METALLOPHILES AND ACIDOPHILES IN METAL-RICH ENVIRONMENTS

Max Mergeay
Laboratory for Microbiology, Center of Studies for Nuclear Energy, Mol, Belgium

Keywords: anthropogenic, bioremediation, copper belt of Austral Africa, efflux, firmicutes, *Geobacter metallireducens*, hydrogenotrophy, metallophytes, minimum inhibitory concentration, mobile genetic elements, natural metal-rich biotopes, New Caledonia, plasmids, proteobacteria, *Ralstonia metallidurans*, reduction, zinc desert

Contents

1. Introduction: industrial biotopes as a reservoirs for extremophiles
2. Bacteria and the periodic table
3. Bacteria and metals
4. How to track metal-resistant bacteria?
5. Metal-rich biotopes as sources of metal-resistant bacteria
5.1. Natural Metal-Rich Soil Biotopes
5.2. Anthropogenic Metal-Rich Soil Biotopes
6. Metal resistance: a role for mobile genetic elements
7. The organization of metal-resistance genes in Ralstonia metallidurans
7.1. A Preliminary View at the Genomic Level
7.2. Regulation Modules in Metal-Resistance Operons
8. Other bacteria involved in biogeochemical processes involving metals
9. Environmental applications of bacteria adapted to heavy metals or able to process heavy metals
10.Conclusions
Acknowledgements
Glossary
Bibliography
Biographical Sketch

Summary

This article describes some bacteria that are specifically adapted to metal-rich biotopes and can therefore be considered as a kind of extremophile. Metal-rich biotopes may be divided up to some extent into natural and anthropogenic biotopes. Natural biotopes correspond to a variety of geological events that often shelter endemic populations of plants (metallophytes). Some deep-sea or terrestrial hydrothermal sources may be also being considered as natural metal-rich biotopes.

In their extreme form, anthropogenic metal-rich biotopes are mainly industrial, and may be found around metal-processing factories or in dumping sites. They are also characteristic of the Industrial Revolution of the last 200–300 years. Their multiplication on the earth's surface has promoted the large-scale redistribution of bacterial populations. The harsh selection pressure, linked to the chemical mixtures they

contain, has likely forced some key genetic rearrangements in these populations, and is therefore significant from the point of view of evolution.

Ralstonia metallidurans and related species are regularly found in such anthropogenic biotopes. Their resistance to high concentrations of heavy metals such as cadmium, chromium, cobalt, copper, mercury, nickel, lead, thallium, and zinc is mainly plasmid-borne. Plasmid-borne resistance to heavy metals in other bacteria also includes resistance to the group of oxyanions formed by arsenite, arsenate, and antimonite, to tellurite and to silver.

The draft sequence of *R. metallidurans* is now available, and makes it possible to identify genes involved in metal resistance, and adaptation on both the chromosome and the large plasmids. Yet plasmid-borne genes seem to be organized in more complex clusters. Some of these genes encode for unique proteins that have, to date, no equivalents in genomic databases.

Other adaptations to heavy metals not necessarily bound to metal-rich biotopes are also mentioned, such as the metal-reducing anaerobic bacteria. The diversity of mechanisms of metal resistances, metal bioprecipitation or mineralization, and of metal solubilization, also provides an arsenal of remediation tools to process polluted soils or effluents, mixed wastes (metals, radionuclides), and recalcitrant synthetic organic compounds (xenobiotics).

1. Introduction: Industrial Biotopes as a Reservoir for Extremophiles

The Industrial Revolution that has lasted for the last 200 years has generated plenty of new biotopes in soils, rivers, lakes, and sediments, mainly via the dumping of metal-rich wastes that were often mixed with recalcitrant synthetic chemical compounds (chloroaromatic compounds). The extreme variety of these industrial sites and their geographical dispersion across the surface of the earth has provoked a huge and yet almost unnoticed redistribution of bacterial populations, probably including those that were already adapted to heavy metals, and has surely exerted evolutionary pressures on their genotypes.

That visible evolutionary processes occurred during the Industrial Revolution may be deduced from the adaptation of various catabolic pathways to recalcitrant synthetic chemicals which are now released in high amounts to the environment. These chemicals did not exist before the Industrial Revolution. Evolutionary pressures have also played a role in the adaptation to new habitats that, because of human activities (the dumping of industrial wastes), poor vegetal coverage, and food scarcity, are constantly exposed to strong variations in physicochemical conditions (chemical toxicity, temperature, moisture, osmotic pressure, and so on) which are rarely balanced.

Therefore, the Industrial Revolution has pushed for the selection of new “extremophiles” among bacteria, with the additional point that the evolutionary trends at work towards this selection can be considered as accessible and even measurable. The current development of comparative genomics, the direct analysis of soil microbial populations with molecular tools, the availability of bacterial strain collections dating from about 1900, and long-term experimental evolution with microbes, will help considerably in this respect.

2. Bacteria and the Periodic Table

Life in prokaryotes (Bacteria and Archaea), as well as in eukaryotes, relies on various elements from the Periodic Table, the basic ones being carbon, nitrogen, oxygen, hydrogen, sulfur, and phosphor. Metals are also required for life: calcium, magnesium, potassium, and sodium are tolerated or required in substantial concentrations. The biological requirements for iron and manganese are satisfied at lower concentrations. Metallic trace elements essential for bacterial life include copper, cobalt, nickel, chromium, molybdenum, vanadium, tungsten, and zinc, but concentrations even slightly exceeding those satisfying the requirements for the trace elements are generally toxic. Tungsten (which can also substitute for molybdenum in various molybdoenzymes) deserves a special mention as far as extremophiles are concerned. Tungsten (W) is essential for the growth of the hyperthermophiles *Pyrococcus furiosus* and *Thermococcus littoralis*, and up to now, looks to be the essential metal with the highest atomic number: 74.

Among the other elements of the Periodic Table, chloride is required for ionic equilibrium and may be tolerated through a broad range of concentration. Silicium content is conspicuous in some biological structures, such as in the diatoms.

The nonmetallic elements boron, fluoride, arsenic, selenium, and iodine may be added to the list of biological trace elements in prokaryotes or in eukaryotes (natural brominated or chlorinated organic molecules have been reported, but mainly look like secondary metabolites). But outside the basic metallic elements that are essential for life, all the other metals and elements are generally toxic when they are bioavailable. (Bioavailable means soluble at biological pH, and/or having access to the various transport pathways that cross the biological membranes.) Cadmium, silver, mercury, thallium, and lead ions are the most toxic for living beings, and constitute major sources of concern in environmental toxicology.

For completeness, a special mention has to be made of metallic species such as U(VI) and Tc(VII) on which some bacteria can respire in anaerobic conditions. In this respect, the case of technetium (a transition element) is remarkable, as this element does not currently exist on earth, even as a natural radioisotope.

3. Bacteria and Metals

For soil microbes, there is a special challenge as they may come in contact with metallic ores or with locally high concentrations of metals. This occurs in a lot of natural situations dictated by the geological history of the earth: deep hydrothermal sources, volcanic areas, and the nickel-rich ultramafic soils of New Caledonia are a few examples of such natural situations. Metal-tolerant or even metal-accumulating plants colonize such terrestrial biotopes, and feed bacterial communities through root exudates.

Anthropogenic situations have to be considered as well: the dumping sites of metallurgical waste or of waste from processed ores, and areas desertified by aerial releases of roasted sulfide. These biotopes may contain very high amounts of metals, of which a part becomes bioavailable.

Metal release in agricultural practices may also affect bacterial communities from soil: copper-based fungicides play a role in this respect in the vineyards that were treated during decennia with "Bordeaux mixture" (mixtures with copper sulfate as the main active agent), but these agriculturally polluted biotopes do not seem to be as selective of extremophiles as industrial biotopes. However, the action of copper-based fungicides allowed the selection of plasmid-borne resistances to copper in bacteria pathogenic for vegetable crops (*Pseudomonas syringae* var *tomato*, *Xanthomonas campestris*, and so on) and the colonizing of plant aerial parts (particularly their leaf surface). In the same way, copper resistant *E. coli* strains have been found in the manure of pigs fed with copper additives. Similarly, the use of arsenic derivatives as a bactericidal agent for veterinary purposes most likely led to the emergence of arsenic-resistant *Yersinia enterocolitica* strains in pigs.

In the metal-rich biotopes that are reservoirs of extremophiles, most of the attention has been focused on metal-resistant heterotrophic bacteria. Nevertheless, these bacteria most likely form only a small part of the biodiversity of these biotopes, and much research has still to be done to provide exhaustive studies of the real biodiversity of these biotopes and their long-term evolution. In the same way, although they do not specifically colonize "extreme" metal-rich biotopes, other bacteria that are remarkable because of functions linked to metal metabolism or processing will briefly be described or mentioned. Therefore, it will be also possible to get a hint of the microbial arsenal that is involved in metal biogeochemistry. or that could be integrated in bioremediation studies.

4. How to Track Metal-Resistant Bacteria?

Selective media for metal-resistant bacteria have to be designed in order to reduce as much as possible the apparent minimal inhibitory concentration. Broth media are not convenient in this respect as they chelate many metals. If broth media have to be used as selective media, the concentration of metals has to be increased in order to make the medium selective enough. Phosphate also has to be reduced to a minimum and, as a buffering agent, has to be replaced by agents such as Tris or MOPS.

A first indication of a selective pressure linked to the presence of bioavailable concentrations of heavy metals may arise when the viable counts of heterotrophic bacteria on selective media (with metals) are similar to those observed in the absence of selection. Further evidence that bacteria carry specific resistance to heavy metals may be provided by the use of molecular techniques such as hybridization with specific probes, or by PCR-mediated amplification of key sequences from metal-resistant colonies.

A complete chemical analysis of the soil samples is also of importance for the phenotypic determination of the bacteria of interest, and data must be produced concerning the total metal content, the bioavailable fraction, and the determination of the main metallic compounds.

5. Metal-Rich Biotopes as Sources of Metal-Resistant Bacteria

5.1. Natural Metal-Rich Soil Biotopes

Natural metal-rich biotopes are often the consequence of geological accidents, and have been studied for their endemic vegetation; they include the copper belt of Austral Africa that extends from Katanga through Zambia and Zimbabwe to South Africa, and the ultramafic soils of New Caledonia that are extremely rich in nickel. It is mainly in New Caledonia that attempts were made to also look at metal-resistant bacteria in the rhizosphere of metal-accumulating plants (Figure 1).

Figure1. In New Caledonia, nickel-rich ultramafic soils shelter around 300 endemic plants adapted to this metal. Latex of *Sebertia acuminata*, shown on the stamp, contains up to 20% nickel. Dry leaves contain up to 1% nickel. The rhizosphere of these plants contains nickel-resistant bacteria with *ncc* genes very similar to those found in bacteria from industrial sites, although the bacteria are taxonomically quite different.

Attention has been given to nickel-resistant Proteobacteria, some of them carrying genes that are very similar to those found in *Ralstonia* or other bacteria characteristic of industrial metal-rich biotopes, and also to Thallobacteria (high GC Gram-positives) belonging to the N_2 fixating *Frankia*, which colonize the roots of various trees of New Caledonia (*Casuerina* sp., *Gymnostoma deplancheanum*) and are studied by Isabelle Navarro in Lyon.

More studies should be done on the rhizosphere of the endemic plants that are characteristic of these areas, and need to focus on the possible synergies between bacteria and plants with respect to metal detoxification or accumulation.

Many metallophytes are also known in temperate areas of the Northern Hemisphere, and act as indicators of metal-rich biotopes, including natural biotopes, but also some anthropogenic biotopes that correspond to abandoned metallurgical plants or disaffected mining sites, such as the site of Plombières (a name that clearly refers to the element Pb, lead) in eastern Belgium. This small site, now arranged as a natural reservation, is extremely rich in lead and zinc, and is extensively colonized by *Thlaspi caerulescens* var *calaminaria* (zinc pennycress), *Viola calaminaria*, *Armeria maritime,* and some *Festuca*.

Volcanic areas should also attract the attention of soil microbiologists, especially soon after eruptive events, at the moment where pioneering bacteria begin to colonize surfaces covered by ash.

Geothermal environments are also a source of interesting microorganisms adapted to heavy metals: bacteria were reported to rapidly oxidize inorganic As(III) to As(V) from arsenic-rich terrestrial geothermal environments or from hot springs in the Yellowstone National Park (USA). Some of these bacteria belong to the genus *Thermus*: one of these *Thermus* strains is able not only to oxidize As(III) to As(V) but also to respire on As(V) in anaerobic conditions.

Deep-sea hydrothermal sources are also environments that are studied for the adaptation of bacteria to heavy metals.

-
-
-

Bibliography

Baldi F., Minacci A., Pepi M., and Scozzafava A. (2001). Gel sequestration of heavy metals by *Klebsiella oxytoca* isolated from iron mat. *FEMS Microbiol Ecol.* **36**, 169–174. [A bioremediation perspective from a bacterium isolated from an industrial biotope.]

Bazylinski D.A. (1999). Synthesis of the bacterial magnetosome: the making of a magnetic personality. *Int Microbiology* **2**, 71–80. [A review of magnetotactic bacteria.]

Bazylinski D.A, Dean A.J., Schuler D., Phillips E.J., and Lovley D.R. (2000). N_2-dependent growth and nitrogenase activity in the metal-metabolizing bacteria, *Geobacter* and *Magnetospirillum* species. *Environmental Microbiology* **2**, 266–273. [A striking feature on subsurface metal-metabolizing bacteria.]

Borremans B., Hobman J.L., Provoost A., Brown N.L., and van Der Lelie D. (2001). Cloning and functional analysis of the *pbr* lead resistance determinant of *Ralstonia metallidurans* CH34. *J. Bacteriol*. **183**, 5651–5658. [The first description of a genetic determinant responsible for the resistance to lead.]

Brandl H. (2001) 8. Microbial leaching of metals. *Biotechnology* Second Edition **10**(8), (eds H.J.Rehm and G.Reed in cooperation with A.Pühler and P.Stadler), 191–224. Weinheim, F.R.Germany: WILEY-VCH. [A comprehensive description of metal-leaching mostly acidophilic micro-organisms and their applications; contains a good oversight of their biodiversity.]

Caccavo F. Jr, Coates J.D., Rossello-Mora R.A., Ludwig W., Schleifer K.H., Lovley D.R., and McInerney M.J. (1996). *Geovibrio ferrireducens*, a phylogenetically distinct dissimilatory Fe(III)-reducing bacterium. *Arch Microbiol*. **165**, 370–376. [This paper and the one below suggest an increasing biodiversity in iron-reducing bacteria.]

Coates J.D., Ellis D.J., Gaw C.V., and Lovley D.R. (1999). *Geothrix fermentans* gen. nov., sp. nov., a novel Fe(III)-reducing bacterium from a hydrocarbon-contaminated aquifer. *Intern. J. Syst. Bacteriol*. **49**, 1615–1622. [see above.]

Coates J.D., Bhupathiraju V.K., Achenbach L.A., Mclnerney M.J., and Lovley D.R. (2001). *Geobacter hydrogenophilus*, *Geobacter chapellei* and *Geobacter grbiciae*, three new, strictly anaerobic, dissimilatory Fe(III)-reducers. *Int. J. Syst. Evol. Microbiol.* 51, 581–588. [Taxonomical description of the best-known group of metal-reducing bacteria.]

Corbisier P., Van Der Lelie D., Borremans B., Provoost A., De Lorenzo V., Brown N.L., Lloyd J.R., Hobman J.L., Csöregi E., Johansson G., and Mattiasson B. (1999). Whole cell- and protein-based biosensors for the detection of bioavailable heavy metals in environmental samples. *Anal. Chim. Acta*. **387**, 235–244. [This paper describes an environmental application of bacterial genes and proteins involved in metal-resistance.]

Diels L., Dong Q., van der Lelie D., Baeyens W., and Mergeay M. (1995). The *czc* operon of *Alcaligenes eutrophus* CH34: from resistance mechanism to the removal of heavy metals. *J. Industr. Microbiol.* **14**, 142–153. [Applications of metal-resistant bacteria in bioremediation.]

Gadd G.M. (2000). Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. *Curr. Opin. Biotechnol*. **11**, 271–279. [An outstanding and comprehensive review.]

Gihring T.M. and Banfield J.F. (2001). Arsenite oxidation and arsenate respiration by a new Thermus isolate. *FEMS Microbiol. Lett.* **204**, 335–340. [One of the recent reports about arsenic compounds as life supporting systems.]

Glasauer S., Langley S., and Beveridge T.J. (2002). Intracellular iron minerals in a dissimilatory iron-reducing bacterium. *Science* **295**, 117–119. [A characteristic paper from a laboratory specialized in the study of the biomineralization/biocristallization phenomena.]

Gupta A., Matsui K., Lo J.F., and Silver S. (1999). Molecular basis for resistance to silver cations in *Salmonella*, *Nat. Med*. **5**, 183–188. [First complete report about a bacterial resistance to silver.]

Kashefi K., Tor J.M., Nevin K.P., and Lovley D.R. (2001). Reductive precipitation of gold by dissimilatory Fe(III)-reducing bacteria and archaea. *Appl. Environ. Microbiol*. **67**, 3275–3279. [An application of microbial metabolism to precious metal recovery.]

Liebert C.A., Hall R.M., and Summers A.O. (1999). Transposon Tn21, flagship of the floating genome. *Microbiol. Mol. Biol. Rev*. **63**, 507–522. [Not only does this attractive review describe a ubiquitous family of mobile genetic elements, it also provides a detailed description of all the mercury genes and their combinations as they appear in a variety of bacteria.]

Lloyd J.R. and Lovley D.R. (2001). Microbial detoxification of metals and radionuclides. *Curr. Opin. Biotechnol.* **12**, 248–253. [An useful, detailed, and comprehensive review.]

Mergeay M., Nies D., Schlegel H.G., Gerits J., Charles P., and van Gijsegem F. (1985). *Alcaligenes eutrophus* is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *J. Bacteriol*. **162**, 328–334. [First isolation and description of *Ralstonia metallidurans* CH34 (then called *Alcaligenes eutrophus*, later *R.eutropha.*)]

Niggemyer A., Spring S., Stackebrandt E., and Rosenzweig R.F. (2001). Isolation and characterization of a novel As(V)-reducing bacterium: implications for arsenic mobilization and the genus Desulfitobacterium. *Appl. Environ. Microbiol*. **67**, 5568–5580. [A bacterium able to reduce a variety of cations and oxyanions that was isolated from arsenic-contaminated environments.]

Schlegel, H.G., Cosson J.P., and Baker A.J.M. (1991). Nickel-hyperaccumulating plants provide a niche for nickel-resistant bacteria. *Bot. Acta* **104**, 18–25. [This report directs attention to metal-adapted plants and their rhizosphere as a source of metal-resistant bacteria.]

Schmidt T. and Schlegel H.G. (1994). Combined nickel–cobalt–cadmium resistance encoded by the ncc locus of *Alcaligenes xylosoxidans* 31A. *J. Bacteriol*. **176**, 7045–7054. [One of the highest resistances to a metal (nickel) described so far.]

Stoppel, R.D. and Schlegel H.G. (1995). Nickel-resistant bacteria from anthropogenically nickel-polluted and *naturally* nickel-percolated ecosystems. *Appl. Environ. Microbiol*. **61**, 2276–2285. [An interesting report about the difference between natural and anthropogenic metal-rich biotopes.]

Storz G., and Hengge-Aronis R. (eds). (2000). *Bacterial Stress Responses*, Washington, D.C., USA: ASM Press. [This readable and exhaustive book covers all aspects of bacterial stress. It includes a chapter on metal-resistant *Ralstonia.*]

Tibazarwa C., Wuertz S., Mergeay M., Wyns L., and van der Lelie D. (2000). Regulation of the cnr cobalt and nickel resistance determinant of Ralstonia eutropha-like CH34 (ex-*Alcaligenes eutrophus*). *J.Bacteriol*. **182**, 1399–1409. [This report describes an unique mechanism of regulation of heavy metal resistance that was only reported in *R.metallidurans* and involves membrane bound proteins.]

Top E., De Smet I., Verstraete W., Dijkmans R., and Mergeay M. (1994). Exogenous isolation of mobilizing plasmids from polluted soils and sludges, *Appl. Environm. Microbiol*. **60**, 831–839. [Application of metal-resistance genes to soil microbial ecology.]

van der Lelie, D. (1998). Biological interactions: the role of soil bacteria in the bioremediation of heavy metal-polluted soils. *Metal-Contaminated Soils: In Situ Inactivation and Phytorestoration*, (eds. J. Vangronsveld and S.D. Cunningham), 31–50. Austin, TX, USA: Landes-Bioscience. [A review about metal bioremediation in soils.]

Wade R. and Di Christina T.J. (2000). Isolation of U(VI) reduction-deficient mutants of *Shewanella putrefaciens*. *FEMS Microbiol. Lett.* **184**,143–148. [The first reported isolation of a respiratory mutant that is unable to use U(VI) as a terminal electron acceptor.]

Zierenberg R.A., Adams M.W., and Arp A.J. (2000). Life in extreme environments: hydrothermal vents. *Proc. Natl. Acad. Sci. USA* **97**, 1296–12962. [Interesting comments about the chemical reactions that could give birth to energy sources in hydrothermal sources and other volcanic environments.]

Biographical Sketch

Max Mergeay (born in Ixelles, Belgium, 1943) received his Ph.D. (Chemistry), under the guidance of Professor Jean-Marie Wiame, in the Laboratory for Microbiology of the University Libre de Bruxelles in 1969. Since July 1968, he has worked in the Belgian Nuclear Research Centre (SCK/CEN) of Mol, Belgium on heterologous gene transfer in bacteria. In 1974–1975 (a Fullbright-Hays grantee), he worked at the Department of Biological Sciences, Stanford, CA, with Professor Charles Yanofsky and then at the University of Washington, Seattle, with Professor Eugene Nester. Since 1976, he has been involved in research on bacterial plasmid-bourne resistance to heavy metals from industrial areas, in parallel with studies on the dissemination of plasmids in soil environments and on genetic instability in bacteria. From 1992 to 1999, he worked in the Environmental Technology department of the Flemish Institute for Technological Research (Mol, Belgium). Since 1999 he has been back to the SCK/CEN, in charge of programs for nuclear bioremediation and of the section Radiobiology. Since 2000, his laboratory is also involved in ESA programs on space microbiology and life support systems.

He has been a teacher (of genetics) in the Université Libre de Bruxelles (since 1993), scientific collaborator of the Université de Liège, of the Vrije Universiteit Brussel and of the Faculté d'Agronomie de Gembloux. He was organizer or co-organizer of workshops including BAGECO-1 in Brussels, and the EMBO Workshop on Genetic Instability in Bacteria (Retie, 1995). He participated in various missions linked to environmental biotechnology and microbiology in Congo (field mission, 1990), Thailand (visiting professor, 1991), Pakistan (1993), Japan (fellow of the Japan Society for the Promotion of Science, 1995), and the French AMISTAD expedition on the deep-sea hydrothermal sources of Eastern Pacific Ocean (1999).

He is a Member of the Board (founder member) of the Belgian Society for Microbiology.