Metal Accumulation and Vanadium-Induced Multidrug Resistance by Environmental Isolates of Escherichia hermannii and Enterobacter cloacae

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Contaminated soils from an oil refinery were screened for the presence of microorganisms capable of accumulating either nickel, vanadium, or both metals. Three strains of bacteria that belonged to the family Enterobacteriaceae were selected. Two of them were Escherichia hermannii strains, and outer membrane profile (OMP) analysis showed that they were similar to a strain of clinical origin; the other one was an Enterobacter cloacae strain that differed from clinical isolates. The selected bacteria accumulated both nickel and vanadium. Growth in the presence of vanadium induced multidrug resistance phenotypes in E. hermannii and E. cloacae. Incubation with this metal changed the OMP profile of E. hermannii but did not produce variations in the expression of the major OMPs of E. cloacae.

The production of heavy metals has increased rapidly since the industrial revolution (2). Toxic metal species are mobilized from industrial activities and fossil fuel consumption and eventually are accumulated through the food chain, leading to serious ecological and health problems (30). Since the natural mineralization of metals is a slow process, pollution by heavy metals constitutes one of the most important environmental problems of industrial societies (2, 30). Different procedures for the removal of toxic metal species from contaminated environments have been developed; most of them are based on ion-exchange technologies and/or precipitation of the cation in an inert form. These methods are expensive and require the use of contaminating products for the desorption of metals and for cleaning up of the inorganic matrix. Within the last decade, biomass metal adsorption has been demonstrated to be a powerful process for the mineralization and concentration of metals from toxic industrial residues (16, 22, 45, 47). Several bacteria and fungi that accumulate an ample range of metal species have been described (13, 46, 47).

Wastewaters released by oil-processing and petrochemical enterprises contain large amounts of toxic derivatives, such as polycyclic and aromatic hydrocarbons, phenols, sulfides, and heavy metals. Vanadium and nickel are constituents of crude oil in the form of inorganic and metallo-organic compounds. Both metals not only are present in residual waters from the oil industry but also can be present as particulate matter in inhaled air (23) and as environmental heavy metals emitted from automotive materials (11). Both metals are also present as aerosols or in the ashes of thermal power stations operating on fuel oil (43).

The recovery of heavy metals from industrial residues is an important task for environmental and economic reasons (2). Nickel and vanadium are toxic even at low concentrations (17, 19, 26, 31) and therefore are an important source of contamination in industrial societies (30); however, heavy metals also are a valuable resource for different industrial applications. In the present work, we selected heavy-metal-resistant microorganisms from contaminated soils at an oil refinery. Among the microorganisms selected, three of them were capable of accumulating nickel and vanadium. All three microorganisms belonged to the family Enterobacteriaceae. The isolation of different nickel-accumulating microorganisms has been reported before (5, 8, 24, 44); however, to our knowledge, this is the first report of vanadium accumulation by a bacterial biomass.

MATERIALS AND METHODS

Isolation of and growing conditions for microorganisms resistant to nickel or vanadium. Twenty-six samples of contaminated soils were obtained at an oil refinery. The samples were pooled, grown in brain heart infusion (BHI) medium (1) at 37°C for 4 h, and seeded in petri dishes containing BHI agar and a 10 mM concentration of either nickel chloride or vanadyl sulfate. Under these selective conditions, the control laboratory strains Escherichia coli MC4100 and Pseudomonas aeruginosa PA01 were unable to grow. Triplicate plates were seeded in all cases and incubated at 30, 37, and 55°C for a maximum of 3 days. Since all selected organisms grew well at 37°C, further culturing was always done at 37°C. The amount of nickel chloride or vanadyl sulfate is always referred as the metal concentration present in the solutions.

Screening for microorganisms capable of accumulating metals. Metal-resistant microorganisms were seeded in sets of duplicate BHI agar plates containing a 10 mM concentration of either nickel chloride or vanadyl sulfate and incubated at 37°C. One set of plates was exposed to sulphydryl acid (resulting from the reaction of sodium sulfide with chlorhydric acid) in a sealed container. Upon formation of the metal sulfide, both sets of plates were carefully screened to detect any change in the region surrounding or inside bacterial colonies (34).

Classification of microorganisms and determination of MICs. The organisms were taxonomically identified with the commercial system PASCÔ (36). The taxonomic classification of the isolates was confirmed with the API 20NE system (20). The MICs of antibiotics were determined with Mueller-Hinton (MH) agar (1) by the E test (3). The MICs of nickel and vanadium were determined with petri dishes containing MH agar and increasing amounts (0 to 5,000 μg/ml) of either nickel chloride or vanadyl sulfate. The clinical strains Enterobacter cloacae RYC70770 and Escherichia hermannii RYC78330 were provided by the Hospital Ramón y Cajal, Madrid, Spain.

Determination of metal accumulation by bacterial isolates. Microorganisms were cultured overnight on Luria-Bertani agar plates without metals. Confluent bacterial lawns were collected and suspended in phosphate-buffered saline, washed once in the same buffer, divided into 1-ml samples, and pelleted by centrifugation at 12,000 rpm in a bench-top microcentrifuge (Biofuge 13; Heraeus). One of the samples was dried in a Speed Vac centrifuge to calculate the dry weight of the bacterial pellet, and the remaining samples were resuspended in sterile polypropylene tubes containing 100 μg of either nickel chloride (1 ml) or vanadyl sulfate (10 ml) per ml dissolved in water. The samples were incubated at room temperature in a roller mixer for 3 h, and the cells were harvested again by centrifugation under the same conditions. The amount of
residual metal present in the supernatant was measured by atomic absorption
with a Perkin-Elmer 3030 atomic absorption spectrophotometer. Values were
the averages of three determinations carried out in parallel.

**Outer membrane isolation and SDS-PAGE.** Outer membranes were obtained
as described by Fukuoka et al. (14) but with 25 mM Tris-Cl (pH 7.2) instead of
HEPES as the washing buffer. Outer and inner membranes were separated by
incubation for 30 min on ice with 1.5% Triton X-100 instead of Sarkosyl NL-97.
The amount of protein in the extracts was determined with a bicinchoninic acid
protein assay kit (Pierce) in accordance with the manufacturer’s instructions. Ten
micrograms of the outer membrane extracts was suspended in 5 μl of water, the
suspension was mixed with the same volume of 2.3 sodium dodecyl sulfate (SDS)
gel loading buffer (40), and the mixture was incubated at 100°C for 10 min in a
dry bath. The outer membrane proteins (OMPs) were analyzed by SDS–12%
polyacrylamide gel electrophoresis (PAGE) as previously described (14). The
gels were stained with GELCODE blue stain reagent (Pierce) in accordance with
the manufacturer’s instructions.

### RESULTS

**Isolation of microorganisms capable of accumulating heavy metals.** Samples of contaminated soils were screened for the
presence of microorganisms resistant to nickel and/or vanadium. Fifty-one microorganisms able to grow in the presence
of nickel chloride or vanadyl sulfate concentrations higher than 10 mM were isolated. A preliminary screening of metal accu-
mulation (34) was carried out as described in Materials and Methods. Isolates forming either a clear halo around the col-
ony (possible sequestration of the metal) or a dark color
around or inside the colony (possible reduction and precipita-
tion of the metal) were considered to be possible metal bio-
sorbents (34).

Three isolates producing dark colonies when grown in the presence of vanadium (Fig. 1) were selected for further char-
acterization. The API 20NE biotype of two isolates was
1144113; these isolates were classified as *E. hermannii*
CNB50 and *E. hermannii* CNB52. The biotype of the third isolate was
3305573; this isolate was classified as *E. cloacae* CNB60. Iden-
tification with the PASCO system confirmed the taxonomic
classifications of the isolates. Two clinical strains were cultured
as controls. The clinical strain *E. hermannii* RYC78330 resisted
up to 0.5 mM nickel and 1 mM vanadium, and the clinical
strain *E. cloacae* RYC70770 resisted up to 1 mM each metal;
neither clear halos nor dark colors were observed around
the colonies when the clinical strains were grown in the presence
of metals.

The MICs of different antibiotics and the two metals used
for the selection of the bacterial isolates are shown in Table 1. The different values observed for *E. hermannii* CNB50
and CNB52 indicate that these isolates are different strains.

**OMPs of heavy-metal-accumulating bacteria.** Analysis of
OMP profiles has been widely used for examining strain vari-

![FIG. 1](http://aem.asm.org/) Metal accumulation by bacterial colonies. A dark coloration of the
bacterial colony was considered a preliminary marker of metal accumulation. C, bacterial cells grown without metal; V, bacterial cells grown in the presence of
100 μg of vanadium per ml. 50, 52, and 60 indicate colonies of *E. hermannii*
CNB50, *E. hermannii* CNB52, and *E. cloacae* CNB60, respectively.
at right are daltons. Vanadium. The arrow indicates the position of the new, 45-kDa OMP. Numbers indicate the two OMPs whose concentrations were reduced in the presence of vanadium. Ten micrograms of protein was loaded per lane. Asterisks of protein was loaded per lane. Numbers at right are daltons.

FIG. 2. OMP profiles for environmental and clinical isolates of E. cloacae. Lanes 1 and 2, E. cloacae RYCT70770 and E. cloacae CNB60 grown in the absence of metals, respectively; lane 3, E. cloacae CNB60 grown in the presence of nickel; lane 4, E. cloacae CNB60 grown in the presence of vanadium. Ten micrograms of protein was loaded per lane. Numbers at right are daltons.

E. cloacae and E. hermannii were lyzed by SDS-PAGE. Liquid broth in the absence of metals were extracted and analyzed within bacterial species (6, 9, 29). To analyze the similarity of the three environmental isolates to clinical isolates of the same species, OMPs from cells grown on Luria-Bertani liquid broth in the absence of metals were extracted and analyzed by SDS-PAGE. E. cloacae CNB60 lacked the 37-kDa porin OmpF (25), which was present in E. cloacae RYCT70770 (Fig. 2, lanes 1 and 2). No clear differences were observed in the OMP profiles of E. hermannii isolates (Fig. 3, lanes 1 to 3). OMPs from this bacterial species have not been analyzed before; five major OMPs with molecular masses of 23 kDa (Omp23), 36 kDa (Omp36), 40 kDa (Omp40), 43 kDa (Omp43), and 48 kDa (Omp48) were detected.

No major differences in OMP profiles were observed when E. cloacae CNB60 was grown in the presence of metals (Fig. 2, lanes 3 and 4), although some minor differences were observed for small proteins (28 to 30 kDa) when the bacteria were grown in the presence of vanadium (Fig. 2, lane 4). The presence of nickel in the culture medium seemed to have no effect on the OMP profiles of E. hermannii CNB50 (Fig. 3, lane 4). However, the presence of vanadium resulted in the appearance of a new, 45-kDa OMP (Omp45) and a severe reduction in the amounts of Omp48 and Omp43 (Fig. 3, lane 5), as was also the case for E. hermannii CNB52 (data not shown).

E. cloacae CNB60, E. hermannii CNB50, and E. hermannii CNB52 was tested. Cells from overnight cultures were suspended in water containing 100 μg of either nickel or vanadium per ml (a value usually found in residual waters from the oil industry) (3a). Metal accumulation was measured as described in Materials and Methods and expressed as nanomoles of accumulated metal per milligram of bacterial dry weight. E. hermannii CNB50, E. hermannii CNB52, and E. cloacae CNB60 accumulated 687.71 ± 29.55 nmol of vanadium and 130.57 ± 3.81 nmol of nickel per mg, 918.07 ± 58.21 nmol of vanadium and 175.12 ± 4.56 nmol of nickel per mg, and 671.70 ± 13.03 nmol of vanadium and 117.12 ± 2.87 nmol of nickel per mg, respectively.

FIG. 3. OMP profiles for environmental and clinical isolates of E. hermannii. Lanes 1 to 3, E. hermannii RYCT8830, E. hermannii CNB52, and E. hermannii CNB50 grown in the absence of metals, respectively; lane 4, E. hermannii CNB50 grown in the presence of nickel; lane 5, E. hermannii CNB50 grown in the presence of vanadium. Ten micrograms of protein was loaded per lane. Asterisks indicate the two OMPs whose concentrations were reduced in the presence of vanadium. The arrow indicates the position of the new, 45-kDa OMP. Numbers at right are daltons.

Accumulation of nickel and vanadium. Metal accumulation by E. cloacae CNB60, E. hermannii CNB50, and E. hermannii CNB52 was tested. Cells from overnight cultures were suspended in water containing 100 μg of either nickel or vanadium per ml (a value usually found in residual waters from the oil industry) (3a). Metal accumulation was measured as described in Materials and Methods and expressed as nanomoles of accumulated metal per milligram of bacterial dry weight. E. hermannii CNB50, E. hermannii CNB52, and E. cloacae CNB60 accumulated 687.71 ± 29.55 nmol of vanadium and 130.57 ± 3.81 nmol of nickel per mg, 918.07 ± 58.21 nmol of vanadium and 175.12 ± 4.56 nmol of nickel per mg, and 671.70 ± 13.03 nmol of vanadium and 117.12 ± 2.87 nmol of nickel per mg, respectively.

DISCUSSION

Three bacterial strains, two E. hermannii strains and one E. cloacae strain, that are capable of accumulating nickel and vanadium have been analyzed. It has been shown that environmental gas-utilizing isolates of P. aeruginosa are indistinguishable from clinical isolates of the same species (12). Our results indicate that this may be the case for E. hermannii as well. Since this organism was first reported in 1982 (4), a few cases of infections by E. hermannii have been described, but the presence of this bacterial species in environmental habitats has not been reported. The isolation of E. hermannii from contaminated soils at an oil refinery suggests that this bacterium also has an environmental habitat. E. cloacae is known to be present in soil samples, to be able to colonize the rhizosphere of plants (35), and to survive strong environmental desiccation (33). Enterobacter species also have been found in heavy-metal-contaminated soils (37), one zinc-tolerant Enterobacter sp. isolate capable of accumulating this metal has been described (18), and the reduction of selenium oxyanions by E. cloacae has been described (21). However, to our knowledge, this is the first time that the accumulation of nickel and vanadium by E. cloacae has been reported.

Multidrug resistance (MDR) has been described for several bacterial species and is currently associated with the expression of efflux pumps that are associated with the expression of efflux pumps. Similar types of pumps are involved in the transport of antibiotics inside bacterial cells. Similar types of pumps are involved in heavy-metal resistance (41, 42). Most MDR systems so far described for gram-negative bacteria are three-component pumps (28, 32, 39); the regulation of their expression still is not completely understood. Some environmental signals can induce an MDR phenotype; for example, the presence of salicylate induces the expression of the efflux pump encoded by the marRAB operon and simultaneously represses the synthesis of the...
OmpF porin (7). Vanadium may have a similar role in the induction of the MDR phenotype, since the MICs of different antibiotics for the three isolates were higher in the presence of vanadium. Additionally, increased amounts of Omp45 and decreased amounts of Omp43 and Omp48 were observed in E. hermannii, and some changes in the amounts of minor bands (in the range of 28 to 30 kDa) were detected in E. cloacae (Fig. 2 and 3). The results obtained suggest the possible existence of functional efflux pump systems in these bacteria.

Metal accumulation or biostimulation is an alternative mechanism for metal detoxification in bacteria (15). The presence of heavy metals affects the ecology of sediments (27) and the biodegradation of organic chemicals by microorganisms (38). The removal of toxic components from industrial effluents is of great importance, not only because of the decontamination of water bodies but also because this recovers the activated sludge of sewage treatment plants from the action of toxic compounds and ensures the functioning of the plants (10). The bacterial isolates described here may be of use for removing nickel and/or vanadium from contaminated effluents.

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