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Precipitation of silver-thiosulfate complex and immobilization of silver by *Cupriavidus metallidurans* CH34

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Abstract

Cupriavidus metallidurans CH34 is a facultative chemolithotrophic bacterium that possesses two megaplasmids (*pMOL28* and *pMOL30*) that confer resistance to eleven metals. The ability of *Cupriavidus metallidurans* CH34 to resist silver is described here. Electronic microscopy, energy-dispersive X-ray (EDX) and X-ray diffractometry (DRX) observations revealed that *C. metallidurans* CH34 strongly associated silver with the outer membrane, under chloride chemical form. Using derivate strains of *C. metallidurans* CH34, which carried only one or no megaplasmid, we show that this resistance seems to be carried by *pMOL30*.

Introduction

Industries use silver because of its electrical and thermal conductivity, malleability, photosensitivity, and antimicrobial proprieties. Silver released in industrial wastewaters is toxic for organisms. At the present time, several processes (precipitation, solvent extraction, electrolysis, ion-exchange resins or chelating agents) are used to treat silver at concentrations above 100 mg/l (about 0.93 mM) (Pethkar & Paknikar 2003). Microorganisms like bacteria, fungi and algae offer an alternative way for heavy metal removal, according to their resistance systems to high heavy metal concentrations (Diels *et al*. 1995a; Chang *et al*. 1997; Chang & Huang 1998; Pethkar & Paknikar 2003). In this way, bacteria like *Thiobacillus thioparus* (Pethkar & Paknikar 2003) are already used for silver bioremediation. The major bacterial resistance to heavy metals mechanisms is based on cation efflux antiporter systems located in bacterial membranes (Nies 1995; Silver & Phung 1996; Van Der Lelie *et al*. 1997; Grosse *et al*. 1999). In the case of silver, this system was studied in *Escherichia coli* and other bacteria (Gupta *et al*. 2001). It possesses two different efflux pumps: SilCBA and SilP (Gupta *et al*, 2001). The first, a well-known CBA efflux pump (Silver & Phung 1996; Van Der Lelie *et al*. 1997; Grosse *et al*. 1999; Gupta *et al*. 2001), is composed of a proton – cation antiporter (SilA), a membrane fusion protein, linking the inner and outer membranes (SilB), and an efflux outer membrane protein (SilC) (Gupta *et al*. 1999). The second, SilP, is a membrane P-type ATPase, which pumps Ag (I) from the cell cytoplasm to the periplasm (Gupta *et al*. 1999; Rensing *et al*. 2000). The system is regulated by two genes, *silS* (a membrane kinase sensor) and *silR* (a transcriptional regulatory responder). Another resistance mechanism to silver, based on a bioaccumulation by bacteria, has

also been reported but the link between the two system was not clear (Simmons & Singleton 1996; Silver 2003).

In addition, homologies between silver resistance and bacterial resistance to other metals have been demonstrated. First, the copper transporting P-type ATPases is also capable of transporting silver (Solioz & Odermatt 1995; Gupta *et al*. 2001; Franke *et al*. 2003; Stoyanov *et al*. 2003). Second, homologies between the *sil* genes and the cadmium, zinc, and copper resistance system (Czc) of *Cupriavidus metallidurans* CH34 were recently reported (Silver 2003).

Cupriavidus metallidurans CH34 (previously *Alcaligenes eutrophus* CH34, also called *Ralstonia metallidurans*, renamed in 2004 *Wautersia metallidurans* and then *Cupriavidus metallidurans*), first isolated from a zinc decontamination tank (Mergeay *et al*. 1985), contains two megaplasmids (*pMOL28* and *pMOL30*), which confer resistance to a broad range of heavy metals (Cd^{2+}, Co^{2+}, Zn^{2+}, Tl^{+}, Cu^{2+}, Pb^{2+}, Ni^{2+}, Hg^{2+} and CrO_4^{2-}). *pMOL28* (163 kb) allows *C. metallidurans* CH34 to resist high concentrations of nickel, cobalt (*cnr* genes), and mercury (transposon *Tn4378*), and *pMOL30* (238 kb) allows *C. metallidurans* CH34 to resist high concentrations of zinc, cadmium, cobalt (*czc* genes), and mercury (transposon *Tn4380*) (Nies *et al*. 1987; Mergeay *et al*. 2003). Moreover, selenium (Se^{4+}) and gadolinium (Ga^{3+}) fixation by *C. metallidurans* CH34 has been demonstrated (Andrès *et al*. 2000; Roux *et al*. 2001). More recently, genes of resistance to copper (*cop*) on pMOL30 of *Cupriavidus* have been described by Mergeay *et al*. (2003). In general, *Cupriavidus metallidurans* CH34 resistances to metals are due to efflux systems (Nies *et al*. 1987; Mergeay *et al*. 2003) followed by metal bioprecipitation or biosequestration (Diels *et al*. 1995b, 1999).

In this paper, the demonstration of the resistance of *C. metallidurans* CH34 to silver is reported for the first time. It has been possible to determine its precipitation kinetics, the minimal inhibition concentration (MIC) of silver, and the ability of *C. metallidurans* CH34 to detoxify the silver-thiosulfate complex used extensively in industrial processing (Pethkar & Paknikar 2003). Finally, it is possible to demonstrate that this resistance is plasmid dependant.

Materials and methods

Strains and growth media

Cupriavidus metallidurans CH34 and derivate strains were grown at 30 °C in a TRIS-Gluconate medium supplemented by a mixture of trace elements and ferric ammonium citrate, according to Mergeay *et al*. (1985). An initial *Cupriavidus metallidurans* CH34 culture (with or without 1.2 mM silver) was realized in a 1-l TRIS-Gluconate medium flask inoculated with 5×10^4 cells/ml of silver non-adapted bacteria in order to verify the bacteria growth. The silver MIC was determined in a TRIS-gluconate liquid medium containing increasing amounts of silver and 19 mM sodium thiosulfate. Cellular growth was determined by counting of colony forming units (CFU) on TRIS-gluconate medium after 5 days of incubation at 30 °C. *C. metallidurans* AE104 (plasmid-free), AE126 (containing *pMOL28*), and AE128 (containing *pMOL30*) were used to determine *C. metallidurans* CH34 localization of gene resistance to silver. Controls were made in the presence of nickel (*pMOL28* positive control), zinc (*pMOL30* positive control), and using dead cells in the presence of 2.5 mM of silver. For this, cells were heated at 60 °C for 45 min before silver addition (at room temperature).

Silver content determination

The concentrations of soluble silver were determined by atomic absorption spectrometry (AAS AA55, Varian).

Electron microscopy, energy dispersive X-ray (EDX) and X-ray diffractometry (DRX) analysis

To determine the presence and the localization of silver in bacterial cells, electron microscopy and energy-dispersive X-rays (EDX) were used on whole cells grown in the presence of silver. Cells were washed in a cacodylate buffer (pH 7.2) and concentrated by centrifugation at $12\,000\times g$ for 15 min. Cells were then fixed in 2.5% glutaraldehyde in the cacodylate buffer for 60 min at room temperature and washed three times in the same buffer. The cell pellets were dehydrated with ethanol and embedded in Epon. Sections were cut

with an ultramicrotome equipped with a diamond knife. Electron microscopes were used at 200 kV (CM20, Phillips) and 80 kV (CM12, Philips). EDX analyses were performed on the same grids, to reveal the presence of silver in cells, using an ultrathin window and a Si–Li detector system (EDAX, Germany).

Cupriavidus metallidurans CH34 outer membranes were obtained by an osmotic shock (Falla *et al.* 1988). Freeze-dried outer membranes were studied by DRX using a cobalt anticathode and a curve detector CPS 120 (Inel) for determining silver complex form. This analysis has been made by the Laboratoire d'Electrochimie des Matériaux UMR CNRS 7555, Metz (France).

Bioreactor operations

The batch experiments were conducted in a 3.5-l fermentor, equipped with temperature, pH, and agitation speed control devices. The incubation temperature and agitation rate were 30 °C and 250 rpm. To limit silver precipitation in the TRIS-gluconate medium, 19 mM sodium thiosulfate was added to form a soluble complex ($[Ag_2(S_2O_3)_2]^{2-}$) before bacterial inoculation.

Silver solubilization was determined at different total silver concentrations. A 1.2 mM silver solution was added at the beginning of the experiment (before bacterial inoculation). Silver-adapted exponential growth phase bacteria were then added at a concentration of 5×10^4 cells/ml. A negative control without bacteria was made in accordance with the conditions above. As the growth medium darkened, the bacterial growth was evaluated on a solid TRIS-gluconate medium and silver concentrations were monitored.

Results

Soluble and non-soluble silver distribution with thiosulfate

Sodium thiosulfate is a silver complexing agent used to remove the unreacted and unexposed silver from photographic films, forming a soluble complex (Pethkar & Paknikar 2003). Soluble silver was determined in the TRIS-gluconate medium, which contained 19 mM sodium thiosulfate. The added silver concentration (total) and soluble silver showed a linear correlation ($R^2 = 0.98$) (data not shown) that confirmed that the reaction between silver and thiosulfate permits the chemical solubilization of at least 70% of total silver ions.

Resistance of C. metallidurans CH34 to silver stress

Cupriavidus metallidurans CH34 grew with 1.2 mM and without (control) silver. When 1.2 mM silver was added, the lag phase was three times longer than that observed for the silver-free medium. This lag phase increased as a function of silver concentration (data not shown). During exponential growth phase, the bacterial growth rate was higher in the presence of silver. Finally, maximal bacteria growth was similar, both with and without silver in the culture medium. From these results, it is reasonable to consider that *Cupriavidus metallidurans* CH34 possesses a silver resistance mechanism.

The MIC of *C. metallidurans* CH34 (both plasmids), AE128, AE126, and AE104 (no plasmids) was determined by culture in liquid TRIS-gluconate medium containing increasing concentrations of silver. After 5 days of incubation, only *C. metallidurans* CH34 and AE128 were able to grow in the presence of silver. The MIC was about 5 mM Ag^{2+} for the two strains (Figure 1). Nevertheless, no CFU were observed from the cultures inoculated with *C. metallidurans* AE126 and AE104. These results indicate that silver resistance is carried on plasmid *pMOL30*. Moreover, a precipitate appeared with *Cupriavidus metallidurans* CH34 and AE128, but no precipitate was observed with *Cupriavidus metallidurans* AE126, AE104, and dead cells.

Batch silver bioremediation

The capacity of *C. metallidurans* CH34 to resist soluble silver could allow the use of this bacterium for silver industrial wastes remediation. *C. metallidurans* CH34 was cultivated in a bioreactor (batch culture) containing a TRIS-Gluconate medium supplemented with 1.2 mM of soluble silver. Bacterial growth and soluble silver concentrations were measured at regular time intervals (Figure 2). Silver concentration in the presence of *C. metallidurans* CH34 decreased. After 6 days, the soluble silver concentration

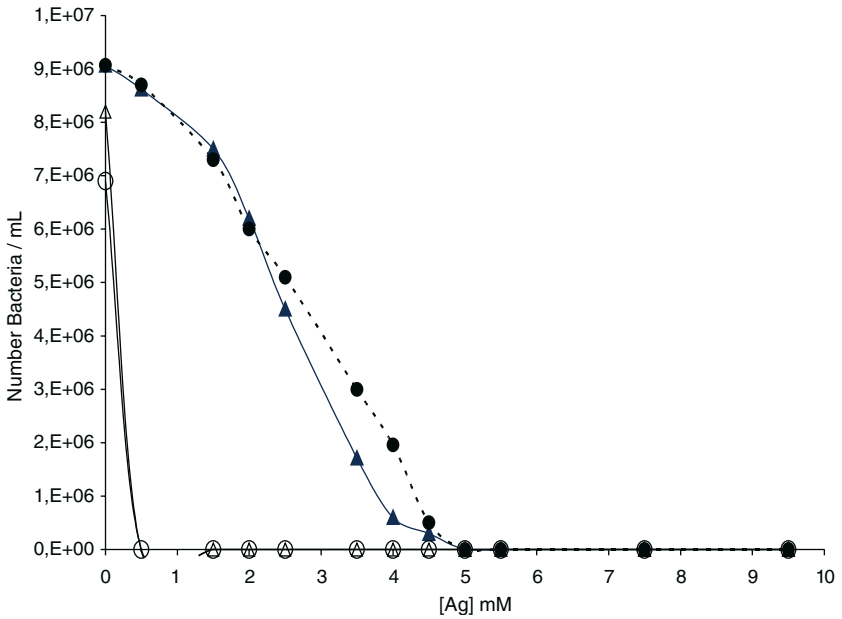

Figure 1. Silver MIC determination for *Cupriavidus metallidurans* CH34 (▲), *Cupriavidus metallidurans* AE128 (●), AE 104 (Δ) and AE 126 (○) grown in liquid TRIS-gluconate medium, containing increasing amount of silver (from 1 to 9.5 mM), at 30 °C after 5 days.

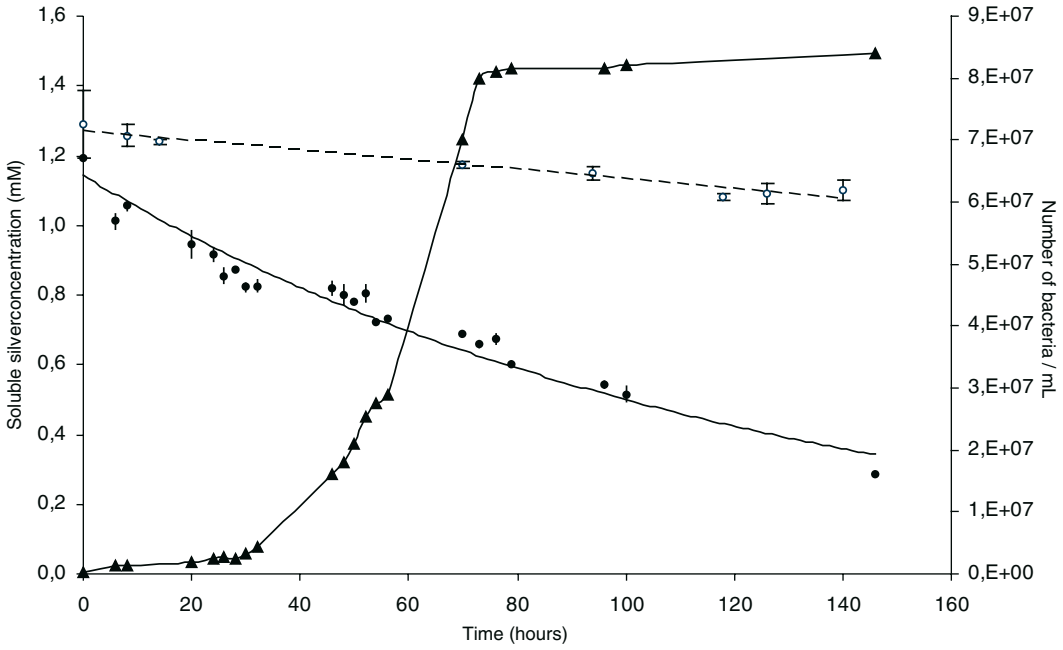

Figure 2. Silver concentration (mM) and growth of *Cupriavidus metallidurans* CH34 as a function of time in a liquid TRIS – gluconate medium batch reactor, containing 1.2 mM silver (○: silver concentration without bacteria, ●: silver concentration with bacteria, ▲: bacterial growth).

was 0.28 mM. Silver was precipitated by *C. metallidurans* CH34 with an efficiency exceeding 75% of the initial soluble silver concentration.

The control without bacteria showed no significant difference between the initial (1.2 ± 0.1 mM) and final (1.10 ± 0.03 mM) concentrations of soluble silver.

Silver elimination pathway

About 30 h after inoculation with *C. metallidurans* CH34, the liquid medium started to blacken. After centrifugation at 6000×*g*, 20 min, the supernatant was colorless.

Washing of the cells with a saline buffer solution did not decrease the black color of the pellet, suggesting that silver was associated to cells.

To investigate the mechanism of resistance and to determine the localization of silver particles in *C. metallidurans* CH34, bacterial cells grown in the presence of 1.2 mM silver were observed by electron microscopy (Figure 3). Electron microscopy micrographs showed silver electron dense particles in *C. metallidurans* CH34 cells (Figure 3). Dark round dense particles were accumulated in the outer membrane. By contrast, no particles were observed in the cytoplasm (Figure 3).

The presence of silver particles in *C. metallidurans* CH34 membrane was confirmed by EDX analysis (Figure 4). This analysis also revealed the presence of carbon and oxygen, probably coming from the outer membrane components and sulphur that remained from the thiosulfate-silver transformation.

To determine the chemical form of silver in the *Cupriavidus metallidurans* CH34 outer membrane,

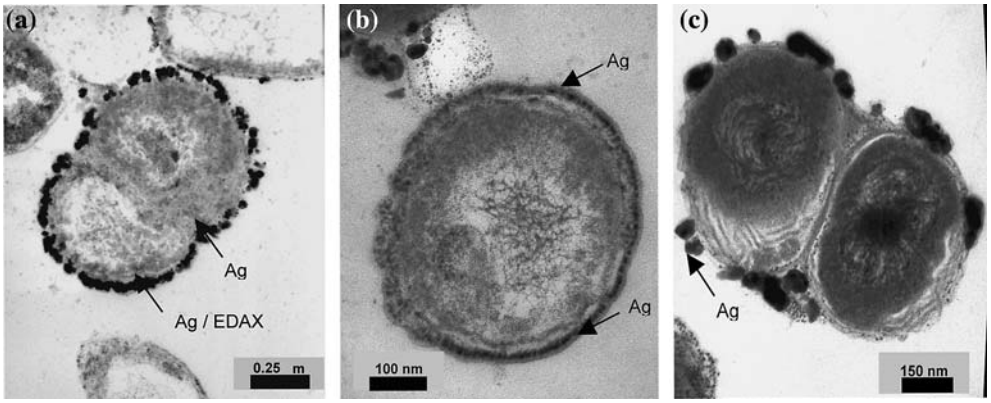

Figure 3. Localization of electron-dense putative elemental silver bodies in the *Cupriavidus metallidurans* CH34 cells after 5 days growth in the presence of 1.2 mM silver.

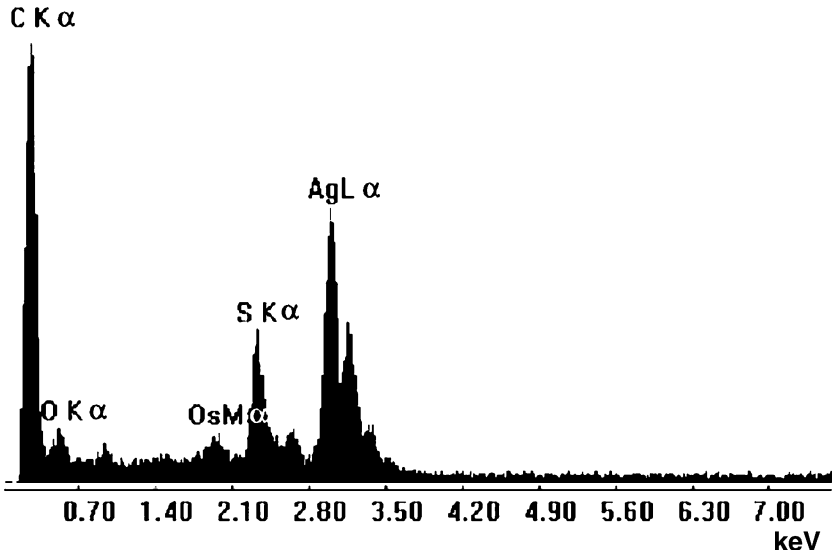

Figure 4. EDX microanalysis spectrum of the electron-dense body contained in a *Cupriavidus metallidurans* CH34 cell, grown in the presence of 1.2 mM silver.

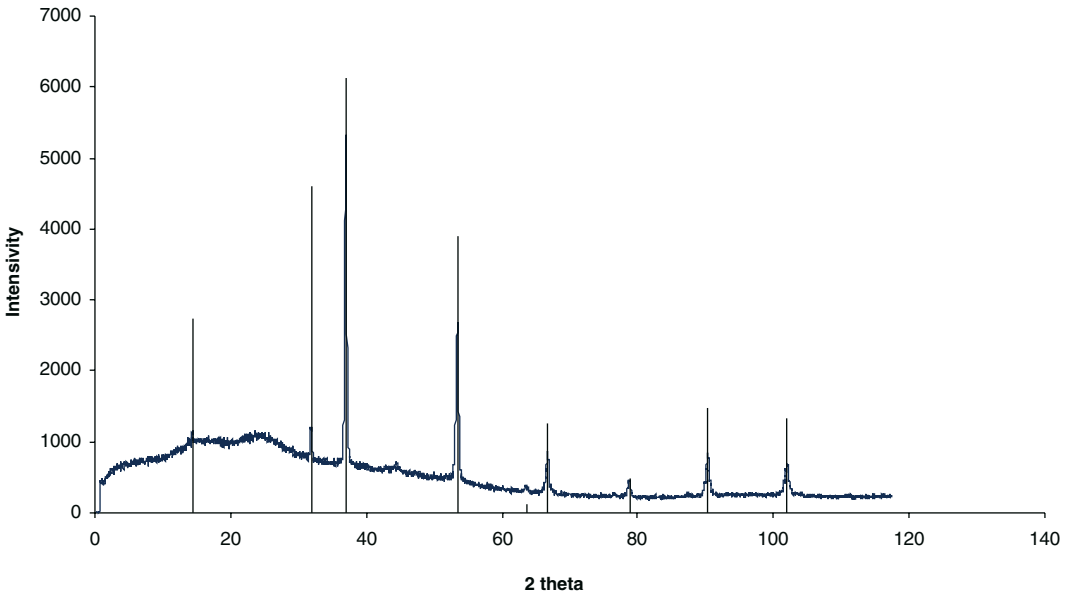

Figure 5. DRX spectrum of *Cupriavidus metallidurans* CH34 outer membrane (cells were grown in a liquid TRIS – gluconate medium supplemented by 1.2 mM silver), corresponding to AgCl spectrum, excluding over Ag forms (ASTM no. 31–1238 report).

X-diffractometry analysis was performed with this bacterial membrane (Figure 5). An experimentally obtained spectrum showed characteristic peaks at 14.4, 31.8, 36.9, 53.4, 63.5, 66.7, 78.7, 90.3, and 102.0 2-theta. This profile corresponds to the theoretical spectrum of AgCl, according to report no. 31–1238 of the ASTM database. This DRX spectrum showed that silver thiosulfate was converted into silver chloride.

Discussion

Cupriavidus metallidurans CH34 was found in a heavy metal-contaminated soil. This bacterial strain contains two megaplasmids (*pMOL28* and *pMOL30*), which confer resistance to a broad range of heavy metals (Cd^{2+}, Co^{2+}, Zn^{2+}, Tl^{+}, Cu^{2+}, Pb^{2+}, Ni^{2+}, Hg^{2+} and CrO_4^{2-}). Recently, *C. metallidurans* CH34 tolerance to gadolinium (Andrès *et al*. 2000) and selenium (Roux *et al*. 2001) have been demonstrated. This report describes for the first time the ability of *C. metallidurans* CH34 to resist soluble silver. Silver (2003) suggested that silver was used by bacteria as a final electron acceptor, but only after the bacterial exponential phase. In any case, details of silver mechanism in the bacterial respiratory chain are not well known.

Several mechanisms of heavy metal resistances are determined by genes of *C. metallidurans*: divalent cation efflux, bioaccumulation, cytoplasmic accumulation, and cation reduction (Nies *et al*. 1987; Nies & Silver 1989; Siddiqui *et al*. 1989). In the case of silver resistance, *C. metallidurans* CH34 seems to absorb monovalent silver from the soluble silver-thiosulfate complex. The EDX analysis showed the presence of silver in the bacterial cell layer. More precisely, the *C. metallidurans* CH34 outer membrane location of dark electron-dense particles suggests the accumulation of silver in this structure. Accumulation mechanisms for other metals such as selenium (Roux *et al*. 2001), zinc, and cadmium (Siddiqui *et al*. 1989; Volesky 1990; Mergeay *et al*. 2003) in *Cupriavidus metallidurans* CH34 have been described. Moreover, silver accumulation has already been described, but the relationship between the silver accumulation and the bacterial efflux resistance has not been established (Simmons & Singleton 1996; Silver 2003).

To determine the genetic location of the silver resistance determinant, *C. metallidurans* derivative strains AE126 (*pMOL30* free), AE128 (*pMOL28*

free), and AE104 (plasmid free) were tested. For *C. metallidurans* AE126 and AE104, growth was inhibited in the presence of silver. These results indicate that the silver resistance in *C. metallidurans* CH34 is localized on *pMOL30*.

To measure the bioremediation capacity of *C. metallidurans* CH34 to remove silver, batch cultures were performed in the presence of 1.2 mM soluble silver and 19 mM sodium thiosulfate. Silver was precipitated by *C. metallidurans* CH34 with an efficiency exceeding 75% of the initial soluble silver concentration after 6 days of culture. This efficiency is comparable with this of Pethkar & Paknikar (2003), which worked on the biosorption of a photographic processing wastewater by bacteria. They obtained a bioremediation efficiency of about 70%, from 0.6 mM silver and 12 mM thiosulfate solution using two micro-organisms *Thiobacillus thioparus* and *Cladosporium cladosporioides*.

Cupriavidus metallidurans CH34 has already been used in bioremediation processes leading to the removal of metal in an insoluble form from soils and effluents (Diels *et al*. 1995b, 1999; Dong *et al*. 1998). It now appears that it can also be used as a biological treatment for waste water contaminated with silver.

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