Journal of Industrial Microbiology, (1995) 14, 142–153

The *czc* operon of *Alcaligenes eutrophus* CH34: from resistance mechanism to the removal of heavy metals

Ludo Diels, Qinghan Dong, Daniël van der Lelie, Wilfried Baeyens and Max Mergeay

Flemish Institute for Technological Research (VITO), B-2400 Mol, Belgium

(Received 16 August 1994; accepted 7 December 1994)

Key words: Soil bacteria; *Alcaligenes*; Plasmid; Cadmium; Metal resistance; Cation efflux; Gene fusion; Bioprecipitation; Bioreactor

SUMMARY

The plasmid-borne *czc* operon ensures for resistance to Cd^{2+}, Zn^{2+} and Co^{2+} ions through a tricomponent export pathway and is associated to various conjugative plasmids of *A. eutrophus* strains isolated from metal-contaminated industrial areas. The *czc* region of pMOL30 was reassessed especially for the segments located upstream and downstream the structural genes *czc* CBA. In cultures grown with high concentrations of heavy metals, *czc*-mediated efflux of cations is followed by a process of metal bioprecipitation. These observations led to the development of bioreactors designed for the removal of heavy metals from polluted effluents.

INTRODUCTION

Soils or sediments that are contaminated by mine or metallurgical wastes with high levels of heavy metals may contain heterotrophic bacteria harboring plasmid-borne resistance to toxic concentrations of these heavy metals. This was the case with numerous samples collected from the Zairian copperbelt of Shaba or from metallurgical factories (non-ferrous metals) in Western Europe [10].

Alcaligenes eutrophus CH34 and related bacteria are characteristic of these biotopes. *A. eutrophus* CH34 is a facultative chemolithotroph that harbors large plasmids hosting various operons responsible for resistances and other responses to heavy metals [29–31]. One of these operons, *czc* (resistance to Cd^{2+}, Co^{2+} and Zn^{2+}), encodes for a chemiosmotic proton antiporter-mediated efflux of cations [34,36,38; (Nies, pers. comm.)]. Gene probes made with *czc* and used in colony or dot blot hybridization allowed the detection of various strains related to *A. eutrophus* CH34 from the biotopes quoted above [10]. The megaplasmids of *A. eutrophus* CH34 contain other well-studied operons for heavy metal resistance such as *cnr* (resistance to Co^{2+}, Ni^{2+} and Zn^{2+} [5,26,45], *chr* [32,35], and mercury transposons [8]. Other genetic determinants that map on these plasmids are involved in resistance or response to Cu^{2+}, Pb^{2+}, Tl^{+} and Mn^{2+} (especially on pMOL30). Recently, the Cu and Pb resistance determinants were cloned but they are not yet understood at the DNA level and physiological studies do not provide clues about the mechanisms involved.

Concern for environmental bioremediation has focused on the possibility of using multiple-resistant bacteria for the removal of heavy metals from polluted effluents or sediments through a form of bioaccumulation that would follow the plasmid-mediated efflux or other resistance mechanism. The following phenomena were observed in cultures grown with organic acids in the presence of 2 mM Cd^{2+} or 5 mM Zn^{2+}: a progressive increase of pH up to a value of 9, and the concomitant removal of metals from culture supernatant fluids through precipitation of carbonates and hydroxides [9]. The pH increase is proportional to the initial concentration of toxic cations and is thought to be the immediate consequence of the *czc*-mediated proton antiporter efflux of cations. Removal of cations from the supernatant medium was so efficient that bioreactors based on the immobilization of *czc* bacteria in composite membranes were designed and these bioreactors removed a variety of metals from polluted effluents [10–12]. Bioprecipitation and the biological sequestration of toxic metals from culture media and polluted effluents are 'post efflux' events and start in the vicinity of the bacterial envelope. The mechanism of these events is still poorly understood as is the mechanism of efflux despite some recent progress [22,38,46]. The possible role of plasmid-mediated resistances in the geochemistry of soils and sediments contaminated with heavy metals waste also deserves further attention.

In the present paper we have: 1) described some *czc*-containing bacteria isolated from a variety of soils and sediments with a high content of heavy metals; 2) reevaluated the genetic structure of the *czc* locus; 3) proposed an efflux mechanism mediated by CzcC, CzcB and CzcA proteins as suggested by the comparison of proposed protein sequence data; and 4) described the bioprecipitation/biological sequestration processes as observed in bioreactors grown in the presence of Cd^{2+} and Zn^{2+} and where *czc* genes were put to full expression.

Plasmid pMOL30 and other czc plasmids

Many strains with pMOL30-like plasmids containing *czc* (as detected by DNA/DNA hybridization with a *czc* probe from pMOL30) were isolated from several contaminated sites.

Correspondence to: M. Mergeay, VITO, Boeretang 200, B-2400 Mol, Belgium.

These soils were characterized by a relatively neutral pH (5–7) and quite high concentrations of bioavailable zinc (leachable with water). Such soils were found in the neighborhood of Belgian non-ferrous industries (five sites) and of Zairian mining sites (four sites). Most of the bacteria were isolated by selection on minimal medium containing 2 mM Zn^{2+} and gluconate as carbon source [10]. Until now, only β class Proteobacteriae related to *A. eutrophus* CH34 were found to bear *czc* DNA fragments (Table 1). Phenotypic variations in the expression of *czc* genes were observed among these strains. In *A. eutrophus* DS185, resistance to Cd^{2+} and Co^{2+} was observed only after induction by zinc. When corresponding plasmids were transferred to a plasmid-free derivative of *A. eutrophus* CH34, full resistance to Cd^{2+} and Co^{2+} occurred. In strain VA47, no resistance phenotype was associated with the *czc* plasmid pMOL214. The *czc*-encoding plasmids are generally very large and can promote their self transfer with variable frequencies (data not shown). Of interest is plasmid pMOL218 (104 kb) from strain VA53, which is self transferable at a frequency of 3.10^{-5} transconjugants per recipient. Some of the hosts of *czc* plasmids are facultative chemolithotrophs that are able to grow with H_2 and CO_2.

The czc *region of pMOL30: a reassessment*

The determinants of Cd, Zn and Co resistances are encoded by the *czc* operon [36]. Related metal efflux systems carried by plasmids include *cnr* which is involved in cobalt and nickel resistances in *A. eutrophus* CH34 [5,26], and *ncc*, which encodes nickel, cobalt and cadmium resistances in *Alcaligenes xylosoxidans* 31A (Schmidt and Schlegel, submitted).

The known structural genes of the *czc* gene cluster are *czc*C, *czc*B and *czc*A [36]. Here we will review the genes of the *czc* region, from *czc*C to *czc*D and continuing to the regions located directly upstream and downstream these genes.

In Fig. 1, we propose some modifications to the Nies et al. [37] map of the *czc* region as a consequence of: (1) sequencing of the *czc*C upstream region; (2) comparison of *czc* sequence date with *cnr* and *ncc*; and (3) discovery of new mutants.

a) *czc*C: When the *czc* DNA sequence was published by Nies et al. [36], CzcC was interpreted to be a polypeptide of 375 amino acids. However, recent analysis of the same sequence suggests the possibility of the open reading frame of *czc*C extending at the 5′ end by adding 72 amino acids. The nucleotide sequence of *czc*C remains unchanged; it is only its presumed open reading frame that has been altered. The reassessed *czc*C ORF begins 29 nucleotides downstream of the *Eco*RI site. Furthermore, the analysis that was based on Von Heijne's method [50] demonstrated clearly that the N-terminal part of the reassessed CzcC protein possesses features typical of a peptide secretion signal (Fig. 2). As we will describe below, there are sequence homologies and topological similarities between CzcC and other accessory proteins of ABC exporter systems, precisely with a family of outer membrane factors (OMF).

b) *czc*B: The second structural gene of the *czc* operon has a gene product (CzcB) containing 512 amino acids. Sequence analysis suggests that CzcB is likely an inner membrane protein which belongs to a family of bacterial membrane fusion proteins (MFP). Besides CzcB displays some homology with calphotin [4]. Calphotin is described as a metal 'mobilizing' protein that sponges Ca^{++} cations from the cytoplasm.

c) *czc*A: The third and largest gene encodes the central component of the whole system. In the three plasmid-encoded heavy metal resistance systems from *Alcaligenes* that have been sequenced so far, *czc*A, *cnr*A and *ncc*A are the most conserved structural genes in the three operons. Very recent experimental data (Nies and Silver, submitted; Nies, pers. comm.) directly demonstrates that CzcA, the center part of the heavy metal

TABLE 1

Overview of some *czc* plasmids in *Alcaligenes eutrophus* strains[a]

Strain	Origin	*czc* plasmid	Size (kb)	Markers	Frequency of self-transfer
CH34	Liège (Belgium)	pMOL30	240	Cd, Co, Zn, Cu, Pb, Tl, Hg	10^{-8}
DS185	Lommel (Belgium)	pMOL85	250	Zn (Co, Cd) Cu, Pb	5×10^{-4}
ER8	Overpelt (Belgium)	pMOL62	160	Zn, Cd, Hg	10^{-5}
ER121	Overpelt (Belgium)	pMOL70	160	Zn, Hg (Cd)	10^{-3}
VA53	Kolwezi (Zaïre)	pMOL218	104	Cd, Co, Cu, Zn, Hg	3×10^{-5}
VA47	Kakanda (Zaïre)	pMOL214	40	Cu?	NT
VA12	Likasi (Zaïre)	pMOL224	>300	Cd, Co, Zn	NT

[a]*czc* plasmids were determined by hybridization with the *czc* probe from pMOL30. All plasmids with mercury resistance hybridized also with *mer* probe made from *mer* transposon Tn*4380* [8]. Self-transfer was assayed by selecting Zn^R transconjugants to a plasmid-free derivative of *A. eutrophus* CH34 (AE229, *phe*-42 *nal*-110) and counterselecting with nalidixic acid. Most of the donor strains contain one or more plasmids in addition to their czc^+ plasmid. Between parenthesis: conditional expression for some metals.

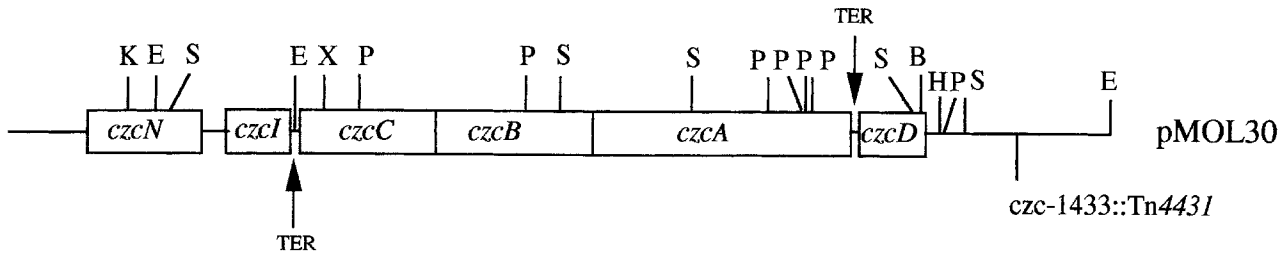

Fig. 1. Gene organization of the *czc* operon. *czc*I and *czc*N are putative ORFs (see Fig. 2). Putative transcription termination sites are shown by arrows. *czc*-1433::Tn*4431* is a *lux*-mediated gene fusion also illustrated in Figs 3 and 4. Capital letters indicate restriction nuclease sites: B, *Bam*HI, E, *Eco*RI, H, *Hind*III, K, *Kpn*I, P, *Pst*I, S, *Sal*I, X, *Xho*I.

efflux pump, is a chemiosmotic cation/H^+ antiporter. The hydropathy pattern of CzcA predicts a 12-transmembrane polypeptide with a symmetry between the first and second halves of CzcA. It is possible that the two halves of *czc*A arose by tandem intragenic duplication during evolution [42]. Such tandem repeat organization of chemiosmotic antiporters resembles strikingly the dimer organization of an ABC transporter where 2×6 transmembrane spanners have also been observed [17,20].

d) *czc*N: Upstream of CzcCBA, two open reading frames have recently been identified, where one was previously predicted [33]. The first one, most distal from *czc*CBA, *czc*N with a putative gene product containing 216 amino acids, begins 1442 nucleotides upstream of *czc*C (Fig. 1). *czc*N was recognized due to a close homology with a gene that was detected in the *ncc* operon region, *ncc*N. The amino acid identity between CzcN and NccN is surprisingly high (66%). Curiously, *ncc*N is located downstream of *ncc*CBA with gene order *ncc*YXHCBAN, while *czc*N (for which no phenotype is known), is found upstream of *czc*CBA. A protein databank search revealed that CzcN shares protein sequence similarities with the ORF7 of the *cob* gene region in *Pseudomonas denitrificans* and the protein-*s*-isophenylcysteine *o*-methyltransferase (STE14) in *Saccharomyces cerevisiae* (28% and 21% identity respectively). Most of the *cob* genes nearby the ORF7 are involved in the biosynthesis of Cob(I)-alamine [7]. However, the function of the ORF7 is still unknown. The STE14 mediates c-terminal methylation of the a-factor and RAS protein in *Saccharomyces cerevisiae* [21]. The hydropathy profile of CzcN and sequence comparisons suggest that CzcN may be a transmembrane protein. No *czc*N mutants are known and their phenotype is not yet predictable. In the *ncc* operon, *ncc*N mutants display decreased resistance to nickel (T. Schmidt, pers. comm.).

e) *czc*I: Starting 318 bp downstream of *czc*N, another putative ORF coding for CzcI, which has 115 amino acids (Fig. 1), was found (Fig. 2). Moreover, by using a DNA probe for this region, a corresponding mRNA band of 400 bp has been detected in Northern blots (Q. Dong, unpublished data). No homology has been found between putative CzcI amino acid sequence and that of any other protein in available databases. It might be suspected that this potential protein would play a regulatory role in the *czc* efflux system, though no experimental evidence has been furnished.

f) *czc*D: *czc*D (gene product: CzcD, 199 amino acids) is located downstream of the three structural genes *czc*CRA [33,36]. A typical rho independent transcription terminator was found between CzcA and CzcD which possibly means that *czc*D is likely not to be cotranscribed with *czc*CBA. The hydropathy plots indicate that CzcD may be a membrane-spanning protein. Moreover, CzcD shares a high amino acid sequence identity with Cot1 [6] and zrc [23] of *Saccharomyces cerevisiae* (37% and 37% respectively). Cot1 confers increased tolerance to cobalt and rhodium in yeast, and zrc is involved in zinc and cadmium resistance in yeast. It was also reported that the CzcD could participate in induction of *czc* efflux [33]. It is possible that CzcD functions to activate the high level efflux system. Another hypothesis is that *czc*D would belong to another operon located downstream of *czc*CBA.

g) A metal-inducible *lux* mediated gene infusion was mapped 1.4 kb downstream of *czc*D. This fusion resulted from insertion of transposon Tn*4431*, which contains the reporter *lux* operon derived from functional *lux*I and *lux*R regulatory genes [48,49] with this fusion, *czc*-1433::Tn*4431*, light was found inducible by zinc, cadmium, cobalt and lead (Fig. 3) and not by copper, nickel, thallium or mercury. This observation shows the presence of additional genes involved in resistance to metals located downstream of *czc*D. It is one of the first clues for the plasmid-borne genes responding to lead, which have long been supposed to be present in pMOL30 [9]; and it is also one of the first clues for genes inducible by cadmium and cobalt. Inducibility by cadmium and cobalt have been suspected from growth studies but not previously demonstrated at the genetic level [34; (Diels, unpublished)]. Deletion mapping [9; (van der Lelie, unpublished data)] revealed the presence of lead and copper resistance genes downstream of *czc*. Fig. 4 shows that zinc and lead induce light at different times in the growth cycle, and that the production of light increases as a function of lead concentration.

czc efflux: a three-component export pathway

A global similarity search of SWISSPROT (Release 28) using an exhaustive algorithm related to that of Smith and Waterman [47] revealed that CzcC belongs to a family of outer membrane factors (OMF). CzcB is part of a family of inner membrane proteins, or membrane fusion protein (MFP); and CzcA is a member of a family of 12-transmembrane helix exporters, which according to Saier et al. [42] belongs to the RND family (Resistance/Nodulation/Division) [14]. Such exporters, which are not driven by ATP (and are thus different

```
      SalI   CzcN
        S  T  W  T  V  H  V  S  S  F  Q  R  N  I  A  D  S  V  W  F
....  GTCGACATGGACGGTCCATGTGTCTTCCTTTCAACGCAATATTGCGGACTCGGTCTGGTT     60

        L  G  L  S  V  V  V  E  S  F  D  L  F  H  D  A  G  V  L  R
 61   TCTCGGCCTGTCGGTGGTCGTGGAGTCGTTTGATCTGTTCCATGACGCCGGCGTCCTCCG    120

        A  V  V  T  L  A  *
121   GGCGGTCGTGACGCTTGCCTGATGCGGGCAACTCCGCATACACGTACAGCCATGCACTAC    180

181   GCTCACGTCTCCGATTCTGTTGTCGAGAGCATGCTCGTGATGCTGGTCGGCCTGatCGTC    240

241   CTGTATGTGGGGTTCGCTGTCTATTTGCGGTGGAAGCATGGGCCCGCCCCCAAGCGTAAG    300

301   ACTGAgtAGGGGCGAAAGCGGCACCCCAAAACGAGCAGGCGAAAGCGATAATCGTAATCT    360

361   GCTCTTAATGCTGGTGATCGAGGATTCATGTAAACTTCGGCGACGGCCCAGCCgtTAGTA    420
                                              CzcI
                                              M  R  R  F  V  L  I  F
421   CTTCAACCCCAAGCCCCCCGCACAGTCTCCCAAGGAATGCGACGTTTCGTTCTGATCTTC    480

        V  L  L  I  L  P  F  Q  F  S  W  A  A  A  A  R  Y  C  Q  H
481   GTGCTGCTCATTTTGCCGTTCCAGTTTTCCTGGGCGGCAgCGGCACGCTATTGTCAGCAC    540

        E  K  A  T  A  T  W  H  L  G  H  H  E  H  R  H  Q  Q  P  E
541   GAGAAAGCCACGGCCACTTGGCACCTTGGGCACCACGAGCATCGTCATCAGCAGCCGGAA    600

        G  K  T  D  A  E  K  K  P  F  V  D  T  D  C  G  V  C  H  L
601   GGTAAAACGGATGCCGAGAAAAAGCCATTCGTGGATACAGACTGCGGGGTATGCCATCTG    660

        V  S  L  P  F  V  Y  G  Q  T  Q  D  V  L  I  A  N  R  V  E
661   GTCTCCCTCCCGTTCGTCTATGGACAGACGCAGGACGTGTTGATAGCGAATCGGGTAGAA    720

        V  T  D  T  Q  H  S  S  E  F  S  S  L  N  A  R  A  P  D  R
721   GTGACCGATACTCAACATTCGTCCGAGTTCTCGTCTCTGAATGCCAGGGCTCCCGACCGT    780

        P  Q  W  Q  R  L  A  *
781   CCTCAGTGGCAGCGTCTCGCTTGATCGGCGAGACGACGACTCTTTTTCTCCTTTCGTCTC    840
                                                    CzcC
            EcorI                                   M  R  R  L  F  L  P
841   TCGCCGAATTCTCCTGGTCACATACCTTGGTGCAATTCGATGCGAAGACTATTTCTGCCG    900

        L  G  L  A  V  A  F  L  S  P  N  F  A  V  A  Q  S  D  T  G
901   CTCGGGCTGGCGGTAGCATTTCTCAGCCCAAACTTTGCCGTAGCGCAATCTGACACCGGC    960

        T  S  M  V  P  V  F  P  R  E  A  A  G  P  L  T  L  E  A  A
961   ACGTCCATGGTGCCCGTCTTCCCAAGGGAAGCGGCGGGACCGTTGACCCTCGAGGCCGCG   1020

        L  S  L  A  A  G  S  N  F  N  L  S  A  A  A  K  E  L  D  S
1021  TTGTCGCTGGCGGCAGGAAGCAATTTCAACCTGTCCGCCGCCGCCAAGGAACTCGATTCC   1080

        T  E  G  G  I  M  Q  A  R  V  I  P  N  P  E  L  K  T  L
1081  ACAGAAGGTGGGATCATGCAGGCCCGGGTTATTCCGAACCCGGAACTCAAGACGCTGG.....
                  *
```

Fig. 2. The partly revised DNA sequence of the *czc* operon. The sequence shown contains the C-terminus of *czc*N and the N-terminus of *czc*C as well as a putative ORF *czc*I. This new sequence (EMBL data base accession number X71400) was made from *czc* fragments cloned in *E. coli* and from PCR product made on pMOL30 extracted from cultures of CH34 grown in the presence of Cd^{2+}. Both sequencing procedures gave the same unequivocal results. The new nucleotide order led to the definition of at least two ORFs where only one (CzcR) was postulated before [33]. The putative *czc*I shown with a possible RBS (underlined) is followed by a putative ρ-independent termination site (bold). *czc*C is shown 216 bp longer than in the formerly-published sequence. Previously-postulated ATG is marked with an asterisk. The aminoacids of the putative signal peptide are in italics. The nucleotides conflicting with the formerly published nucleotide sequence are shown in lower case.

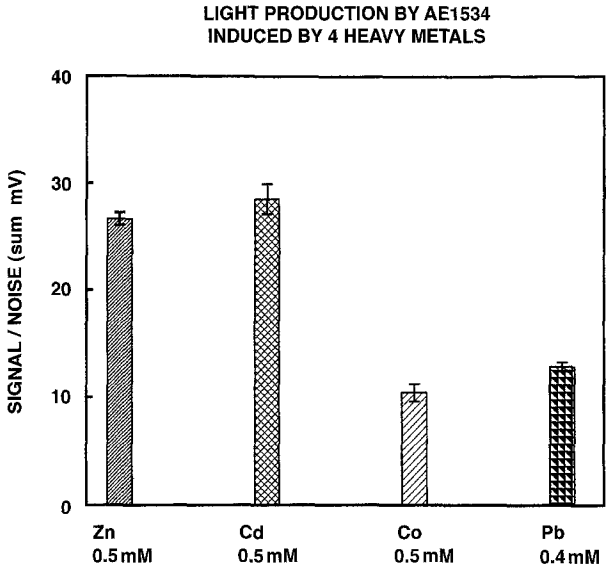

Fig. 3. *czc*-1433::*lux* gene fusion. Effects of metals on light production. Cultures (0.5 ml) of *A. eutrophus* strain AE1534 (pMOL30-*czc*-1433::Tn*4431*) in minimal medium + gluconate + tetracycline (20 μg ml^{-1}) were incubated at 25 °C in the presence of different heavy metal salts. The total amount of light produced during a 72-h period was measured, using an automated luminometer LKB type 1251. The results are expressed as the ratio between the light produced in the presence of the metals and the light produced by a control culture without heavy metals added (signal/noise ratio). The results represent the average of duplicate samples (± 1 standard deviation).

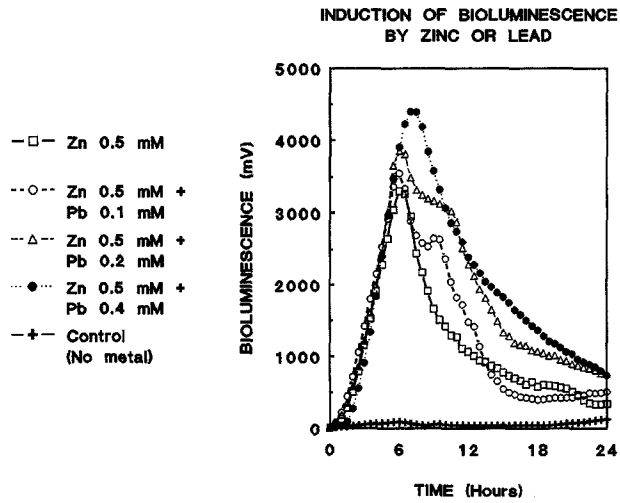

Fig. 4. *czc*-1433::*lux* gene fusion. Light production as a function of time. Cultures (0.5 ml) of *A. eutrophus* strain AE1433 (pMOL28, pMOL30-*czc*1433::Tn*4431*) in minimal medium containing gluconate and tetracycline (20 μg ml^{-1}) were incubated at 25 °C in the presence of 0.5 mM Zn^{2+} alone or in the presence of 0.5 mM Zn^{2+} together with increasing concentrations of Pb^{2+}. The amount of light produced was simultaneously registered as a function of time, using an automated luminometer LKB type 1251. The results represent the average of duplicate samples and are expressed in arbitrary bioluminescence units (mV).

from traffic ATPases) transport various metabolites, antibiotics or drugs to the extracellular space.

The gene organization of *czc*CBA and the functions associated with these genes are strikingly similar to a range of bacterial export pathways (Table 2) and include:

- OrfABC (also named MexAB and OprK [39], which mediate the secretion of siderophores and resistances to antibiotics in *Pseudomonas aeruginosa* [40,41]. OprK is the outer membrane component of the system and shares 24% amino acid identity with CzcC; MerA is an inner membrane component and shares 24% amino acid identity with CzcB; and MerB is the central 12-transmembrane helix pump and has 26% sequence identity with CzcA.
- AcrAB [27] which is involved in acriflavine resistance in *E. coli* and in which AcrA is the homologue of CzcA (24% identity). No associated outer membrane component (homologue of CzcC) has been identified in this system.
- AcrEF of *E. coli* which has the similar function and high sequence similarity with AcrAB, are counterparts of CzcBA [24].
- NolFGHI [3], which is assumed to secrete a nodulation factor in *Rhizobium meliloti*, has a similar and quite interesting organization vis-a-vis CzcB and CzcA. NolF is, based on sequence similarity, the homologue of CzcB (inner membrane protein). NolGHI codes for three short polypeptides which together are colinear with CzcA [42], and together they may also perform an export function.
- CyaBDE, which participates in the cyclolysin secretion in *Bordetella pertussis*, in which CyaE is an OMF, CyaD is a MFP, and CyaB is the central exporter. Interestingly, the exporter CyaB is a traffic ATPase [19].
- HlyBD-TolC, which are involved in the hemolysin secretion in *E. coli* [18] and in which TolC is a member of OMP family, HlyD is a member of MFP family, and HlyB is an ABC transporter [51]. Fig. 5 summarizes the gene organization of different bacterial export pathways and Fig. 6 shows evolutionary relationships between the different members of every family of proteins involved in the tricomponent efflux systems. These trees show the strong relationships between the various *Alcaligenes* genes implied in plasmid-borne cation efflux (*czc*, *cnr* and *ncc*) but also quite unexpectedly the strong relationship of *czc*CBA with *hel*CBA. *hel*CBA might be involved in virulence factor transport in a *Legionella* strain (a γ-Proteobacterium) [(C. Engleberg, personal communication); 2].

According to our sequence analysis and by analogy with the structural organization of other ABC transporters, we propose a modified Czc efflux model (Fig. 7) : CzcA, the central protein, functions as a pump driven by a H^+ gradient. CzcB, a MFP, possibly acts to connect the inner and outer membranes and to facilitate the export of ions across both membranes without release in the periplasm. It might also function in providing specificity for heavy metals [35]. In the case of homologous ABC transporters, the membrane fusion proteins are anchored in the inner membrane and span the periplasm. CzcC, the outer membrane factor, is also required in the *czc*

TABLE 2

Three-component export pathway

Operon and organism	Translocator organization			Function	Reference
	OMF	MFP	Exporter (type)		
czc in *A. eutrophus* CH34	CzcC	CzcB	CzcA (H-antiporter)	Co^{2+}, Zn^{2+}, Cd^{2+} efflux	[36]
cnr in *A. eutrophus* CH34	CnrC	CnrB	CnrA (H^+-antiporter)	Co^{2+}, Ni^{2+} Zn^{2+} efflux	[5,26]
ncc in *A. xylosoxidans*	NccC	NccB	NccA (H^+-antiporter?)	Ni^{2+}, Co^{2+}, Cd^{2+} efflux	[Schmidt, submitted accession number L31363]
orfABC in *Pseudomonas aeruginosa*	OprK (OrfC)	MexA (OrfA)	OrfB MexB	Siderophore secretion, antibiotic efflux	[40,41]
acrAB in *Escherichia coli*	?	AcrA	AcrB	Acriflavine resistance	[27]
nol in *Rhizobium meliloti*	?	NolF	NolGHI	Nodulation factor secretion	[3]
cya in *Bordetella pertussis*	CyaE	CyaD	CyaB (ABC exporter)	Cyclolysine secretion	[19]
hly in *Escherichia coli*	TolC	HlyD	HlyB (ABC exporter)	Hemolysin secretion	[18]
apr in *Pseudomonas aeruginosa*	AprF	AprE	AprD (ABC exporter)	Alkaline protease secretion	[15]
prt in *Erwinia chrysanthemi*	PrtF	PrtE	PrtD (ABC exporter)	Protease A,B,C secretion	[25]

efflux process to complete the efflux of ions into the extracellular medium. Transport data [37] suggest that the export of very toxic Cd^{2+} to the extracellular medium requires the participation of CzcC, which is however dispensable for the efflux of Zn^{2+}. Since Zn^{2+} is an essential metal, it is conceivable that a chromosomal OMF involved in the homeostasis of Zn^{2+} can act to replace CzcC for the efflux of Zn^{2+}.

In conclusion, several bacterial export pathways have newly recognized and similar three-component constitutions, although in some systems the outer membrane components have not yet been identified. The function of the central pump may be different from one system to another. In other words, certain ABC transporters (ATPases) and 12-transmembrane helix transporters (chemiosmotic) have the same overall structural organization and use the same classes of accessory proteins to complete their export pathways.

Metal efflux and bioprecipitation

The *czc*- and *cnr*-mediated heavy metal resistance mechanisms are based on the three-component, chemiosmotic, energy-dependent, cation efflux systems composed of the CzcABC and CnrABC proteins, respectively. During the late log phase and the stationary phase of cultures grown in the presence of high concentrations of Cd^{2+} (2 mM) or Zn^{2+} (4–10 mM), a decrease in concentration of Cd^{2+} or Zn^{2+} ions in solution was observed [13]. Up to 99% of the initial metal concentration was removed from culture supernatant fluids. Metal sequestration and metal precipitation were observed. This bioprecipitation increased with initial metal concentration and was influenced by the nature and the concentration of carbon sources. The pH of the growth medium increased as a function of increasing metal concentration, as shown for Cd^{2+} in Table 3. This effect of increasing concentrations of Zn^{2+} or Cd^{2+} on the final pH of the culture was observed in any growth condition including chemolithotrophy (Diels, unpublished data) and only when active resistance mechanisms take place. We think the slow build up of alkaline pH may be related to the proton influx during the *czc*-mediated efflux of cations.

The highest metal precipitation rate was found with 0.8% lactate or with 1.0% acetate as carbon source. Higher concentrations led to substrate inhibition of growth, lower concentrations resulted in too low a CO_2 production. Carbonates could precipitate with Cd^{2+} ions to form $Cd(HCO_3)_2$ and $CdCO_3$. This was shown by X-ray diffraction spectrometry of the bioprecipitated material. The precipitation of cadmium seems to occur at defined nucleation foci around the cell surface as shown by transmission electron microscopy (Fig. 8).

Search of functions involved in post-efflux events

This search is not easy because it requires distinctions between unknown plasmid-mediated functions, pH or acute stress-mediated responses, and the physicochemical properties

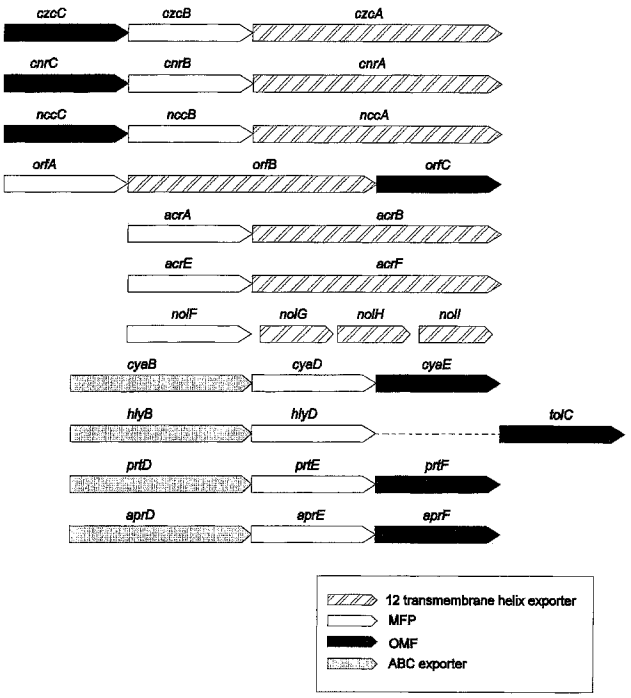

Fig. 5. Gene organization of selected bacterial export pathways including *A. eutrophus* CH34 *czc*CBA- and *cnr*CBA-mediated efflux, *A. xylosoxydans* *ncc*CBA-efflux, *P. aeruginosa* *opr*K *mex*AB (*orf*ABC)-efflux and secretion system, *E. coli* acriflavine-resistance (*acr*AB, *acr*EF), *R. meliloti* nodulation factor secretion (*nol*GHI), *B. pertussis* cyclolysin secretion (*cya*BDE), *E. coli* hemolysin secretion (*hly*BD-*tol*C), *E. chrysanthemi* protease secretion (*prt*DEF) and *P. aeruginosa* alkaline protease secretion (*apr*D,E,F). Open symbols: genes encoding membrane fusion proteins (MFP); Dark gray: genes for outer membrane factors (OMF); Hatched: genes responsible for 12-transmembrane helix exporters; Light gray: genes for ABC ATPases.

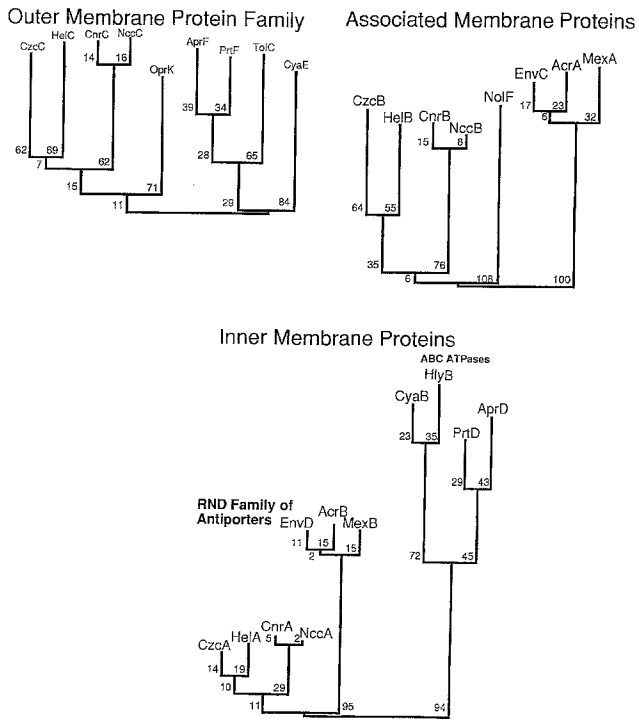

Fig. 6. Dendrogram alignment of protein sequences showing relationships between members of the three families of proteins involved in tricomponent efflux systems: outer membrane protein including CzcC, Associated Membrane Proteins (also called Membrane Fusion Proteins) including CzcB, Inner Membrane Proteins (12-transmembrane helix transporters) including CzcA (by courtesy from Dr S. Silver). Hel proteins are involved in the expression of virulence by a *Legionella* strain. Accession number for *hel*C is U11704.

of the bacterial cell envelope. Some physiological responses occur as a consequence of Cd^{2+} resistance. First, an increase in extracellular polymers was observed (Table 3 and Fig. 8). Extracellular polymers were isolated from the supernatant medium by precipitation with two volumes of ethanol. The concentration was measured by determining their sugar content. Sugars were lysed with sulfuric acid and, after reaction with phenol, measured spectrophotometrically. Polysaccharides can bind high amounts of metals (up to 50% of the polymer weight). A related observation is on electrophoretic mobility of *A. eutrophus* cells grown with Cd^{2+}, which decreased substantially as the pH increased in the culture following *czc*-mediated efflux. This increased negative charge also indicates a high cation binding capacity for *A. eutrophus* CH34 (F. Glombitza, personal communication).

Another observation is the appearance of a 27-kDa protein in the supernatant fluid which was found in the presence of Cd^{2+}, Co^{2+} or Zn^{2+} but not in the presence of Cu^{2+} or Pb^{2+} (P. Corbisier, G. Nuyts and L. Diels, in preparation). This protein was not observed during the stationary growth phase, although this may be an artifact due to the interaction of bioprecipitation with the protein extraction method. Working hypotheses are that this protein could play a role either in the nucleation process or in a kind of metal sequestration required to minimize the metal cation reentry. This soluble protein of 27 kDa is apparently not compatible with any of the known *czc* gene products (*czc*C or *czc*D), which seem to be mainly membrane-bound. Finally, it would be very interesting to examine which component of the outer cell membrane is involved in the nucleation foci that are visible in Fig. 8.

A bioreactor concept to manage continuous bioprecipitation

The observation of bioprecipitation as a physiological consequence of plasmid-mediated efflux of cations, especially with some carbon sources, led to the possible application of metal sequestration/bioprecipitation as a tool to remove heavy metals from polluted effluents. In order to use the bioprecipitation process for the removal of heavy metals from wastewater, *A. eutrophus* cells must be kept viable. This requires a special reactor concept involving immobilization of the cells, a crystal collection system, and a feeding system to keep bacteria alive. Furthermore, the reactor needs to be controlled for optimal metal removal.

In order to fulfil all these conditions, a new reactor named BICMER (Bacteria Immobilized Composite MEmbrane Reactor) was designed [11]. The concept is based on the separation of the waste stream to be treated and a small nutrient stream to keep the bacteria alive. The two streams are separated from each other by a membrane inside and on the surface

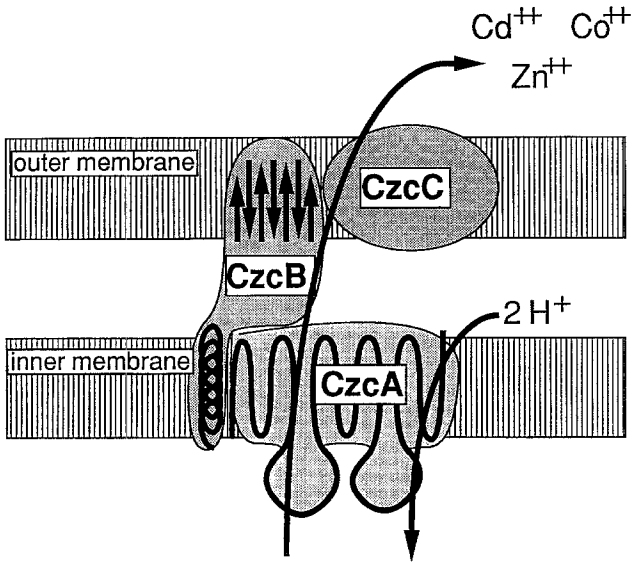

Fig. 7. *czc* efflux model. This model is based on protein sequence comparisons [14; (S. Silver, personal communication)]. CzcC is shown as an outer membrane protein, CzcB as a protein encompassing both membranes, perhaps containing β sheet regions in the outer membrane and an X helical hydrophobic region in the inner membrane. The dimeric structure of the 12-transmembrane helix-CzcA cation pump is suggested on the Figure. The arrows indicate the directions of the cation and proton fluxes. The figure was provided by Simon Silver.

TABLE 3

pH and extracellular polysaccharides in function of the initial Cd concentration[a]

Initial Cd concentration (mM)	pH in stationary phase	Sugar concentration in the extracellular polymers (mg sugar L^{-1} growth medium)
0.00	7.69	3.0
0.50	8.16	3.5
1.00	8.43	6.5
2.00	8.97	8.1

[a]*A. eutrophus* CH34 was grown in Tris minimal medium supplemented with 0.8% lactate as a carbon source. Final pH was measured 48 h after the end of exponential phase. Extracellular polymers were harvested 10 h after the end of the exponential phase. The initial pH was always 7.00.

of which the bacteria are immobilized. The bacteria form a biofilm on the effluent side of the membrane. The metals of the effluent are bioprecipitated by the bacteria on the biofilm, and the crystals formed are released from the biofilm and recovered on a column filled with glass beads. This column can be regenerated by an acid treatment without disturbing the biofilm on the membrane. This membrane reactor concept is novel in a way that nutrient and effluent were separated by a membrane which forms the carrier for the immobilization of the cells. Both streams (effluent and nutrient) flow tangentially along the membrane. Conventional membrane bioreactors do contain a membrane system outside the bioreactor for the separation of biomass from the liquid.

The membrane itself is composed of an organic polysulfone matrix with ZrO_2 (Zirfon® membranes, VITO, Mol, Belgium). ZnO, Sb_2O_3 or Al_2O_3 are used as pore formers to prepare membranes with 60% total porosity, and pore diameters between 4 μm (at the open side) and 0.2 μm (at the skin side). The bubble point of the membranes is between 0.3 and 0.6 bar and the thickness is around 100 μm. The flux is around 200 L h^{-1} per m^2. These characteristics allow for membranes with a low flux to avoid transport of too high a volume of liquid from one side to the other. On the other hand, there is a need for a good diffusion of nutrients through the membrane into the biofilm and the biofilm needs a good anchor in the membrane.

This concept was used in a Flat Sheet Reactor (FSR) configuration as a test system for bacteria and membranes. Scale up was done in Tubular Membrane Reactors (TMR) used in a continuous way. The BICMER reactor consisted of multipipe TMR reactors with a glass bead column and two sandfilters in order to remove very small crystallites that are not retained by the column [11,12]. A scanning electron microscope photo (Fig. 9) shows crystals induced by bacteria in a membrane reactor. At an early stage (4 days) of the biofilm colonization, some large precipitates were observable and spread on the biofilm. These structures were only observable in the beginning of colonization. The tiny brilliant points (Fig. 9(A)) are mixtures of small crystals and bacteria.

After 6 days of incubation the biofilm was much more developed and nice crystals of around 10 μm are formed. After 10 days the crystals form a crust embedded in the biofilm. Bacteria colonize the faces of the crystals. Although the strains are also resistant to Cu^{2+}, Co^{2+} and Ni^{2+} ions, their sequestering activity was only induced by Cd^{2+} or Zn^{2+}. Y and Ge could also be removed after induction by Cd^{2+}, although no special resistance or plasmid-mediated response to these metals has been observed (Table 4).

OVERVIEW

czc$^+$ bacteria have been found via phenotypic analysis and dot blot or colony hybridization in a variety of soils or sediments polluted by mine or metallurgical wastes. Such contaminated soils or sediments contain up to 5% Zn^{2+}, Cu^{2+}, Co^{2+} and Pb^{2+} by weight and traces of Cd^{2+} from which a fraction is sufficiently bioavailable to exert a toxic effect on all heterotrophic bacteria, with the exception of those that are equipped with specific resistance mechanisms. All *czc*-containing bacteria isolated display a carbon source utilization spectrum very similar to that of *Alcaligenes eutrophus* strain CH34. The plasmid composition of these strains is more variable than might be expected [10]. The size of *czc*$^+$ plasmids (some of which are able to self-transfer at high frequencies, based on transfer of metal resistance markers to a plasmid-free derivative of *A. eutrophus* (CH34) varies from 40 kb to 350 kb.

An efflux mechanism was recognized as the mechanism of resistance for *czc* on plasmid pMOL30 [36], and also for the evolutionarily-related *cnr* system (plasmid pMOL28) [44].

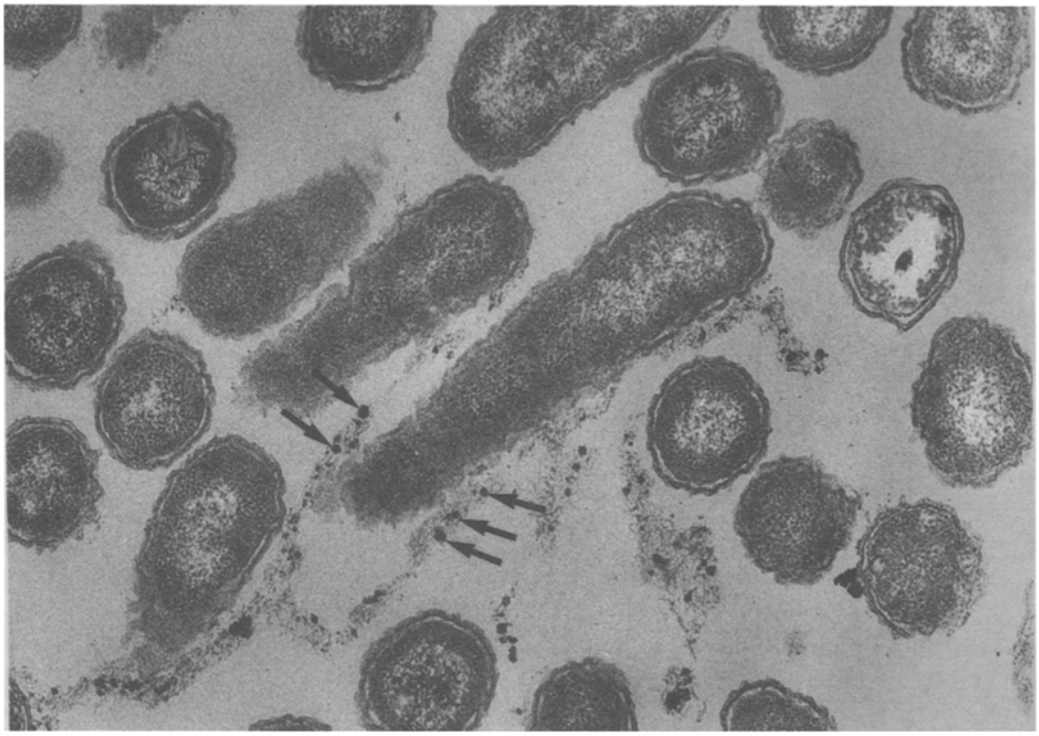

Fig. 8. Cadmium precipitation by CH34 cells. A transmission electron micrograph showing *A. eutrophus* cells after growth with 1 mM Cd^{2+} for 120 h. Cd precipitates (black spots indicated by arrows) are seen on structures surrounding the bacterial rods and partially released from cells.

Both efflux systems involve three proteins CzcCBA and CnrCBA, with CzcA (and CnrA) working as the central cation pump. CzcA has been shown to be a chemiosmotic pump working as a cation/proton antiporter [36,38]. The functions for CzcC and CzcB have also been discussed in other papers of this issue, but a reexamination of the sequences led us to propose a longer open protein reading frame for czcC (417 aa product instead of 375 aa; Fig. 1) making it more similar to the proposed CnrC polypeptide in length and to a family of Outer Membrane Factors. All these OMFs are in fact components of a tri-transporter involved in the efflux of a variety of molecules: extracellular proteins, siderophores, metal and mutagenic substances.

The reassessment of the *czc* region was extended to the upstream region, where the new gene *czc*I is proposed on the basis of reappraised sequence data and of Northern blotting data. Although *czc*I was found to be inducible by zinc its possible role in the regulation of *czc* is still unknown. The same is the case for *czc*N. The latter was identified through sequence analysis and analogies with a very similar gene found in the *ncc* operon (resistance to Ni^{2+}, Co^{2+} and Cd^{2+}) of *A. xylosoxidans* (Schmidt and Schlegel, submitted). The regulation of the expression of *czc*CBA remains to be elucidated, although the *czc*D gene downstream of *czc*A may play a role [33]. The insertion *czc*-1433::Tn*4431*, which was inducible not only by Zn^{2+} but also by Cd^{2+} and Co^{2+}, gave a clue for a possible genotropic effect of Cd^{2+} and Co^{2+}. Lead also induced the production of light with this insertion. Therefore, it seems that new interesting genes are present on both sides of *czc*ICBAD that are either involved in the resistance to Cd^{2+}, Zn^{2+} and Co^{2+} or to other heavy metals including Cu^{2+} and Pb^{2+}.

The *czc*-mediated efflux allows *A. eutrophus* cultures to process heavy metals at the millimolar level (1–10 mM). Cd^{2+} and Zn^{2+} were efficiently removed from fully grown cultures. Metal sequestration occurred by bioprecipitation, especially in the presence of carbon sources such as lactate or acetate, and was associated with an increase of pH. Clearly, in the *czc*-mediated resistance mechanism, post-efflux events have to be addressed to answer the question of how the cells would avoid or minimize reentry of exported metal especially at such high concentrations of heavy metals. Bioprecipitation offers an answer and has been exploited in bioreactors specifically designed for environmental biotechnology.

Other microbial bioprecipitation processes include the formation of $CaCO_3$ by *Synechococcus* [43], $CdHPO_4$ formation by *Citrobacter* N14 [28], CdS intracellular precipitation in *Klebsiella aerogenes* [1], and the formation of manganese nodules in oceans [16].

In *A. eutrophus* CH34, bioprecipitation is strongly dependent on available carbonates and more generally on active heterotrophic growth conditions, and therefore does not seem to be the exclusive strategy used by *A. eutrophus* to answer the problem of reentry. The avoidance or the minimization of reentry of toxic metals is crucial in the very oligotrophic ecological conditions where all *czc* bacteria are found (zinc deserts, etc.).

The phenomenon of bioprecipitation observed as a consequence of *czc*-mediated efflux leads to important physiological

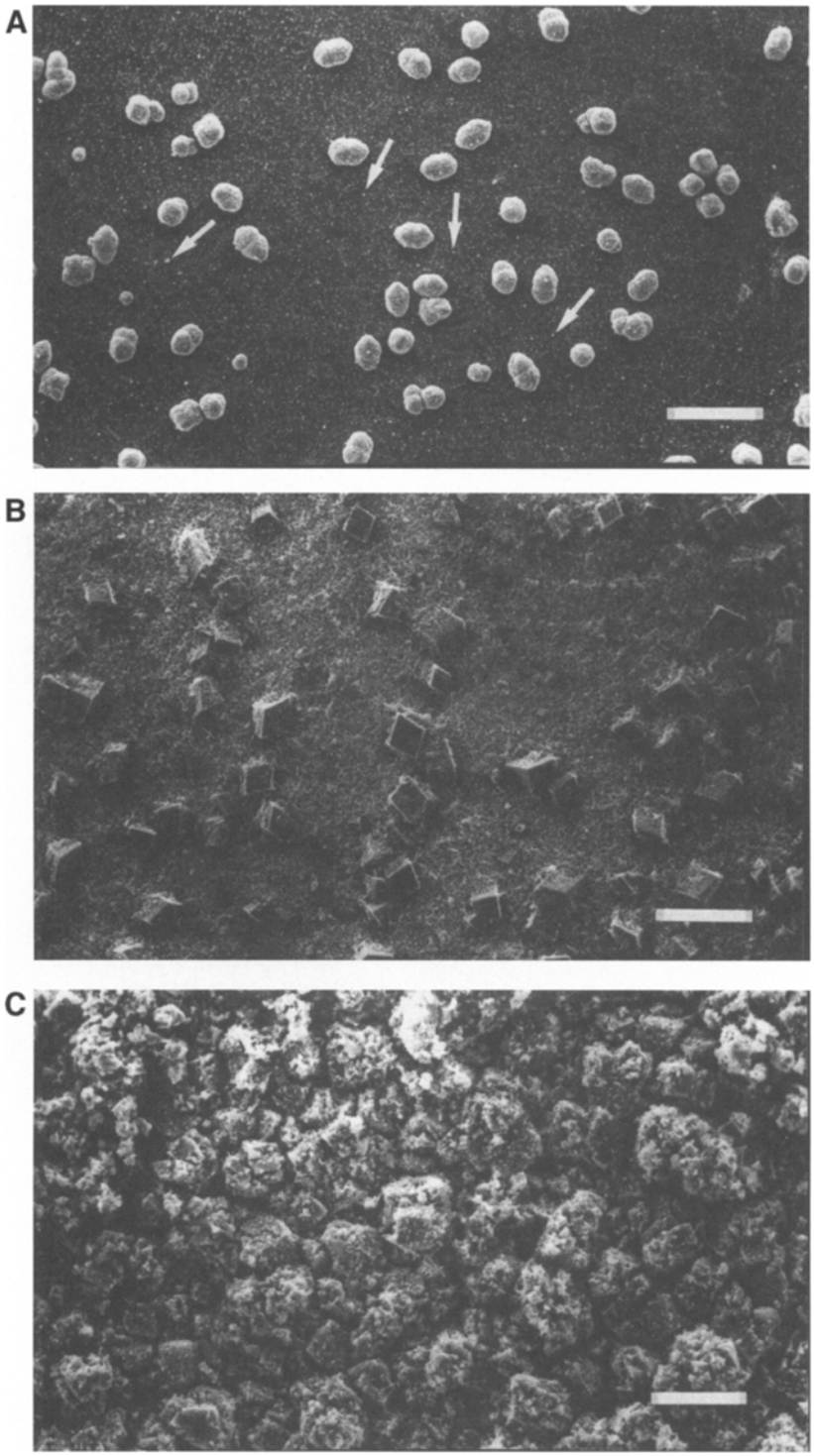

Fig. 9. Cd crystals formed on *A. eutrophus* CH34 biofilm in a tubular membrane reactor TMR. Surface views of membranes colonized by *A. eutrophus* CH34. The white bars = 100 μm. Three incubation times are represented: (A) Early stage (4 days) incubation of the biofilm. Some large precipitates are spread on the biofilm. The tiny brilliant points (white arrows) are mixtures of small crystals and bacteria. (B) Biofilm after 6 days of colonization. The colonization between the cubic structures is much more developed. (C) Biofilm after 10 days of colonization. Formation of a crust made of crystals embedded in the biofilm.

questions such as: i) the nature and the role of cellular components in the nucleation of crystals, as suggested by Fig. 8; ii) the nature and evolution of crystals formed from apparently amorphous structures; iii) the formation and the evolution of biofilms and microbial communities in such conditions. The *czc* system offers a special opportunity to study the effect of a rather small group of genes and functions on the formation and the evolution of a biofilm; and iv) the role of the pH

TABLE 4

Removal of several heavy metals from effluents[a]

Metal	Initial metal concentration (p.p.m.)	Final metal concentration (p.p.m.)
$CdCl_2$	120	0.05
$ZnSO_4$	60	0.05
$Cu(NO_3)_2$	10	0.05
$CoCl_2$	8	0.10
$NiCl_2$	10	0.10
$Pb(CH_3COO)_2$	97	0.05
YCl_3	44	0.05
$GeCl_4$	140	0.10

[a]*A. eutrophus* CH34 was grown to stationary phase. Twelve hours after the end of the exponential phase, the biomass was diluted 7–10 times in media containing known amounts of different heavy metals and allowed to grow for an additional 48 h. Then, the final heavy metal concentration in the effluent was determined.

increase and the correlated stress reactions on biofilm formation (polysaccharides, etc...).

ACKNOWLEDGEMENTS

This work was supported by an EEC grant (CEC BRITE-EURAM) and by a grant from the Flemish Government (VLAB-ETC-003). We thank S. Silver for Figs 6 and 7 and continuing exchange of information, T. Schmidt and C. Engleberg for communicating information prior to publication and S. Wuertz for useful criticisms and reviewing the manuscript.

REFERENCES

1 Aiking, H., K. Kok, H. van Heerikhuizen and J. van't Riet. 1982. Adaptation to cadmium by *Klebsiella aerogenes* growing in continuous sulfide. Appl. Environ. Microbiol. 44: 938–944.

2 Arroyo, J., M.C. Hurley, M. Wolf, M.S. McClain, B.I. Eisenstein and N.C. Engleberg. 1994. Shuttle mutagenesis of *Legionella pneumophila*: identification of a gene associated with host cell cytopathicity. Infect. Immun. 62: 4075–4080.

3 Baev, N., G. Endre, G. Petrovics, Z. Banfalvi and A. Kondorosi. 1991. Six nodulation genes of *nod* box locus 4 in *Rhizobium meliloti* are involved in nodulation signal production *nod*M codes for D-glucosamine synthetize. Mol. Gen. Genet. 228: 113–124.

4 Ballinger, D.G., N. Xue and K.D. Harschman. 1993. A *Drosophila* photoreceptor cell specific protein. Calphotin, binds calcium and contains a leucine zipper. Proc. Natl Acad. Sci. USA 90: 1536–1540.

5 Collard, J.M., A. Provoost, S. Taghavi and M. Mergeay. 1993. A new type of *Alaligenes eutrophus* CH34 zinc resistance generated by mutations affecting regulation of the *cnr* cobalt-nickel resistance system. J. Bacteriol. 175: 779–784.

6 Conklin, D.S., J.A. McMaster, M.R. Culbertson and C. Kung. 1992. *Cot1*, a gene involved in cobalt accumulation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 12: 3678–3688.

7 Crouzet, J., S. Levy-Schil, B. Cameron, L. Cauchois, S. Rigault, M.C. Rongez, F. Blanch, L. Debussche and D. Thibaut. 1991. Nucleotide sequence and genetic analysis of a 13-1-kilobase-pair *Pseudomonas denitrificans* DNA fragment containing five *cob* gene and identification of structural genes encoding Cob(I) alamin adenosyl transferase cobytic acid synthase and bifunctional cobinamide kinase – cobinamide phosphate guanylytransferase. J. Bacteriol. 173: 6074–6187.

8 Diels, L., M. Faelen, M. Mergeay and D. Nies. 1985. Mercury transposons from plasmids governing multiple resistance to heavy metals in *Alcaligenes eutrophus* CH34. Arch. Int. Physiol. Biochim. 93: B27–B28.

9 Diels, L., A. Sadouk and M. Mergeay. 1989. Large plasmids governing multiple resistances to heavy metals: a genetic approach. Toxic. Environ. Chem. 23: 19.

10 Diels, L. and M. Mergeay. 1990. DNA probe-mediated detection of resistant bacteria from soils highly polluted by heavy metals. Appl. Environ. Microbiol. 56: 1485–1491.

11 Diels, L., S. Van Roy, M. Mergeay, W. Doyen, S. Taghavi and R. Leysen. 1993a. Immobilization of bacteria in composite membranes and development of tubular membrane reactors for heavy metal recuperation. In: Effective Membrane Processes: New Perspectives (R. Paterson, ed.), pp. 275–293, Mechanical Engineering Publications Limited, London, UK.

12 Diels, L., S. Van Roy, S. Taghavi, W. Doyen, R. Leysen and M. Mergeay. 1993b. The use of *Alcaligenes eutrophus* immobilized in a tubular membrane reactor for heavy metal recuperation. In: Biohydrometallurgical Technologies, Vol. II (A.E. Torma, M.L. Apel and C.L. Brierley, eds), pp. 133–144, The Minerals, Metals & Materials Society, Warrendale, PA, USA.

13 Diels, L. 1990. Accumulation and precipitation of Cd and Zn ions by *Alcaligenes eutrophus* strains. In: Biohydrometallurgy 89 (J. Salby, R.G.I. McCready and P.Z. Wichlacz, eds), pp. 369–377, Proceedings of the International Symposium of Jackson Hole (Wyoming), August 13–18, 1989.

14 Dong, Q. and M. Mergeay. 1994. Czc/Cnr efflux: 3 component-export pathway with 12 transmembrane helix exporter. Mol. Microbiol. 14: 185–187.

15 Duong, F., A. Lazdunski, B. Cami and M. Murgier. 1992. Sequence of a cluster of gene controlling synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationship to other secretory pathways. Gene 121: 47–54.

16 Ehrlich, H.L. 1981. Microbial formation and decomposition of carbonates. In: Geomicrobiology, pp. 101–123, Marcel Dekker, New York.

17 Fath, M.J. and R. Kolter. 1993. ABC transporters: bacterial exporters. Microbiol. Rev. 57: 995–1017.

18 Felmlee, T., S. Pellett, E.Y. Lee and R.A. Welch. 1985. *Escherichia coli* hemolysin is released extracellularly without cleavage of a signal peptide. J. Bacteriol. 163: 88–93.

19 Glaser, P., H. Sakamoto, J. Bellalou, A. Ullmann and A. Danchin. 1988. Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis*. EMBO J. 7: 3997–4004.

20 Higgins, C.F. 1992. ABC transporter: from micro-organisms to man. Annu. Rev. Cell Biol. 8: 7–113.

21 Hrycyna, C.A., S.K. Sapperstein, S. Clarke and S. Michaelis. 1991. The *Saccharomyces cerevisiae* STE14 gene encodes a methyltransferase that mediates C-terminal methylation of a-factor and RAS proteins. EMBO J. 10: 1699–1709.

22 Ji, G. and S. Silver. 1994. Review: bacterial resistance mechanisms for heavy metals of environmental concern. J. Ind. Microbiol. (this issue).

23 Kamizono, A., M. Nishizawa, Y. Ternishi, K. Murata and A. Kimura. 1989. Identification of a gene conferring resistance to

zinc and cadmium ions in the yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. 219: 161–167.

24 Klein, J.R., B. Henrich and R. Plapp. 1991. Molecular analysis and nucleotide sequence of the *env*CD operon of *Escherichia coli*. Mol. Gen. Genet. 230: 230–240.

25 Létoffé, S., P. Delepelaire and C. Wandersman. 1990. Protease secretion by *Erwinia chrysanthemi*: the specific secretion functions are analogous to those of *Escherichia coli* α-haemolysin. EMBO J. 9: 1375–1382.

26 Liesegang, H., K. Lemke, R. Siddiqui and H.G. Schlegel. 1993. Characterization of the inducible nickel and cobalt resistance determinant *cnr* from pMOL28 of *Alcaligenes eutrophus* CH34. J. Bacteriol. 175: 767–778.

27 Ma, D., D.N. Cook, M. Alberti, N.G. Pon, H. Nikaido and J.E. Hearst. 1993. Characterization of *acr*A and *acr*E genes of *Escherichia coli*. J. Bacteriol. 175: 6299–6313.

28 Macaskie, L.E. 1990. An immobilized cell bioprocess for the removal of heavy metals from aqueous flow. J. Chem. Tech. Biotechnol. 49: 357–379.

29 Mergeay, M., C. Houba and J. Gerits. 1978. Extrachromosomal inheritance controlling resistance to cadmium, cobalt and zinc ions: evidence from curing in a *Pseudomonas*. Arch. Int. Physiol. Biochim. 86: 440–441.

30 Mergeay, M., D. Nies, H.G. Schlegel, J. Gerits and F. Van Gijsegem. 1985. *Alcaligenes eutrophus* CH34, a facultative chemolithotroph displaying plasmid bound resistance to heavy metals. J. Bacteriol. 162: 328–334.

31 Mergeay, M. 1991. Towards an understanding of the genetics of bacterial metal resistance. Trends Biotech. 9: 17–24.

32 Nies, A., D. Nies and S. Silver. 1990. Nucleotide sequence and expression of a plasmid-encoded chromate resistance determinant from *Alcaligenes eutrophus*. J. Biol. Chem. 265: 5648–5653.

33 Nies, D. 1992. CzcR and CzcD, gene products affecting regulation of resistance to cobalt, zinc, and cadmium *czc* system in *Alcaligenes eutrophus*. J. Bacteriol. 174: 8102–8110.

34 Nies, D., M. Mergeay, B. Friedrich and H.G. Schlegel. 1987. Cloning of plasmid genes encoding resistance to cobalt, zinc and cadmium from *Alcaligenes eutrophus* CH34. J. Bacteriol. 167: 4865–4868.

35 Nies, A., D. Nies and S. Silver. 1989a. Cloning and expression of plasmid genes encoding resistance to chromate and cobalt in *Alcaligenes eutrophus*. J. Bacteriol. 171: 5065–5070.

36 Nies, D.H., A. Nies, L. Chu and S. Silver. 1989b. Expression and nucleotide sequence of a plasmid determined divalent cation efflux system from *Alcaligenes eutrophus*. Proc. Natl Acad. Sci. USA 86: 7351–7355.

37 Nies, D. and S. Silver. 1989c. Plasmid-determined inducible efflux is responsible for resistance to cadmium, zinc and cobalt in *Alcaligenes eutrophus*. J. Bacterial. 171: 896–900.

38 Nies, D. and S. Silver. 1994. Ion efflux systems involved in bacterial metal resistances. J. Ind. Microbiol. (this issue).

39 Nikaido, H. 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 264: 382–388.

40 Poole, K., D.E. Henrichs and S. Heshat. 1993a. Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. Mol. Microbiol. 10: 529–544.

41 Poole, K., K. Krebes, C. McNally and S. Neshat. 1993b. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. 175: 7363–7372.

42 Saier, M.H. Jr, R. Tam, A. Reizer and J. Reizer. 1994. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. Mol. Microbiol. 11: 841–847.

43 Schultze-Lam, S., G. Harauz and T.J. Beveridge. 1992. Participation of cyanobacterial S layer in fine-rain mineral formation. J. Bacterial. 174: 7971–7981.

44 Sensfuss, C. and H.G. Schlegel. 1988. Plasmid pMOL28 encoded resistance to nickel is due to specific efflux. FEMS Microbiol. Lett. 55: 295–298.

45 Siddiqui, R.A., K. Benthin and H.G. Schlegel. 1989. Cloning of pMOL28 encoded nickel resistance genes and expression of the genes in *Alcaligenes eutrophus* and *Pseudomonas* spp. J. Bacteriol. 171: 5071–5078.

46 Silver, S. and M. Walderhaug. 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev. 56: 195–228.

47 Smith, T.F. and M.S. Waterman. 1981. Identification of common molecular subsequences. J. Mol. Biol. 147: 195–197.

48 Shaw, J.J., L.G. Settles and C.I. Kado. 1988. Transposon Tn*4431* mutagenesis of *Xanthomonas campestris* pv. *campestris*: characterization of a non pathogenic mutant and cloning of a locus of pathogenicity. Molecular Plant–Microbe Interactions 1: 39–45.

49 van der Lelie, D., P. Corbisier, W. Baeyens, S. Wuertz, L. Diels and M. Mergeay. 1994. The use of biosensors for environmental monitoring. Res. Microbiol. 145: 67–74.

50 Von Heijne, G. 1986. A new method predicting signal sequence cleavage sites. Nucl. Acids Res. 14: 4683–4690.

51 Wandersman, C. and P. Deleplaire. 1990. TolC an *Escherichia coli* outer membrane protein required for hemolysin secretion. Proc. Natl Acad. Sci. USA 87: 4776–4780.