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Effect of the siderophore alcaligin E on the bioavailability of Cd to *Alcaligenes eutrophus* CH34

A Gilis, P Corbisier, W Baeyens, S Taghavi, M Mergeay and D van der Lelie

Milieutechnologie, Vlaamse Instelling voor Technologisch Onderzoek (VITO), Boeretang 200, B-2400 Mol, Belgium

Alcaligin E, the siderophore of the heavy metal-resistant *A. eutrophus* strain CH34, was shown to interact with Cd and consequently affect its bioavailability and toxicity. The addition of alcaligin E markedly stimulated the growth in the presence of Cd of an alcaligin E-deficient CH34 derivative. Using bioluminescence assays, this effect could be assigned to a decrease in bioavailability of Cd in the presence of alcaligin E. However, Cd-uptake studies showed no influence of alcaligin E on the cellular concentration of Cd. Furthermore, by scanning electron microscopy, the morphology of precipitated Cd crystals was shown to be altered by alcaligin E. These data suggest that alcaligin E, besides its function in iron supply to the cell, provides a protection against heavy metal toxicity. A link between the *A. eutrophus* CH34 siderophore system and the *czc*-mediated Cd-efflux system is hypothesized.

Keywords: siderophore; *Alcaligenes eutrophus*; heavy metal resistance; *czc*; bioavailability; biosensor

Introduction

In virtually all microorganisms, iron plays an irreplaceable role as cofactor for a variety of functional proteins and enzymes. Due to the formation of insoluble ferric hydroxide complexes under aerobic conditions and at neutral pH, the bioavailability of iron is severely restricted. In response to iron limitation, microorganisms have evolved specialized high affinity transport systems. A common strategy is the production and excretion of siderophores, high affinity iron-ligating compounds that transport ferric iron into the cell via specific ferric iron-siderophore receptors. Siderophores do not only interact with ferric iron, they also display a weaker but still significant affinity for other metal ions [16]. This interaction can affect the bioavailability and consequently the toxicity of metals to microorganisms. Therefore, a correlation is thought to exist between siderophore production and heavy metal resistance.

Metal-tolerant *Alcaligenes eutrophus* strains are often encountered in environments containing high levels of heavy metals. The type strain CH34 was shown to possess two megaplasmids, pMOL28 and pMOL30, conferring resistance to a variety of heavy metals [4,15]. Among the best characterized determinants are the *cnr* operon (resistance to Co and Ni) on pMOL28 and the *czc* operon (resistance to Cd, Zn and Co) on pMOL30. The resistance mechanism in both cases is based on cation efflux [6]. This efflux of heavy metals, which includes a proton antiporter system, results in a pH increase outside the cell. In correlation with the progressive alkalization of the medium, *A. eutrophus* CH34 can accumulate and precipitate heavy metals like Cd and Zn. The sequestration appears to be associated with the outer membrane [6] and it is thought to be initiated on carbonates and functional groups of outer membrane polysaccharides and proteins [7]. This phenomenon of bioprecipitation has been exploited in the design of bioreactors for the removal of metals from effluents [7]. Also, metal-responsive genes involved in these resistance mechanisms have been used for the development of metal biosensors, based on the fusion of the former genes to a reporter system [5]. Although the concentration of heavy metals can be determined from an environmental sample, the amount of the metals, which are biologically available, cannot be determined by simple chemical analysis. Since resistance genes are induced in response to the bioavailable concentration of heavy metal, metal biosensors based on resistance genes can be used to determine the bioavailability of heavy metals in the environment.

Recently, the synthesis of a novel phenolate type siderophore, named alcaligin E, was observed in *A. eutrophus* CH34 and the ferric iron-alcaligin E receptor was identified [12]. Therefore, *A. eutrophus* CH34 is an excellent model system for the study of the interactions between siderophore-mediated iron uptake and heavy metal resistance.

In this work, the relationship between alcaligin E production and Cd bioavailability to *A. eutrophus* CH34 is investigated.

Materials and methods

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are described in Table 1. *A. eutrophus* and *E. coli* were grown at 30°C and 37°C, respectively. Growth in the presence of heavy metal salts was determined on Tris-minimal-medium [15] by following the OD_{660}. Fe(III) was omitted from this medium in order to screen for siderophore production in iron-limiting conditions. The chrome azurol S (CAS) shuttle solution [23] was used for routine testing of siderophore production and for semi-quantitative determination of the concentration of siderophores in liquid media. Antibiotic-resistant clones were selected on media supplemented with 20 μg ml^{-1} tetracycline, 100 μg ml^{-1}

Correspondence: D van der Lelie, Milieutechnologie, Vlaamse Instellung voor Technologisch Onderzoek (VITO), Boeretang 200, B-2400 Mol, Belgium
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Table 1 Bacterial strains and plasmids used

Strain/plasmid	Relevant characteristics	Source/reference
Strains		
Alcaligenes eutrophus		
CH34	Wild-type, pMOL28, pMOL30	[15]
AE104	Plasmid-free derivative of CH34	[15]
AE1152	*Ale*B1152::Tn*5*-Tc, Sid$^-$, Tc^R, Km^R	[12]
AE1153	*Ale*O1153::Tn*5*-Tc, Sid^c, Tc^R, Km^R	Gilis *et al*, in preparation
AE1433	pMOL28, pMOL30-*czc*S::Tn*4431*, Tc^R	[5, 25]
AE1595	*ale*-1595::miniTn*5*-Km1, Sid$^-$,Km^R	[12]
AE2263	pMOL28, pMOL30-*czc*S::Tn*4431*, *ale*O::Km, Sid^c, Km^R, Tc^R	This study
AE2350	pMOL28, pMOL30, *czc*S::Tn*4431*, *ale*-1595::miniTn*5*-Km1, Sid$^-$, Km^R, Tc^R	This study
Escherichia coli		
DH10B	plasmid free	Gibco BRL
CM404	pRK2013	
CM1570	pUC18	
Plasmids		
pRK2013	Km^R,Tra$^+$	[10]
pUC18	Ap^R, lac$^+$	[17]
pMOL1013	Ap^R, based on pUC18	This study
pMOL1056	Tc^R, *ale*O$^+$, hybrid pLAFR3 derivative	This study
pMOL1077	Ap^R, *ale*O$^+$, based on pUC18	This study
pMOL1083	Ap^R, *ale*O::Km^R-genblock, based on pMOL1077	This study

ampicillin, 25 μg ml^{-1} chloramphenicol, or 50 (for *E. coli*) to 1000 (for *A. eutrophus*) μg ml^{-1} kanamycin. Schatz lactate medium [22] was chosen for experiments with Cd under precipitating conditions. Due to the large amount of phosphate in this medium, Cd precipitation occurs immediately upon addition of $CdCl_2$, forming a visible Cd-phosphate precipitate.

So far, no strategy has been found to obtain alcaligin E in pure form. Therefore, alcaligin E was added to the growth medium in the form of culture supernatant of the alcaligin E-overproducing CH34 derivative AE1153 (Gilis *et al*, in preparation). This strain was grown during 24 h in iron-limited Schatz lactate medium. After growth, the concentration of alcaligin E in the medium was estimated to be about 80 μM by means of the CAS-shuttle solution [23]. The AE1153 culture was then centrifuged (20 min, $3000 \times g$) and passed through a 0.45-μM filter to remove all bacteria. Subsequently, the alcaligin E-containing culture supernatant was added to the growth medium to obtain a 1/10, 1/50 and 1/100 dilution. To rule out matrix effects, iron-limited culture supernatant of AE1595, a siderophore-deficient mutant of CH34, was always included as control.

Molecular cloning techniques and electroporation

Restriction enzymes, phosphatase, and T4 DNA ligase were purchased from GIBCO BRL (Merelbeke, Belgium) and used as recommended by the supplier. Molecular cloning techniques and plasmid DNA isolation from *E. coli* were performed as described by Sambrook *et al* [21]. Genomic DNA was isolated from *A. eutrophus* as described for *Bacillus subtilis* according to the method of Bron and Venema [2]. Electrocompetent cells of *E. coli* were prepared and transformed with the BioRad Gene Pulser (BioRad Laboratories, Richmond, CA, USA) according to the manufacturer's instructions. Electrocompetent cells of *A. eutrophus* were prepared and transformed with linearized DNA as described previously [24].

Bioluminescence assay

Biosensor strains were precultured overnight in iron-limited Schatz lactate medium. Subsequently, the cultures were diluted to an OD_{660} of 0.1 in fresh iron-limited Schatz lactate medium to which increasing concentrations of $CdCl_2$ were added and grown on a rotary shaker for 4 h. Then, a 500-μl sample was transferred into a Lumacuvette-P#9200-0 tube, which was incubated for 30 min at 23°C. The bioluminescence was measured during 10 s in a Lumac M2010 luminometer (Landgraaf, The Netherlands).

Cd-uptake assay

The study of the alcaligin E-mediated uptake of Cd was based on a previously described protocol [13]. *A. eutrophus* CH34, its heavy metal-sensitive derivative AE104 and the *ale*B (ferric iron-alcaligin E receptor) mutant AE1152 were grown in 2-L flasks in iron-limited Tris minimal medium with acetate, β-glycerophosphate and MOPS buffer. In this medium, no precipitation of Cd occurs. When the OD_{660} reached a value of approximately 1.0, the cells were washed twice with fresh medium and were five-fold concentrated into the new medium.

To follow the Cd uptake in function of time, alcaligin E-containing concentrated culture supernatant which was obtained by freeze-drying, was first incubated during 1 h with $CdCl_2$ and subsequently added to the medium. The final concentration of alcaligin E was approximately 15 μM and that of Cd 4 μM. Under these conditions, an excess of siderophores compared to Cd is present in the system. As negative control, the experiment was also performed with the same concentration of siderophore-free AE1595 culture supernatant. Five-fold concentrated cells were added to this

medium. Samples were collected after 2, 5, 10, 20, 30, 60, 120 and 240 min.

To study the effect of different alcaligin E concentrations on the Cd uptake, the same alcaligin E concentrations (0, 0.8, 1.6 and 8.0 μM) were used as in the growth and bioluminescence experiments. The cadmium concentration was chosen to be equal to the free Cd concentration when 1 M $CdCl_2$ was added to Schatz lactate medium. This concentration was estimated by passing a 1 M $CdCl_2$ supplemented Schatz lactate solution through a 0.2-μm filter and by subsequently measuring the Cd concentration in the filtrate using inductivity coupled plasma emission spectrometry (ICP). The free Cd concentration was estimated to be 221 μM. The samples were collected after 4 h of incubation.

Immediately after collection of the samples, they were centrifuged ($3000 \times g$, 10 min) and washed twice with 0.2 M EDTA (to remove unbound Cd) and once with distilled water. The cells were dried by lyophilisation and weighed. The amount of Cd was determined by ICP analysis.

Scanning electron microscopy

Strain AE1595 (Sid^-) was grown in minimal medium with different concentrations of Cd (0, 1, 2, 4 and 8 mM) with or without the addition of approximately 0.8 μM alcaligin E. Alcaligin E was added in the form of culture supernatant derived from AE1153. After growth, 200 μl of culture (and precipitate) were collected on a 0.45-μm filter. The samples were fixed by vacuum-drying, gold-stained and examined by scanning electron microscopy (SEM).

Results

Effect of alcaligin E on the toxicity of Cd

Because siderophores are known to interact with divalent heavy metals, alcaligin E is likely to affect the resistance of *A. eutrophus* CH34 against these metals.

To study the effect of alcaligin E on the toxicity of Cd, the growth of the alcaligin E-deficient CH34 derivative AE1595 [12] was studied under increasing concentrations of Cd in the absence and presence of different concentrations of alcaligin E (Figure 1), added in the form of iron-limited AE1153 culture supernatant. As a control, the same concentration of iron-limited culture supernatant of an alcaligin E-deficient mutant (AE1595) was included. In the growth medium (Schatz lactate), there exists an equilibrium between Cd in solution (toxic) and Cd precipitated in the form of Cd phosphate (inert). Alcaligin E can potentially solubilize the precipitated Cd, opening the way to two possible scenarios. Cd can become toxic to the cell if AleB (the ferric iron-alcaligin E receptor) can take up the Cd-alcaligin E-complex and if this complex is subsequently dissociated. Alternatively, when the internalized Cd-alcaligin E complex is not dissociated or if AleB does not internalize the complex, Cd will become less toxic to the cell by complexation to alcaligin E. The results presented in Figure 1 were representative for three independently performed experiments. In the absence of alcaligin E, growth was repressed in 1 mM added $CdCl_2$. In the presence of approximately 0.8 μM alcaligin E, the bacteria were able to grow in the presence of up to 4 mM added Cd. In the presence of approximately 1.6 μM alcaligin E, the growth is slightly increased compared to that observed with 0.8 mM. When approximately 8 μM of alcaligin E was added to the growth medium, the bacteria were able to grow in the presence of 8 mM added $CdCl_2$. With the iron-limited AE1595 culture supernatant, no effect on the growth could be observed (data not shown). The beneficial effect of alcaligin E was also observed with Cd under non-precipitating conditions. Therefore, it can be concluded that alcaligin E helps the bacteria to survive in the presence of high Cd concentrations, favouring the second hypothesis.

The WT strain (CH34) was able to grow in the presence of up to 4 mM added Cd. It has to be taken into account that alcaligin E was not present in the growth medium from the start of the experiment (as it was for AE1595 with added alcaligin E). The production of alcaligin E had to be initiated by the WT strain. This process requires time (during which Cd can exert its toxic effect) and energy (for the synthesis of alcaligin E and the ferric iron-alcaligin E uptake machinery), causing retardation of growth.

The beneficial effect of alcaligin E on the growth of strain AE1595 in the presence of Cd might also be explained by the fact that alcaligin E helps the bacteria to take up trace amounts of iron that are present in the iron-limited growth medium, thereby resolving the iron stress. In iron-replete conditions (30 μm $FeCl_3$), the growth of strain AE1595 in the presence of Cd is comparable to that observed in iron-limiting conditions in the absence of alcaligin E. However, high Cd concentration can lead to precipitation of iron (M Hofte, personal communication). Therefore, relief of the iron stress by alcaligin E cannot be excluded to participate in the growth stimulation. If alcaligin E would interact directly with Cd to decrease its toxic effect, this would be reflected in a bioavailability decrease of Cd to the bacterium.

Construction of an alcaligin E-overproducing and an alcaligin E-deficient mutant of strain AE1433

To study the effect of alcaligin E on heavy metal bioavailability of Cd, it was decided to construct both a constitutively alcaligin E-overproducing (*ale*O) and an alcaligin E-deficient mutant of the Cd, Zn, Co and Pb biosensor strain AE1433 [25]. In order to construct the *ale*O-mutant of strain AE1433, it was decided to clone and subsequently inactivate the *ale*O-gene by introducing a Km-resistance marker, followed by inactivating the *ale*O-gene of AE1433 by gene replacement. The *ale*O-gene was cloned as a 3.5-kb *Pst*I fragment from pMOL1056 into pUC18. This plasmid was called pMOL1077. Subsequently, the *ale*O-gene on pMOL1077 was inactivated by introducing the Kanamycin Resistance GenBlock (Pharmacia, Uppsala, Sweden). The restriction map of pMOL1077 showed two *Bgl*II sites, one of which was located in the *ale*O-gene. A partial digestion of pMOL1077 with *Bgl*II was performed. Linearized pMOL1077 was dephosphorylated and ligated with the *BamH*I-digested Kanamycin Resistance GenBlock. After electroporation into DH10B, transformants were selected for Km-resistance. Restriction analysis was performed to select those transformants that contained the Kanamycin Resistance GenBlock in the *ale*O gene. The plasmid

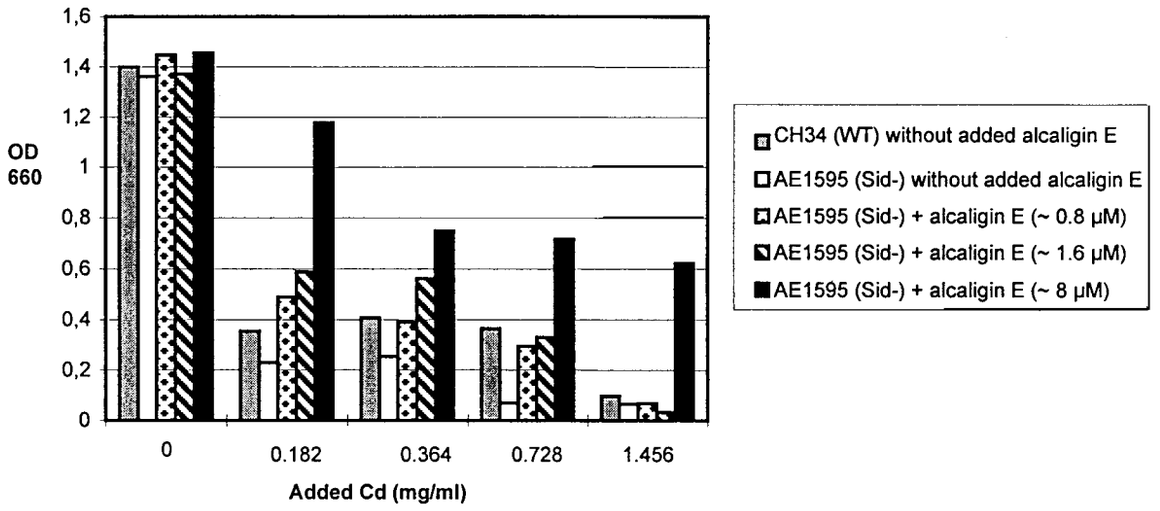

Figure 1 Influence of alcaligin E on the growth of the alcaligin E-deficient strain AE1595 in the presence of different amounts of Cd in precipitating conditions. The OD_{660} was determined after 44 h of growth in Schatz lactate medium. As a control, the growth of the wild-type strain *A. eutrophus* CH34 was determined without the addition of alcaligin E. The results presented are representative for three independently performed experiments.

obtained in this way was called pMOL1083. Gene replacement was performed by transforming AE1433 cells with the 3.1-kb *Bgl*II-*Sph*I fragment of pMOL1083, containing the Kanamycin Resistance GenBlock with 0.8 kb right- and 1.3 kb left-flanking DNA. Transformants were selected for Km and Tc resistance, and their siderophore constitutive phenotype was checked in minimal medium without iron or with 10 μM iron. The ability to function as biosensor was checked by comparing the light production in minimal medium with or without Cd. The *ale*O mutant of strain AE1433 obtained in this way was called AE2263.

For the construction of the AE1433 alcaligin E-negative mutant, a triparental mating was performed with AE1595 (*ale*-1595::miniTn*5*-Km1) as the acceptor strain [12], AE1433 (pMOL30-*czc*S::Tn*4431*) as the donor strain and CM404 (with pRK2013) as the helper strain. Transconjugants were selected on minimal medium containing tetracycline and kanamycin. Their Sid$^-$ phenotype was checked after growth in minimal medium without iron, and their ability to function as biosensor was checked by comparing the light production in minimal medium with or without Cd. The *ale*-1595 mutant of strain AE1433 obtained in this way was called AE2350.

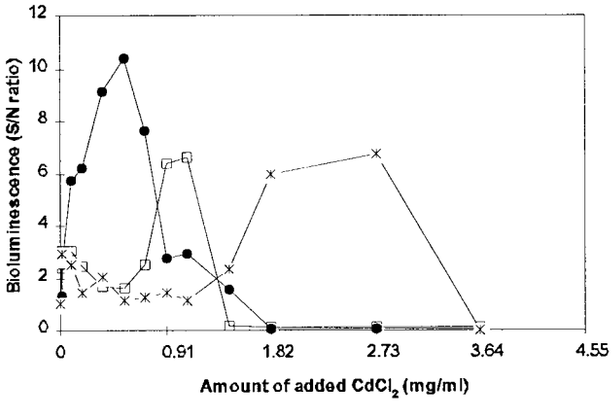

Figure 2 Bioluminescence profile of the *ale*-1595 (Sid$^-$) Cd, Zn, and Pb biosensor (AE2350) in the presence of increasing amounts of Cd in precipitating conditions, with and without addition of alcaligin E-containing supernatant. (●) Without alcaligin E; (□) with approximately 1.6 μM alcaligin E-containing supernatant; (*) with approximately 8 μM alcaligin E-containing supernatant. The light production, expressed in RLU (relative light units) was measured after 4 h and related to the OD_{660} of the culture. The results presented are representative for three independently performed experiments.

Effect of alcaligin E on the bioavailability of Cd

In order to determine a possible effect of alcaligin E on the bioavailability of Cd, the wild-type (AE1433), *ale*O$^-$ (AE2263) and *ale*-1595$^-$ (AE2350) biosensor strains were subjected to bioluminescence assays. Strain AE2350 was incubated in the presence of increasing concentrations of Cd (50 μM to 20 mM under precipitating conditions) in the absence or presence of added alcaligin E (approximately 1.6 and 8 μM, which are the same concentrations of alcaligin E as used in the growth experiment). Figure 2, which is representative for the data of three independently performed experiments, shows that in the absence of alcaligin E, the maximum light production occurs when 3 mM Cd was added. In the presence of alcaligin E, the bioluminescence profile was shifted towards higher Cd concentrations and the maximum light production occurred at about 6 mM added Cd with 1.6 μM alcaligin E, and at about 12 mM added Cd with 8 μM alcaligin E-containing supernatant. Also, the toxicity phase (S/N ratio < 1), leading to a decrease of the bioluminescence was obtained at higher Cd concentrations. This indicates that the bioavailability of the Cd is decreased in the presence of alcaligin E. This result is in agreement with the earlier observation of growth stimulation in the presence of alcaligin E. The growth stimulation could thus be explained by a bioavailability decrease of the Cd.

In a second experiment the wild-type (AE1433), the *ale*O$^-$ (AE2263), and the *ale*-1595 (AE2350) biosensor strains were incubated in the presence of increasing concentrations of Cd (50 μM to 20 mM under precipitating

conditions). Figure 3, which is representative for three independently performed experiments, shows that the bioluminescence profile of the wild-type strain (able to produce alcaligin E) was shifted towards lower Cd concentrations as compared to the *ale*-1595 mutant. The maximum light production of the wild-type biosensor occurred at about 1 mM, whereas that of the *ale*-1595 mutant appeared at about 3 mM. The toxicity phase was also displaced to lower Cd concentrations for the wild-type strain. For the *ale*O$^-$ biosensor strain, a similar bioluminescence shift was obtained (data not shown). This suggests that alcaligin E (produced by the wild-type and the *ale*O$^-$ strain) can make Cd more bioavailable. At first sight, this seems to be in contradiction to the former bioluminescence experiment and the earlier observation of growth stimulation by alcaligin E in the presence of Cd. However, the amount of siderophores present in the growth medium during these experiments is of a completely different order of magnitude. In the growth experiment and the first bioluminescence experiment, alcaligin E is added directly to the culture medium in a relatively high concentration (from approximately 0.8 to 8 μM). This cannot be compared to the alcaligin E production after 3.5 h growth in the second bioluminescence experiment, which is too low to be detected by the CAS test.

Alcaligin E-mediated Cd uptake

To study the effect of alcaligin E on Cd uptake, strains CH34, AE1152 (a CH34 mutant deficient in alcaligin E uptake), and AE104 (a plasmid-free CH34 derivative, lacking the *czc*-system) were concentrated to a calculated OD_{660} of 5 and incubated with Cd (4 μM) and alcaligin E-containing (approximately 15 μM) culture supernatant. As a control, parallel experiments were performed with culture supernatant of an alcaligin E-deficient strain. At different time intervals, samples were taken and the cells were analyzed for their Cd content. The results, which are representative for three independently performed experiments, are presented in Figure 4. Apparently, there is no significant difference in Cd uptake of cells incubated with or without alcaligin E. Also, the curves for CH34 and AE1152 seem to evolve similarly. However, the Cd uptake by strain AE104 (*czc*$^-$) is significantly lower than for CH34 and AE1152 (both *czc*$^+$). This might be explained by the functioning of the *czc*-system that is accompanied by an increased bioprecipitation [6].

The experiment was also performed for CH34, AE1152 and AE104 using the same conditions as for the growth and bioluminescence experiments. The Cd concentration was 221 μM, since this was estimated to be the free Cd concentration for Schatz lactate medium to which 1 M $CdCl_2$ was added. No difference could be observed between the Cd concentrations for cells incubated with different concentrations of alcaligin E (data not shown). As in the former experiment, the Cd content of AE104 was lower than that of CH34 and AE1152. These results indicate that alcaligin E does not influence the uptake of Cd by *A. eutrophus* CH34.

Influence of alcaligin E on the crystal structure of precipitated Cd

Scanning electron microscopy was used in order to observe possible changes in the morphology of precipitated Cd crystals in the presence of alcaligin E. It was observed that the morphology of the $CdHPO_4$ precipitate in the culture markedly changed due to bacterial activity (Figure 5). One of the important parameters was the presence or synthesis of alcaligin E. At low Cd concentrations (up to 4 mM added $CdCl_2$), the cultures grew rapidly, and amorphous Cd precipitates were formed, both in the presence and absence of alcaligin E. At high Cd concentrations (8 mM $CdCl_2$ added), bacterial growth was only possible in the presence of low concentrations of alcaligin E. Under these conditions, where only slow bacterial growth was observed, the orthorhombic $CdHPO_4$ crystals disappeared and monoclinic

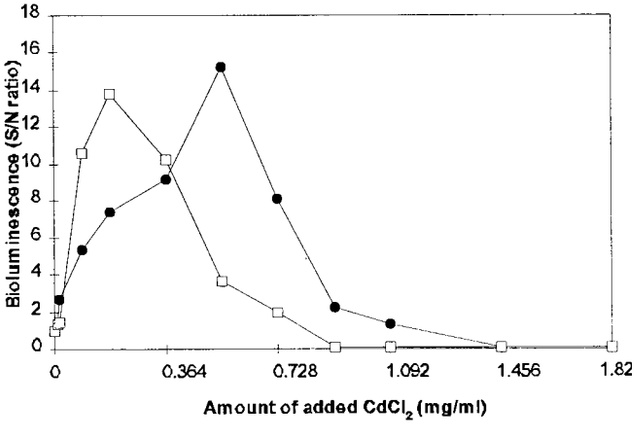

Figure 3 Bioluminescence profile of the *ale*-1595 (Sid$^-$) (AE2350) (●) and the wild-type (AE1433) Cd, Zn, and Pb biosensor (○) in the presence of increasing amounts of Cd in precipitating conditions. The light production, expressed in RLU (relative light units) was measured after 4 h and related to the OD_{660} of the culture. The results presented are representative for three independently performed experiments.

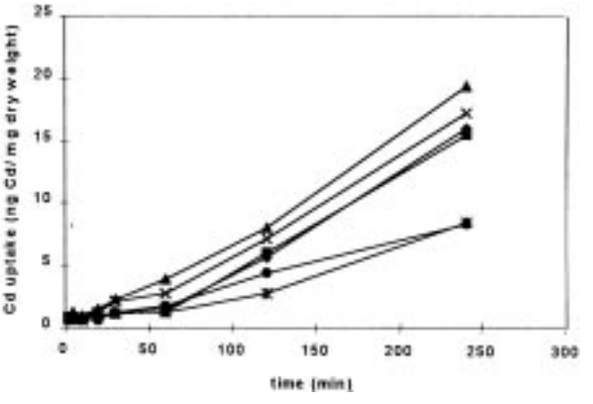

Figure 4 Cd uptake as a function of time by *A. eutrophus* CH34, AE1152 (deficient in alcaligin E uptake) and AE104 (plasmid-free), grown under iron-deficient conditions in medium containing 4 μM Cd and iron-limited supernatant of an alcaligin E-overproducing (AE1153) and an alcaligin E-deficient (AE1595) mutant. Each point represents the average of three replicates. (◆) CH34 + AE1153 supernatant; (■) CH34 + AE1595 supernatant; (▲) AE1152 + AE1153 supernatant; (×) AE1152 + AE1595 supernatant; (*) AE104 + AE1153 supernatant; (●) AE104 + AE1595 supernatant. The results presented are representative for three independently performed experiments.

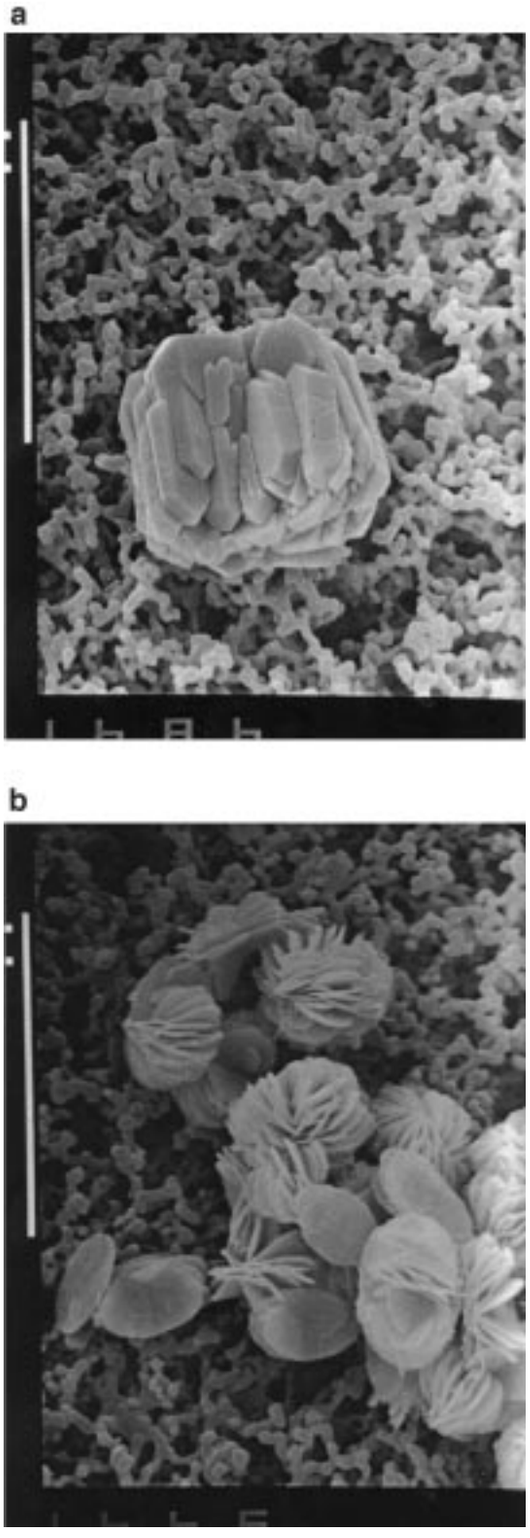

Figure 5 (a) Scanning electron micrograph of orthorhombic $CdHPO_4$ crystals formed when 8 mM $CdCl_2$ was added to iron-limited Schatz lactate medium. (b) Scanning electron micrograph showing monoclinic 'desert-rose'-like structures formed when a Sid^- derivative of CH34 was grown for 4 days in this culture medium in the presence of alcaligin E. The bar represents 10 μm.

'desert-rose'-like structures were formed (Figure 5). In case $FeCl_3$ was added to overcome the iron limitation and no alcaligin E was added or synthesized, no monoclinic structures were found. Instead, an amorphous Cd precipitate was formed. This indicates that alcaligin E directly influences the structure of the Cd precipitate that is formed due to bacterial activity.

Discussion

Siderophores are known to possess very high binding constants for ferric iron. Although with lower affinity, they also form complexes with metals other than iron. For example, the stability constants for complexes of the hydroxamate siderophore desferriferrioxamine B with Ga^{3+}, Al^{3+} and In^{3+} are in the range of 10^{20} to 10^{28} [9]. The stability constants between pseudobactin PSB3 and Zn^{2+}, Cu^{2+}, and Mn^{2+} range between 10^{17} and 10^{22} [3]. Both siderophores have a stability constant for ferric iron of about 10^{30}. In *Azotobacter vinelandii* competition between molybdate and Fe(III)hydroxide for the siderophore *N,N*′-bis(2,3-dihydroxybenzoyl)-l-lysine (LYSCAM) results in a delay of iron solubilization in the presence of molybdate [8]. Also, actinides have been shown to be bound and solubilized by microbial siderophores [1].

In this study, the interaction of alcaligin E, the siderophore of the heavy metal-resistant bacterium *A. eutrophus* CH34, with Cd was investigated. The lack of purified alcaligin E (up till now no effective strategy for the purification of alcaligin E was found) necessitated the use of alcaligin E-containing culture supernatant. Control experiments were included with culture supernatant of an alcaligin E-deficient CH34 derivative. Alcaligin E was found to decrease the toxic effect of Cd to *A. eutrophus* CH34, as was shown by a significant increase in growth of a Sid^- mutant in the presence of Cd, when alcaligin E was added (Figure 1). Scanning electron microscopy showed the modification of Cd precipitate crystals in the presence of alcaligin E (Figure 5). Bioluminescence assays with Cd biosensor strains, performed under the same conditions as the growth experiment, revealed that alcaligin E provokes a decrease in the bioavailability of Cd to the bacterium (Figure 2), evidencing a direct interaction between alcaligin E and Cd. This can explain the protective effect of alcaligin E on the growth in the presence of Cd. However, relief of the iron stress might also be partly responsible for the growth stimulation by alcaligin E in the presence of Cd.

In *A. eutrophus* CH34, Cd is taken up by means of the aspecific Mg-transport system. By performing Cd uptake studies in the absence and presence of an excess alcaligin E, no effect of alcaligin E on the uptake of Cd by *A. eutrophus* CH34 could be observed. If the ferric iron-alcaligin E receptor (AleB) would not recognize the Cd-alcaligin E complex, the Cd uptake would be expected to decrease in the presence of alcaligin E. Conversely, a ferric iron-alcaligin E receptor that internalizes Cd-alcaligin E would initially provoke an increase in cellular Cd concentration (if the rate of internalization is equal or higher than that of the Mg transport system). What happens thereafter depends on the fate of the Cd siderophore complex. Since

alcaligin E appears to have no influence on the intracellular Cd concentration, it might be possible that the *czc*-system effluxes the Cd that is internalized by alcaligin E. In this scenario, the observed toxicity decrease of Cd in the presence of alcaligin E, suggests a direct link between the *czc*-efflux system and the alcaligin E-mediated detoxification of Cd. One hypothesis could be that the *czc*-system is involved in the direct excretion of the Cd-alcaligin E-complex. The gene organization in *czc*CBA and the functions associated with these genes are strikingly similar to a range of bacterial export pathways including *orf*ABC of *Pseudomonas aeruginosa*, which is involved in resistances to antibiotics [20] and possibly in the secretion of the siderophore pyoverdine [19]. In *Pseudomonas fluorescens* ATCC 17400, a gene *cyt*A, resembling *czc*C, was shown to be involved in the export of heme and pyoverdine [11]. Another hypothesis could be that Cd is first dissociated from alcaligin E and then excreted by the *czc*-system. In this case, a mechanism that brings the Cd from the siderophore-complex to the *czc*-system, so that free Cd cannot exert its toxic effect, is needed. It can be expected that a bacterium that evolved such efficient heavy metal resistance mechanisms, will make sure that the produced siderophores do not turn the heavy metal more toxic to the cell. Rather, it seems that alcaligin E might function as a second protective mechanism against heavy metal toxicity.

Strikingly, when *A. eutrophus* CH34 biosensor strains with an intact alcaligin E system were subjected under iron-limiting conditions to 4-h bioluminescence assays in the presence of different Cd concentrations, their bioluminescence profile pointed to a bioavailability increase of the Cd, compared to the alcaligin E-deficient biosensor strains (Figure 3). The conclusion that can be drawn from this experiment, ie alcaligin E makes the Cd more bioavailable to the cell, seems to be in contradiction to the results that were previously found when alcaligin E was added in different concentrations to the Sid^- biosensor strains (Figure 2). However, the amount of siderophores present in both experiments cannot be compared: after 4 h of growth in iron-limited conditions, the alcaligin E concentration is so low that it cannot be detected with the CAS test, this in contrast to the experiment where alcaligin E was added to the culture from the beginning of the experiment. We postulate that alcaligin E plays a role in solubilizing the precipitated Cd. Due to the equilibrium 'Cd^{2+}-alcaligin E $\leftrightarrow$ Cd^{2+} + alcaligin E', an increase in free Cd-ions will occur that can be transported into the cell via the Mg transport system. There it can activate the *lux*-reporter system and, at higher concentrations, result in a toxic effect. In the situation where an excess of alcaligin E is present, the chance that free Cd ions will encounter a free siderophore increases, making them less toxic.

The observed interaction between alcaligin E and Cd opens the way to application perspectives in the field of bioremediation. Immobilized alcaligin E could be used to remove Cd or other heavy metal ions from contaminated waste-waters. As an alternative for acid leaching using acidophilic bacteria [14,18], non acid leaching processes based on increased solubilization of heavy metals by alcaligin E could evolve from further studies of the interaction between this siderophore and heavy metals. This leaching could be used for the treatment of industrial wastes, like catalysators and metal-containing wastes from non-ferro industries. In this way precious or rare metals could be recycled or toxic metals could be removed from wastes that cannot be recycled to guarantee a safe deposit.

Further research should address purification of alcaligin E, the dissociation constants between alcaligin E and different heavy metals and the regulation of alcaligin E by different heavy metals. Also, the link between siderophore production and heavy metal resistance deserves further attention.

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