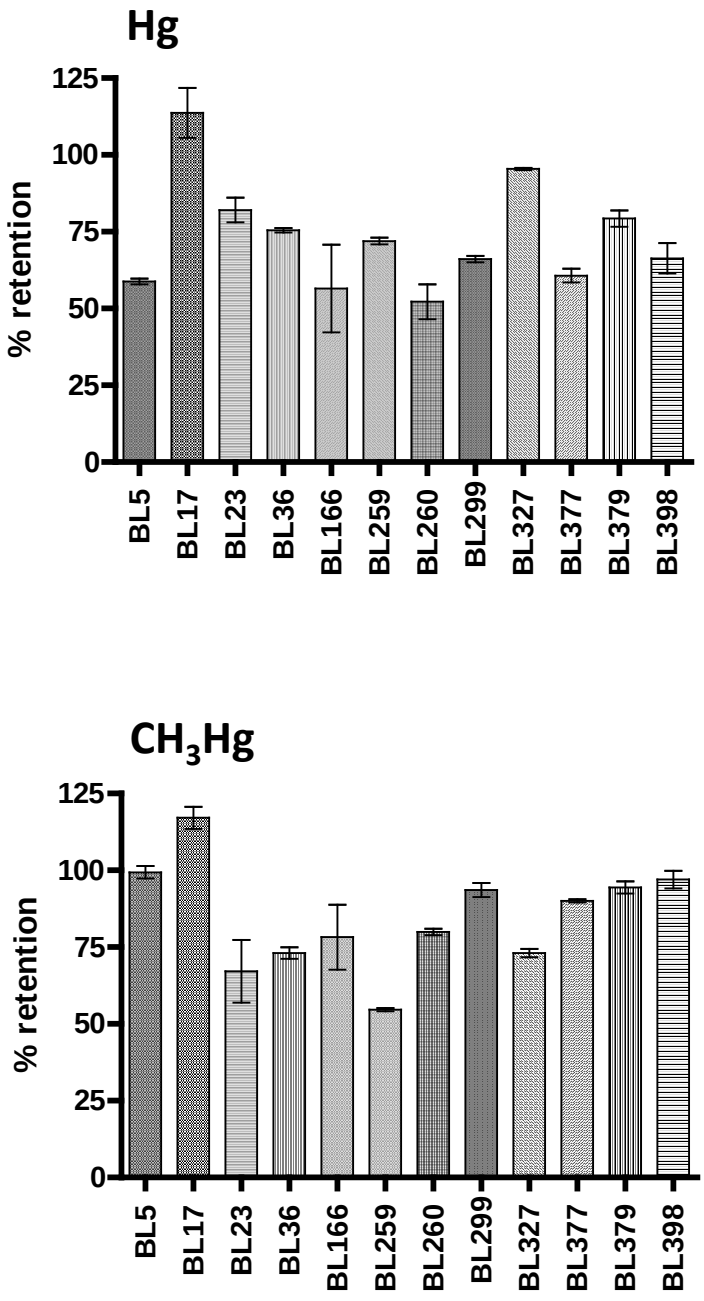
Hg
% retention
125
100
75
50
25
0
BL5
BL17
BL23
BL36
BL166
BL259
BL260
BL299
BL327
BL377
BL379
BL398
CH3Hg
% retention
125
100
75
50
25
0
BL5
BL17
BL23
BL36
BL166
BL259
BL260
BL299
BL327
BL377
BL379
BL398

Figure 1

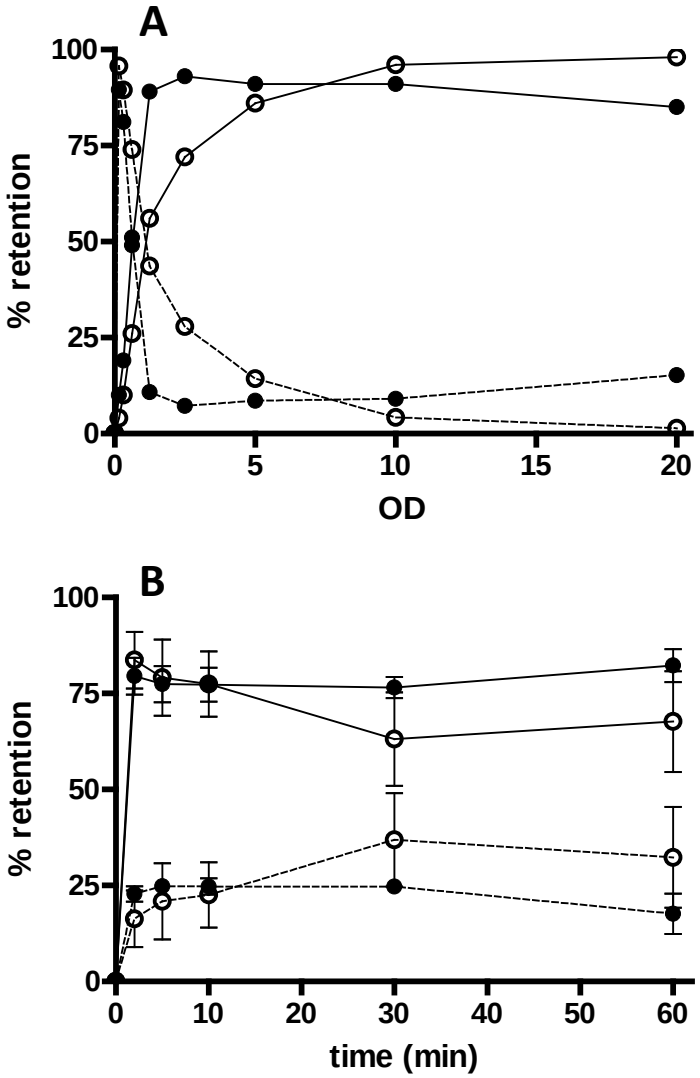
A
100
75
50
25
0
% retention
0
5
10
15
20
OD
B
100
75
50
25
0
% retention
0
10
20
30
40
50
60
time (min)

Figure 2

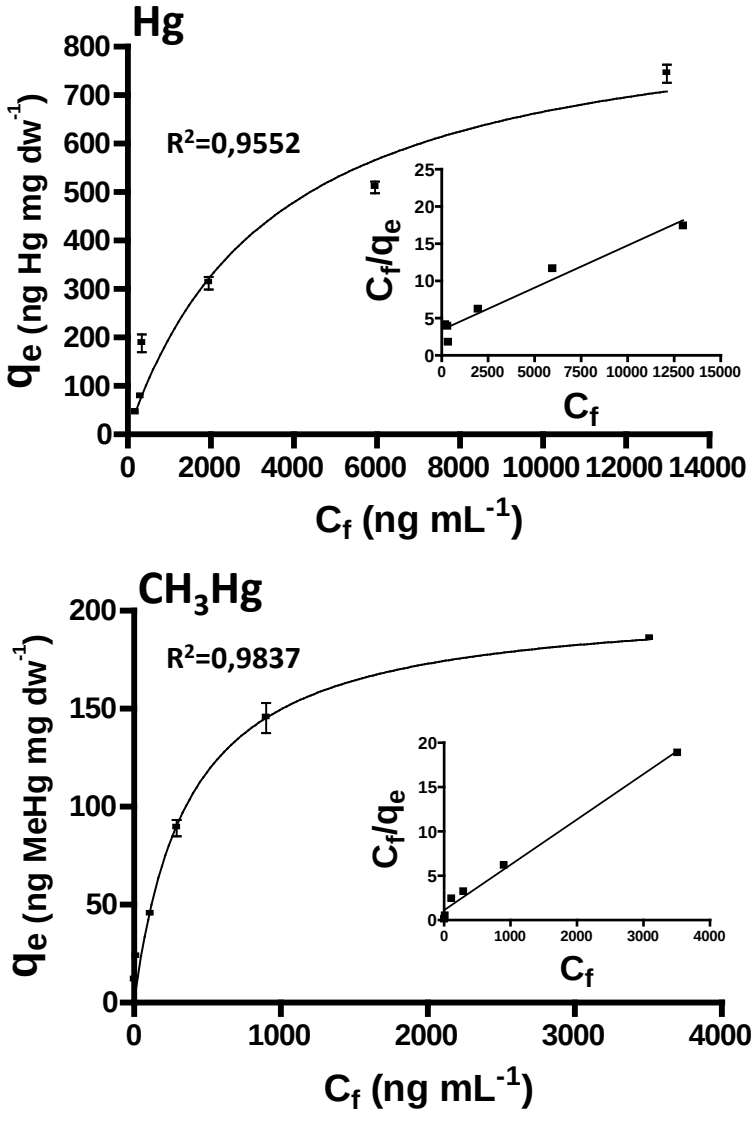
Hg
R²=0,9552
qe (ng Hg mg dw-1)
Cf (ng mL-1)
Cf/qe
Cf
CH3Hg
R²=0,9837
qe (ng MeHg mg dw-1)
Cf (ng mL-1)
Cf/qe
Cf

Figure 3

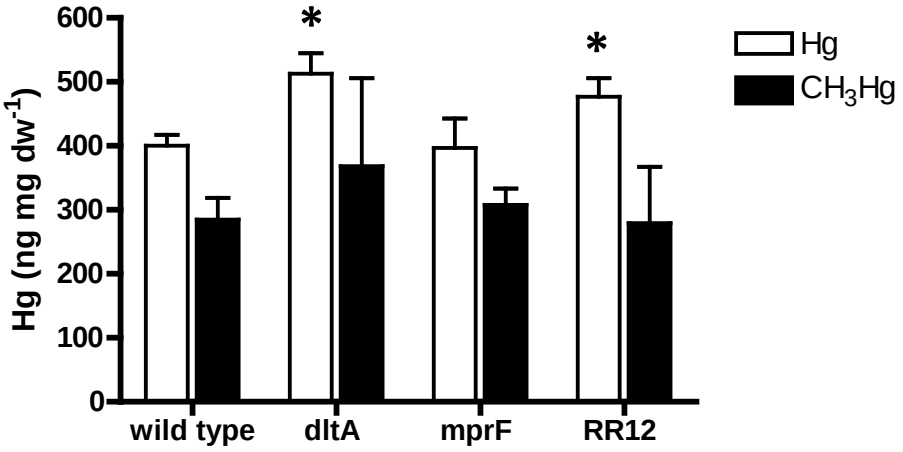
600
500
400
300
200
100
0
Hg (ng mg dw-1)
*
*
Hg
CH3Hg
wild type
dltA
mprF
RR12

Figure 4

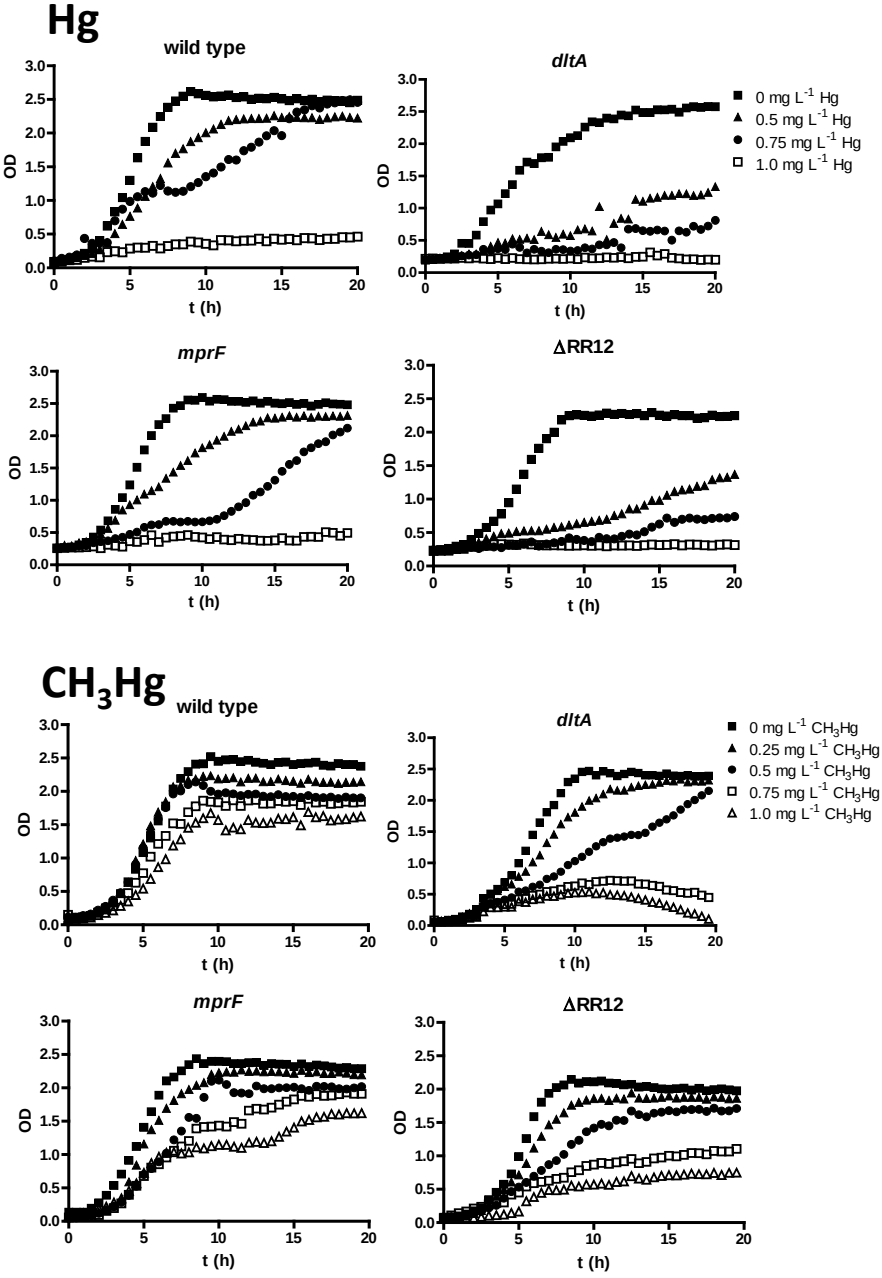
Hg
wild type
dltA
mprF
ΔRR12
OD
t (h)
0 mg L-1 Hg
0.5 mg L-1 Hg
0.75 mg L-1 Hg
1.0 mg L-1 Hg
CH3Hg
wild type
dltA
mprF
ΔRR12
OD
t (h)
0 mg L-1 CH3Hg
0.25 mg L-1 CH3Hg
0.5 mg L-1 CH3Hg
0.75 mg L-1 CH3Hg
1.0 mg L-1 CH3Hg

Figure 5