Elemental and Redox Analysis of Single Bacterial Cells by X-ray Microbeam Analysis

Kenneth M. Kemner,[1]* Shelly D. Kelly,[1] Barry Lai,[1] Joerg Maser,[1] Edward J. O'Loughlin,[1] Deirdre Sholto-Douglas,[1] Zhonghou Cai,[1] Mark A. Schneegurt,[2] Charles F. Kulpa Jr.,[3] Kenneth H. Nealson[4]

High-energy x-ray fluorescence measurements were used to make elemental maps and qualitative chemical analyses of individual *Pseudomonas fluorescens* strain NCIMB 11764 cells. Marked differences between planktonic and adhered cells were seen in the morphology, elemental composition, and sensitivity to Cr(VI) of hydrated cells at spatial scales of 150 nm. This technology can be applied to natural geomicrobiological systems.

Attachment of prokaryotic cells to surfaces during biofilm formation not only leads to major changes in metabolism, resistance, and survivability (*1*), but the substrates metabolized by such communities (such as iron or manganese oxides) and their products (such as uraninite or chromium oxides) often are insoluble and associated with the attached bacteria (*2–4*). Thus, quantifying and specifying the locations of cellular components, metabolic reactants, and metabolic products of prokaryotes is technologically challenging.

There are no techniques currently available that use noninvasive, nondestructive analytical techniques with the spatial resolution to examine living and hydrated samples at the nanometer scale and that are capable of both imaging and chemical determinations. In particular, most analytical methods with high spatial resolution (typically with electron or proton microprobes) use high-vacuum systems, almost certain to alter the cellular material itself and the locations of key associated elements.

To address this issue, we have used high-energy x-ray fluorescence (XRF) methods with a spatial resolution of 150 nm to examine elemental compositions of single hydrated bacterial cells (*5*). We determined elemental compositions of single cells of *P. fluorescens* NCIMB 11764, an aquatic Gram-negative bacterium that exists naturally both in planktonic form and in surface-adhered biofilms. We saw changes in cell morphology and elemental composition of single cells when attached to a solid substrate. Many studies have investigated the interactions of metals and ions with bacteria (*6–8*) and aquatic protists (*9*), but not with hydrated bacteria at the single-cell level.

Zone plate fabrication technology for the production of high-energy x-ray microprobes (*10*) combines high spatial resolution with high elemental sensitivity (better than 1×10^4 atoms within a spot size of 150 nm) (*11*) and x-ray absorption spectroscopy to probe chemical interactions at this scale (*12*). The high-energy x-ray microprobe has better elemental sensitivity than most charged-particle probes and allows the investigation of heterogeneous hydrated samples. The x-ray microprobe also allows investigation of structures tens of microns beneath solid-phase surfaces.

High-energy XRF microscopy showed planktonic cells were rod shaped and contained high concentrations of virtually all elements expected in living cells (Fig. 1, table S1); the attached cells were rounded and had notable excesses of Ca and P associated with them (Fig. 2, table S1). With surface adherence, [P] associated with the cells increased from ~16,000 to ~650,000 ppm, and [Ca] increased from ~4,000 to ~550,000 ppm, yielding ~1:1 stoichiometry for these two elements (table S1). Other elements showed only minor changes in the attached cells.

The greater [Ca] and [P] in the adhered cells versus the planktonic cells remains un-

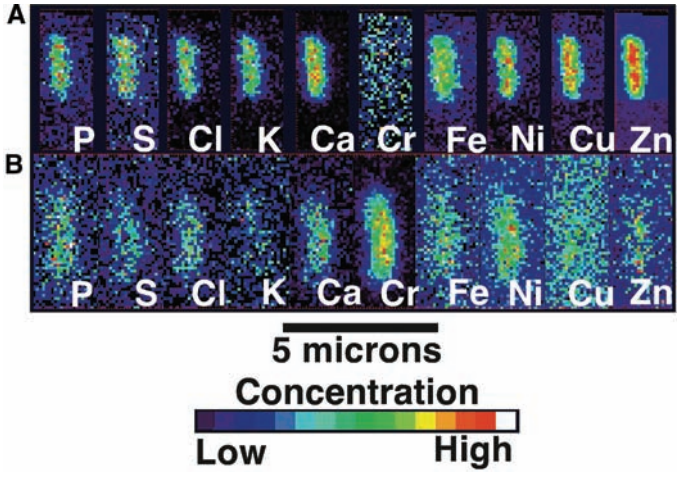

Fig. 1. False-color micro-XRF maps of qualitative spatial distributions and concentration gradients of elements in and around planktonic *P. fluorescens* microbes harvested before (**A**) and after (**B**) exposure to potassium dichromate [Cr(VI)] solution (1000 ppm) for 6 hours.

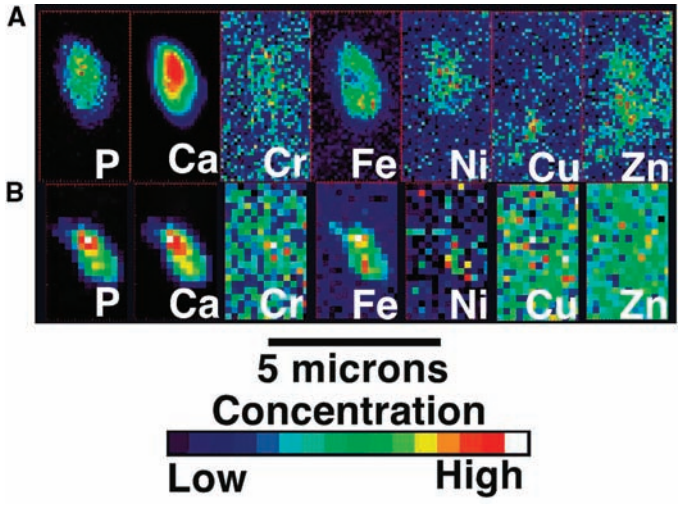

Fig. 2. False-color micro-XRF maps of qualitative spatial distributions and concentration gradients of elements in and around surface-adhered *P. fluorescens* microbes harvested before (**A**) and after (**B**) exposure to potassium dichromate [Cr(VI)] solution (1000 ppm) for 6 hours. Because of the extremely high concentrations of Ca and P in the surface-adherent microbes, the intensities of neighboring XRF signals from S, Cl, and K could not be determined accurately. Therefore, spatial distributions for these elements are not illustrated.

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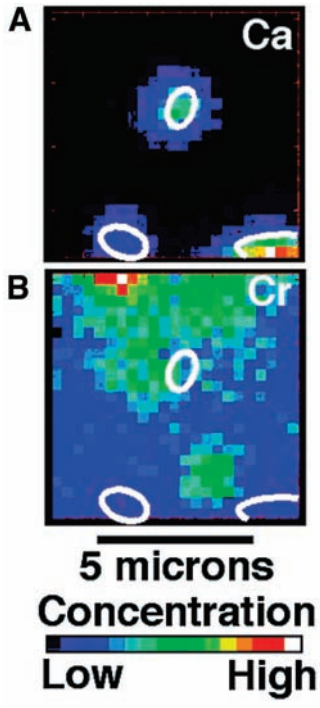

Fig. 3. False-color micro-XRF maps of Ca (**A**), identifying the locations of surface-adherent microbes exposed to Cr(VI) solution (1000 ppm), and Cr (**B**), depicting the locations of elevated concentrations of Cr within 1 to 3 μm of surface-adherent *P. fluorescens*. Cell positions [determined from Ca distribution in (A)] are drawn in white.

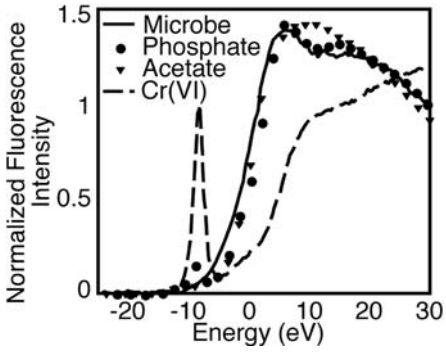

Fig. 4. Cr K-edge micro-XANES spectra representing potassium dichromate [Cr(VI)] solution added to growth medium; a chromium phosphate dihydrate [Cr(III)] standard; a chromium acetate [Cr(III)] standard; and the elevated microbe-associated Cr concentrations illustrated in Fig. 3B.

explained, but it probably involves the extracellular polysaccharide (EPS) matrix laid down by the *P. fluorescens* cells and the precipitation of apatite (*5*). Strains of this species produce various acidic EPSs (*13–16*) that can bind calcium ions preferentially from solution (*17–19*). SEM imaging of the cells from our experiments indicated the formation of an extracellular matrix.

Because attached cells are thought to have enhanced resistance to stress, we challenged the planktonic cells with Cr(VI) at 1000 and 25 ppm for 6 hours and the attached cells with Cr(VI) at 1000 ppm for 6 hours. After this treatment, planktonic *P. fluorescens* cells were markedly altered; they stained as dead cells in the live or dead stain reaction (*5*), lost almost all of their cellular transition elements, and accumulated substantial amounts of Cr (Fig. 1). In contrast, the attached cells showed virtually no change in elemental composition and no uptake of Cr into the cells (Fig. 2), and living cells were abundant.

Finally, we used the XRF microscopy system to measure x-ray absorption near edge spectra (XANES) (*12*) to determine the redox state of the Cr in this system. With micro-XANES we investigated the chemical speciation of Cr in the Cr-enriched areas seen in Fig. 3. Comparison of these Cr K-edge spectra to standards (Fig. 4) indicates reduced Cr [i.e., Cr(III)], consistent with association of Cr with a phosphoryl functional group.

The XRF approach also revealed that, when attached to a surface, the cells changed shape slightly, lost some transition elements (Co, Cu, Ni, and Zn), gained others (Fe and Mn), and became resistant to exposure to high levels of Cr(VI), which shows the utility of this technique for investigating a cell's metabolic state. Combining elemental analysis with the ability to measure redox state and local chemistry is advantageous for dissecting the activity of metal-active bacteria in geomicrobiological systems.

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Supporting Online Material
www.sciencemag.org/cgi/content/full/306/5696/686/DC1
Materials and Methods
SOM Text
Table S1

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A Chromosome 21 Critical Region Does Not Cause Specific Down Syndrome Phenotypes

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The "Down syndrome critical region" (DSCR) is a chromosome 21 segment purported to contain genes responsible for many features of Down syndrome (DS), including craniofacial dysmorphology. We used chromosome engineering to create mice that were trisomic or monosomic for only the mouse chromosome segment orthologous to the DSCR and assessed dysmorphologies of the craniofacial skeleton that show direct parallels with DS in mice with a larger segmental trisomy. The DSCR genes were not sufficient and were largely not necessary to produce the facial phenotype. These results refute specific predictions of the prevailing hypothesis of gene action in DS.

Trisomy 21 is among the most complex genetic insults compatible with human survival past term. The genetic complexity and individual variability of DS phenotypes pose a considerable challenge to understanding mechanisms by which development is dis-

Science Supporting Online Material

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Material and methods

A 1-ml aliquot of a 48-h culture of *P. fluorescens* str. NCIMB 11764 was inoculated into 100 ml of autoclaved Luria-Bertani (LB) growth medium. The concentrations of trace elements in the growth medium as determined by ICP-OES analysis are shown in Table S1. Prior to inoculation, the growth medium had a pH of 6.90. Autoclaved solution sample chambers with Kapton™ film windows (0.3 mm thick) were mounted in the beakers to enable harvesting of surface-adhered *P. fluorescens* cells from the same culture that produced the planktonic cell samples. These sample chambers enable XRF analysis of adhered and hydrated microbes. Cultures were grown aerobically on a rocking stage for 3 days to stationary phase (as determined by optical density readings). An additional set of cultures was treated with potassium dichromate solution [Cr(VI) at 1000 ppm and 25 ppm] for 6 hours. Sample holders with adhered cells were removed from the growth chamber, rinsed in 18-Mohm water to remove growth medium and cells loosely attached to the Kapton™, filled with 18-Mohm water, and sealed with another piece of Kapton™ film. At harvest, the cell suspension had a pH of 8.33. After centrifugation and rinsing with 0.1 M $NaClO_4$, the biomass representing planktonic *P. fluorescens* cells was chemically fixed with 2.5% glutaraldehyde for 1 hour, rinsed in autoclaved 18-Mohm water, deposited on 200-mesh Formvar-coated transmission electron microscopy Au grids, and allowed to air-dry in a dust-free environment. Fixation of planktonic microbes ensured that the cells remained stationary during the micro-XRF measurements. Additional elemental analyses were performed on surface-adhered cells, prepared in the same manner as the chemically fixed planktonic cells. Results from these studies were similar to those for the hydrated samples. Cr XANES measurements were not performed on the chemically fixed surface-adhered cells.

The viability of *P. fluorescens* cells after exposure to Cr(VI) was determined by using the LIVE/DEAD® *Bac*Light™ kit (Molecular Probes, Eugene OR) as described by the manufacturer. Briefly, cell suspensions were stained with two nucleic acid stains, SYTO® 9 (a green fluorescent stain) and propidium iodide (a red fluorescent stain). Because of changes in permeability, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red when examined by fluorescence microscopy using the appropriate optical filters.

To maximize detection of Kα XRF radiation from low-atomic-number elements and to remove background XRF radiation from atmospheric Ar, samples measured in the XRF microscope were maintained in a free-flowing He gas environment. Because the focusing efficiency of the zone plate is reduced with increasing x-ray probe energy in the present configuration, production of 150-nm-sized x-ray beams (and hence spatial resolution) is limited to x-ray probe energies no greater than 10 keV. This limits excitation of 1s-orbital electrons to elements with atomic number no greater than 30 (Zn). Hence, the x-ray microscope in its present configuration is optimized for determining the spatial distribution of elements (via Kα fluorescence radiation

intensities) with atomic numbers ~14 to 30, inclusive. Typically, the distributions (i.e., position, size, morphology, and orientation) of elevated XRF signals correlates well with previous optical microscopy characterizations of the microbes in the sample. Once precise (~150-nm resolution) x-ray microscope coordinates of the microbes were determined, the x-ray microbeam was positioned within 150 nm of the center of the microbe, and an XRF spectrum was measured for 500 s. An XRF spectrum was also measured ~5 μm from the microbe to determine the intensity of the background radiation of the experimental setup. Comparison of the XRF intensities for the microbes and thin glass film standards [National Institute of Standards and Technology (NIST), 1832 and 1833] — measured under the same experimental conditions, (experimental geometry, Kapton^{TM} film attenuators, etc.) and normalized to incident x-ray beam intensity — enabled the determination of the average area concentration of elements in each microbe. Under the assumption that the density of a microbe is very similar to that of water(1 gm/cm^3) and has a known thickness (assumed to be equal to the width of the cell, as determined from the XRF image), area elemental concentrations (mg/cm^2) were converted to average volumetric elemental concentrations (ppm in solution) for each microbe. The attenuation of the fluorescent x-rays by 1 μm of water is less than 10% for Ca fluorescence and less than 50% for P fluorescence. However, comparable self-absorption effects (4% for Ca and 14% for P) were introduced with the NIST thin film standard. Final results in Table S1 were corrected for these effects.

After the XRF measurements of the microbes, samples were observed optically with an Olympus BX60 microscope (50× magnification). No visible damage to the cells was detected. XRF imaging experiments of Kapton^{TM} film without surface-adhered microbes did not indicate high concentrations of Ca and P with sizes and morphologies similar to the *P. fluorescens* cells used in these experiments.

Supporting Text

Surface-adhered cells typically outnumber planktonic cells 200 to 1000 times in natural environments (*S1*) and have been routinely isolated from chromium-contaminated sites (*S2*). A detailed discussion of *P. fluorescens* and its relevance in environmental systems can be found elsewhere (http://genome.jgi-psf.org/draft_microbes/psefl/psefl.homelhtml).

Comparison of the distribution and gradients of Ca, P, and Fe (an element usually closely associated with cells because of its importance in a variety of biochemical processes) for the surface-adhered cells indicates high concentrations and steep gradients for Ca and P *outside* the cell, where most of the Ca and P are found. It is also possible to estimate the effective thickness of a $CaPO_4$ moiety associated with the attached cells if (i) the density of the $CaPO_4$ moiety is assumed to be ~3 gm/cm^3 and (ii) the vast majority of the Ca and P associated with the cell is present as a $CaPO_4$ moiety. With these assumptions and with calculations similar to those explained above, the effective thickness of the $CaPO_4$ moiety is estimated at ~0.4 μm.

Radiation-induced reduction of Cr(VI) to Cr(III) could have occurred in our experiments. To investigate the extent of such an effect, the x-ray probe energy was set to the value consistent with the pre-edge feature commonly used to identify Cr(VI) (~5994 eV), and a sample of hydrated and centrifuged *P. fluorescens* biomass previously exposed to a solution of Cr(VI) was placed in the x-ray beam. Partial reduction of additional Cr(VI) occurred, but at a rate that allowed us to estimate conservatively that, during the Cr XANES measurement of the surface-

adhered samples (~10min.), the effect of radiation-induced chemical reduction of the Cr was less than 25% in our samples.

A control experiment was performed to distinguish between active and passive biotic reduction of Cr(VI). Because heat or chemical treatments can cause significant alternations to the cell wall structure, planktonic cells were made metabolically inactive by exposure to gamma radiation from a Cs source for 1.5 hours at 35,000 rads/hours. This procedure has been demonstrated previously to kill over 90% of *B. subtilis* cells, with minimal disturbance to the cell wall structure or integrity. (*3*) Two experiments were run: one with irradiated cells, and another with nonirradiated cells. In both cases, the cells were exposed to Cr(VI) solutions at the same concentrations as those used in the x-ray microscopy studies. Cr k-edge XANES studies of the centrifuged biomass resulting from all of these experiments indicated 80% ± 15% of the Cr(VI) was reduced to Cr(III). These results are consistent with our x-ray microspectroscopy measurements of individual planktonic cells and our conclusion that under these conditions the reduction of Cr(VI) by planktonic *P. fluorescens* cells is not an active metabolic response.

Table S1. Results of quantitative analysis of x-ray fluorescence spectra. Spectra were collected from the center positions of cells to determine the average and standard deviation of elemental concentrations for the cells measured and ICP-OES elemental analysis of LB growth media used in these experiments. Values are in ppm (by mass) with the assumption that the density of the microbes is 1.0 g/cm^3. The number in parentheses in each row heading is the number of either microbes measured with XRF or LB growth solutions measured with ICP-OES. The number in parentheses in each concentration is the standard deviation for that average value. ND, Because of the high intensities of the Ca and P fluorescence radiation, the S, Cl, and K concentrations could not be determined accurately for the surface-adhered cells.

	[P]	[S]	[Cl]	[K]	[Ca]	[Cr]	[Mn]	[Fe]	[Co]	[Ni]	[Cu]	[Zn]
Planktonic (5)	16,048 (2,446)	6,625 (1,117)	8,421 (2,628)	3,604 (1,173)	3,815 (392)	9 (2)	22 (4)	156 (23)	190 (37)	120 (33)	201 (46)	1,175 (176)
Planktonic, Cr(VI) added at 1000 ppm (6)	6,156 (1,034)	3,719 (1,516)	3,908 (1,814)	2,201 (1668)	673 (230)	949 (323)	22 (4)	58 (29)	13 (12)	26 (18)	105 (76)	94 (30)
Planktonic, Cr(VI) added at 25 ppm (12)	8671 (4097)	3201 (1092)	994 (421)	19 (9)	42 (36)	34 (15)	3 (1)	195 (68)	7 (2)	2 (1)	ND*	11 (5)
Surface-adhered(8)	661,032 (139,416)	ND	ND	ND	570,855 (92,831)	32 (10)	40 (7)	360 (216)	14 (7)	26 (10)	0 (14)	25 (13)
Surface-adhered, Cr(VI) added at 1000 ppm (10)	419,034 (362,728)	ND	ND	ND	427,987 (147,983)	24 (15)	23 (8)	326 (177)	12 (7)	18 (9)	2 (5)	15 (7)
LB growth mediim solution (3)	17.8168 (0.1619)	3.028 (0.006)	NA	228.91 (3.37)	7.5915 (0.0986)	0.0194 (0.0002)	0.0181 (0.0002)	0.4034 (0.0049)	0.0138 (0.0001)	0.0036 (0.0001)	0.0253 (0.0004)	5.4162 (0.0064)

*[Cu] could not be determined accurately due to elevated [Cu] in the grid for this particular sample.

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