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A 200-Antibody Microarray Biochip for Environmental Monitoring: Searching for Universal Microbial Biomarkers through Immunoprofiling

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Environmental biomonitoring approaches require the measurement of either unequivocal biomarkers or specific biological profiles. Antibody microarrays constitute new tools for fast and reliable analysis of up to hundreds of biomarkers simultaneously. Herein we report 150 new polyclonal antibodies against microbial strains and environmental extracts, as well as the construction and validation of an antibody microarray (EMCHIP200, for "Environmental Monitoring Chip") containing 200 different antibodies. Each antibody was tested against its antigen for its specificity and cross-reactivity by a sandwich microarray immunoassay. The limit of detection was 0.2 ng mL^{-1} for some proteins and 10^4-10^5 cells mL^{-1} for bacterial cells and spores. Partial biochemical characterization allowed identification of polymeric compounds (proteins and polysaccharides) as some of the targets recognized by the antibodies. We have successfully used the EMCHIP200 for the detection of biological polymers in samples from extreme environments around the world (e.g., a deep South African mine, Antarctica's dry valleys, Yellowstone National Park, Iceland, and Rio Tinto surface and subsurface). Clustering analysis permitted us to associate similar immunoprofiles or patterns to samples from apparently very different environments, indicating that they indeed share similar universal biomarkers. Our EMCHIP200 constitutes a new generation of immunosensors for biomarker detection and profiling, for either environmental, industrial, biotechnological, or astrobiological applications.

The detection and identification of microorganisms or molecules of biological origin from environmental samples is of general interest (e.g., in biomedical, bioterrorism, environmental, industrial, or even astrobiological applications[1−3]). Antibodies, and in particular optical immunosensors, have been used for many years for clinical (e.g., serotyping) and environmental purposes.[4−6] One of the first fluorescent antibody tests for bacterial detection was reported in 1967 for the ecological study of some *Bacillus* spp. in soil.[7] Antibodies against environmentally key metabolic enzymes have also been produced for the detection of the microorganisms that produce them.[8,9] Also, immunoreactive material have been obtained from ancient samples like mammoth fossils[10] or from a 65-million-year-old *Tyranosaurus rex* fossilized bones,[11] and reactive antibodies against natural biofilms and sediments have been reported.[12] The identification and selection of key unequivocal molecular biomarkers is essential for biomonitoring purposes.

Antibody microarray technology[13] is an excellent platform for environmental applications, and a broad range of molecular size biomarkers can be detected simultaneously by microarray immunoassays, from the amino acid size level to whole cells.[14] A number of two-dimensional array-based biosensors using fluoroimmunoassays as their detecting systems have been described for rapid analysis of several analytes in complex samples,[15,16] and instrumentation for several applications is being developed.[17,18] In addition, antibodies or other bioaffinity receptor molecules have been also proposed during the past years as sensors for the

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detection of biomarkers in planetary exploration.[3,12,19,20] Recently, the European Space Agency (ESA) evaluated very positively an antibody array-based instrument (Life Marker Chip-LMC) as one of the instruments of the "Pasteur" Payload for the *ExoMars* mission (http://esamultimedia.esa.int/docs/Aurora/Pasteur_Newsletter_5.pdf.). We have previously reported a LMC-like instrument called SOLID (for "Signs Of LIfe Detector"[3]) and a field prototype (SOLID2) has been recently successfully tested in a Mars drilling simulation experiment.[21,22]

The aim of this work is the development of antibody microarrays against universal and specific biomarkers and the use the immunoprofile outputs as a new analytical tool for environmental biomonitoring processes. First, we propose a shotgun strategy for the identification of key molecular biomarkers, which basically consists of going to the environment, investigate whether there is preserved immunogenic material, characterize it, design the method for its detection, and validate its degree of universality or specificity. We report the production and testing of 150 new polyclonal antibodies against different natural samples (water, sediments, soil, biofilms, etc.) and bacterial strains, as well as the construction and validation of an antibody microrray for monitoring the presence of such bacteria or some of their remains in samples collected from extreme environments around the world.

EXPERIMENTAL SECTION

Detailed methods and procedures are provided in Supporting Information (SI).

Strains and Culture Conditions. Microbial strains used as source of biomass for antibody production and immunological tests were obtained from our laboratory collection, from the American Type Culture Collection (ATCC), and from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). A list of strains as well as of culture conditions are described in SI Experimental Section and SI Table S1.

Preparation of Immunogenic Extracts. Crude extracts and several fractions from natural samples (water, sediments, biofilms, salt precipitates, soil, etc.) and microbial cultures were carried out for the preparation of complex antigenic mixtures as described in SI Experimental Section.

Antibody Production, Purification, and Fluorescent Labeling. Polyclonal rabbit antibodies against the antigenic fractions prepared as indicated above were produced, and the IgG fraction was purified and fluorescently labeled (Alexa 647, Molecular Probes) as described previously.[3]

Arraying Antibodies, Immunoassays, Image Processing. Antibody microarrays were performed as described[3] with some modifications: (i) the spotting solution contained 0.5× protein printing buffer (TeleChem International, Sunnyvale, CA), 1× of an antibody stabilizing mixture (SM1, Biotools, Madrid, Spain), and 0.02% Tween 20. (ii) Printing was done in a duplicate pattern on epoxy-activated glass slides (TeleChem International) using a MicroGrid II TAS arrayer (Biorobotics, Genomic Solutions). See SI Experimental Section for details. The slides were scanned for fluorescence at 635 nm in a GenePix 4100A scanner, and the images were analyzed and quantified by Genepix Pro Software (Genomic Solutions). Clustering analysis was done with Acuity software (Genomic Solutions).

Biochemical Characterization of the Antigens. Bulk biochemical determination of proteins and carbohydrates, as well as a partial biochemical characterization of some of the antigens by SDS-PAGE, Western blotting, thin-layer chromatography (TLC), immuno-TLC, immunoprecipitation experiments, and protein fingerprint analysis, were carried out as described in SI Experimental Section.

RESULTS AND DISCUSSION

Production of Antibodies against Environmental Samples for Biomarker Detection. The aim of this work is the development of an antibody microarray for quick and reliable environmental monitoring, for example, by following temporal or spatial variations of main biological components, the presence of pollutants or natural toxins, bioremediation or bioindustrial processes, or for biomarker detection in astrobiology. In all these cases, it is critical to have a set of good-quality and high-performance antigen−antibody pairs capable of recognizing specific biomarkers. However, multianalyte-containing samples, like environmental ones, may render multiple cross-reaction events that complicate the interpretation of the results. To overcome this, herein we propose that, in spite of the cross-reactions, an antibody microarray with a high number of capturing antibodies generates a specific immunoprofile, a kind of fingerprint, which can help to classify environmental samples.

Why produce antibodies against environmental extracts? It is well-known that an appropriate antigen presentation is fundamental for a sensitive immunoassay. Many antigens from bacteria are hindered under a thick cell wall composed of lipopolysaccharides capsules in Gram-negative bacteria or S layers from Gram-positives. For example, *Escherichia coli* cells must be boiled in order to get access to some bacterial antigens for serotyping.[23] Antibodies raised against structures difficult access, to which a highly stringent antigen presentation procedure has to be applied, may be useless for the analysis of complex samples. And this is the case for many environmental samples, defined by a complex antigenic mixture, where a stringent treatment cannot be applied without destroying or masking other antigens. Moreover, biological polymers in nature are very often chemically modified (phosphorylation, acethylation, methylation, etc.),[24] and these chemical groups may be removed during elaborated extraction procedures in the laboratory.[25] The antibodies produced against these chemically altered biomarkers would not necessarily recognize their natural counterparts. Consequently, the antigen presentation in the immunoassay could be predetermined by the antigen preparation for immunogenization.

In addition to the commercially available antibodies, or the production of antibodies against purified compounds, we also

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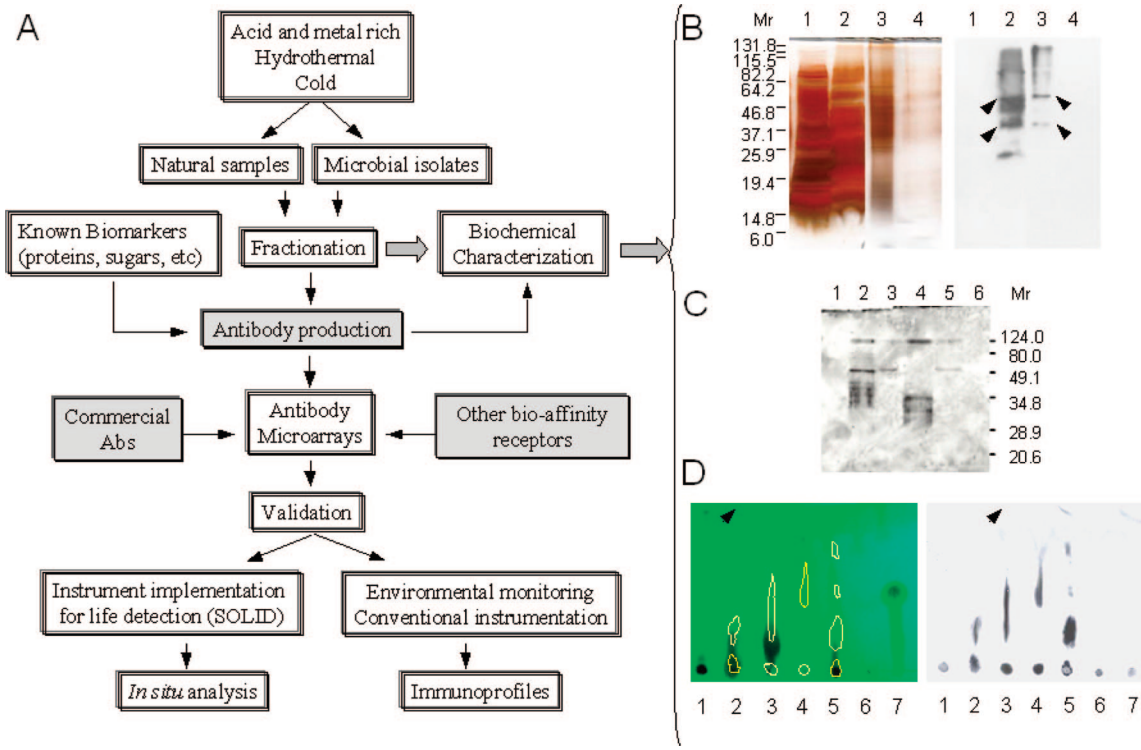

Figure 1. (A) Global approach and shotgun strategy for antibody production against natural extracts for antibody microarray-based environmental monitoring and life detection (see text for explanation). (B−D) Partial characterization of the antigenic extracts using the produced antibodies. (B) "Environmental antibodies" reacted against high molecular weight compounds in both environmental and bacterial cell extracts. Samples of whole cell lysates from *Leptospirillum ferrooxidans* (1), *Acidithiobacillus ferrooxidans* (2), and natural extracts from IC7C1 (3) and IA2C1 (4), were fractionated in 15% SDS-PAGE (silver stained, left), blotted, and incubated with IC7C1 antibody (right). Positive and common reactive bands were detected in *A. ferrooxidans* and IC7C1 extracts (arrowheads). (C) SDS-PAGE fractionation and Western blot with the environmental antibody ID4S2, raised against a 162-m-deep Río Tinto subsurface sample, showed reactions with proteins from *Burkholderia fungorum* (1−2), and *Geobacter metallireducens* (3−4). Up to 20 μg of whole bacterial lysate or a total mass from ID4S2 natural extract were loaded into the gel: (1) proteinase K treated *B. fungorum* cell lysate, (2) *B. fungorum* cell lysate, (3) proteinase K treated *G. metallireducens* cell lysate, (4) *G. metallireducens* cell lysate, (5) ID4S2 extract, and (6) proteinase K treated ID4S2 extract. Preimmune antiserum did not show any band (not shown). (D) Antibodies specifically bind to TLC fractionated compounds from different environmental extracts. Immuno-TLC assays were performed after running in the solvent isobutiric: ammonium hydroxide (1N): water (5:3:2, by volume). Two TLC plates were run in parallel, one (right) was assayed with a pool of 8 antibodies against natural samples (IA2C1, IA1C1, IA3C1, IC1C1, IC4C1, IC7C1, IC8C1, and IC9C1; see SI Table S1), and the other one (not shown) was incubated with the pool containing the 8 corresponding preinmune sera (no positive spots were detected). Before immunoassay, the TLC plate was visualized under short-wave UV (left). The fractionated samples corresponded to extracts IA3S1 (lanes 1), IA3C1 (lanes 2), IA2C1 (lanes 3), IC9C1 (lanes 4), IC8C1 (lanes 5), ID4S2 (lanes 6), and an extract from a Río Tinto sediment called CH15 (lanes 7). The immunoreactive spots (right image) were mapped (yellow figures) on the short-wave UV light image (left). The arrows show the front of the solvent.

propose a shotgun strategy consisting of the use of the pool of biomarkers in a natural sample extract as immunogen for the random production of high-affinity polyclonal antibodies (Figure 1). In this way, only the most antigenic compounds in the complex extracts will induce a good immunogenic response. A further characterization of the antigen will identify the nature of the preserved environmental biomarker. Even the same produced antibodies can be used for antigen characterization, for example, by affinity purification. The antibodies against natural extracts are giving extra information: they tell us which antigen is in fact present in the sample, not which one it is supposed to be there.

Three extreme environments (acidic and metal-rich, hydrothermal, and cold) were first considered in this work for several reasons: (i) the relative low prokaryotic diversity in the water column of acidic environments[26,27] allowed a high representation on a microarray with a low number of antibodies; (ii) the prokaryotes dominating in each of them are different enough to expect a differentiable immunological pattern; (iii) all of them are in different aspects good terrestrial analogues of hypothetical habitats on the planet Mars and, therefore, have an extraordinary astrobiological interest; and (iv) acidophile microorganisms are very important both in biomining industry and in environmental pollution, as they are responsible for the acid mine drainage. Antibodies were raised against natural extracts obtained from an acidic and iron-rich environment (Río Tinto, Spain), against environmental bacterial isolates, or against microbial strains isolated from any of the above-mentioned environments or similar habitats from type collections (SI Table S1). A total of 150 new polyclonal antibodies against whole environmental extracts, whole cells, extracellular material, or S100 extracts (Table S1 and SI Experimental Section), were produced and the IgG fraction of each one purified.

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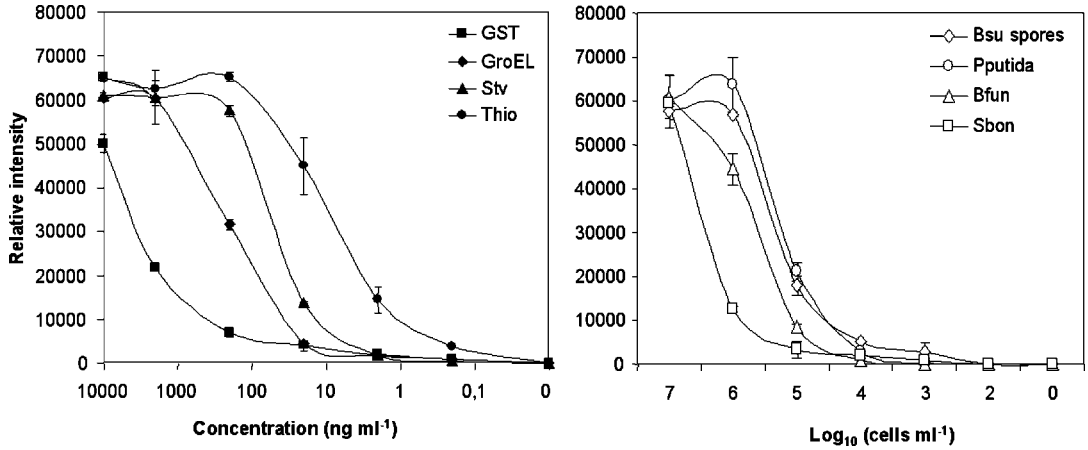

Figure 2. Limit of detection of the antibody sandwich microarray immunoassay. Sandwich immunoassays were done as described in SI Experimental Section by using 20 μL of the different proteins (left) and bacterial cell (right) concentrations as antigenic samples and 20 μL of the corresponding fluorescent labeled antibody (4 μg mL^{-1}). The error bars corresponded to the standard deviation of two replicate experiments and 4 replicate spots. Thio, thioredoxin from *E. coli*; Stv, streptavidin; GroEL, GroEL chaperone from *E. coli*; GST, glutathione-*S*-transferase; Sbon, *S. bongori*; Bfun, *B. fungorum*; Pputida, *P. putida*; Bsu spores, *B. subtilis* spores. The relative intensity values were corrected by their local background and by the negative control array (first incubation with buffer, no antigen).

Identification of Environmental Biomarkers Recognized by the Antibodies. A shotgun or random strategy was followed by using whole extracts or cells to obtain polyclonal antibodies and, consequently, the exact target molecules that are being recognized by the antibodies are unknown. In order to investigate the nature of the antigens responsible for the immunoreactivity, we analyzed several environmental and bacterial extracts by different analytical techniques. Protein and carbohydrates were detected in the environmental extracts (SI, Table S2), although the amount of protein was very low in many samples, probably due to a very low cell number. Silver staining SDS-PAGE gels revealed the presence of high molecular weight compounds in different environmental extracts (SI, Figure S1), and Western blot analysis showed specific bands for high molecular weight compounds present either in natural extracts or in bacterial whole lysates when revealed either with environmental or antibacterial antibodies (Figures 1 and S1 and S2 in SI). Interestingly, a Western blot with the environmental antibody ID4S2 (raised against an extract from a 162 m deep pyrite rich rock from Río Tinto subsurface) showed specific bands on its own extract as well as on cell lysates from *B. fungorum* and *G. metallireducens* (Figure 1C). These bands disappeared when the extracts were treated with proteinase K, indicating their protein nature. Also, specific bands from some microorganisms like *L. ferrooxidans* and *A. ferrooxidans* were recognized by A139 and IVE3C1 antibodies, respectively (SI, Figure S2). A positive universal protein band was observed in most of the strains when reacted against A139 (anti-*L. ferrooxidans*), indicating that either it was the same protein or it showed common epitopes (SI, Figure S2, middle). In order to identify this protein, a whole lysate of *Pseudomonas putida* was used for immunoprecipitation with A139. The immunoprecipitate was fractionated by SDS-PAGE, stained, and the corresponding band recovered for a peptide fingerprint (not shown). The results showed that this band corresponded to the universal Fo subunit of the bacterial ATPase, indicating that part of the polyclonal A139 recognized this evolutionary well-conserved protein. In addition, several natural extracts from Rio Tinto soils, containing proteins and sugars, gave positive reactions against A139.[21] Immunoprecipitation experiments with the anti-*Bacillus subtilis* spore antibody allowed us to identify the proteins OppA and flagellin as some of the antigenic biomarkers in *B. subtilis* biofilms (SI, Figure S3). Further analysis by TLC, intended to fractionate polysaccharides or peptidoglycan fragments, showed clear spots in environmental extracts (Figures 1, SI S4 and S5). Preliminary analysis of some of these spots by liquid gas chromatography−mass spectrometry (LGC−MS, not shown) indicated the presence of monosaccharides like glucose or rhamnose as components of the samples, indicating that polysaccharides (mostly exopolysaccharides or EPS) are major components of the environmental extracts.

To investigate whether the TLC spots contained immunoreactive material, we subjected several TLC plates to an immunodetection assay with some of the antibodies. The results indeed showed specific imnunoreactive spots in several extracts (Figure 1C), indicating that polysaccharides, lipopolysaccharides, or both are targets of the antibodies. Observe that not all the spots detected by UV light were reactive to the antibodies, which means that the extracts are in fact heterogeneous multianalyte-containing samples where only some compounds are specifically recognized by the antibodies.

Production and Testing of a 200-Antibody-Containing Biochip for Environmental Monitoring. In order to develop environmental monitoring tools, we constructed antibody microarrays containing 200 different antibodies (EMCHIP200, for "Environmental Monitoring CHIP") including those produced by us and other commercially available ones (SI, Figure S6 and Table S1). Apart from the antibodies against natural samples, the microarray covered several degrees of specificity, from suprageneric groups (Gram-positive or Gram-negative bacteria), genus or species specific, as well as against some proteins, peptides, and metabolites (SI, Table S1). The limit of detection for purified proteins, bacterial cells, and spores was determined by a sandwich microarray immunoassay (SMI). In a typical SMI, the antigenic sample is incubated with the antibody microarray, and after a washing, the positive antigen−antibody reactions (and possible biomarkers) are revealed by either one or a mixture of fluores-

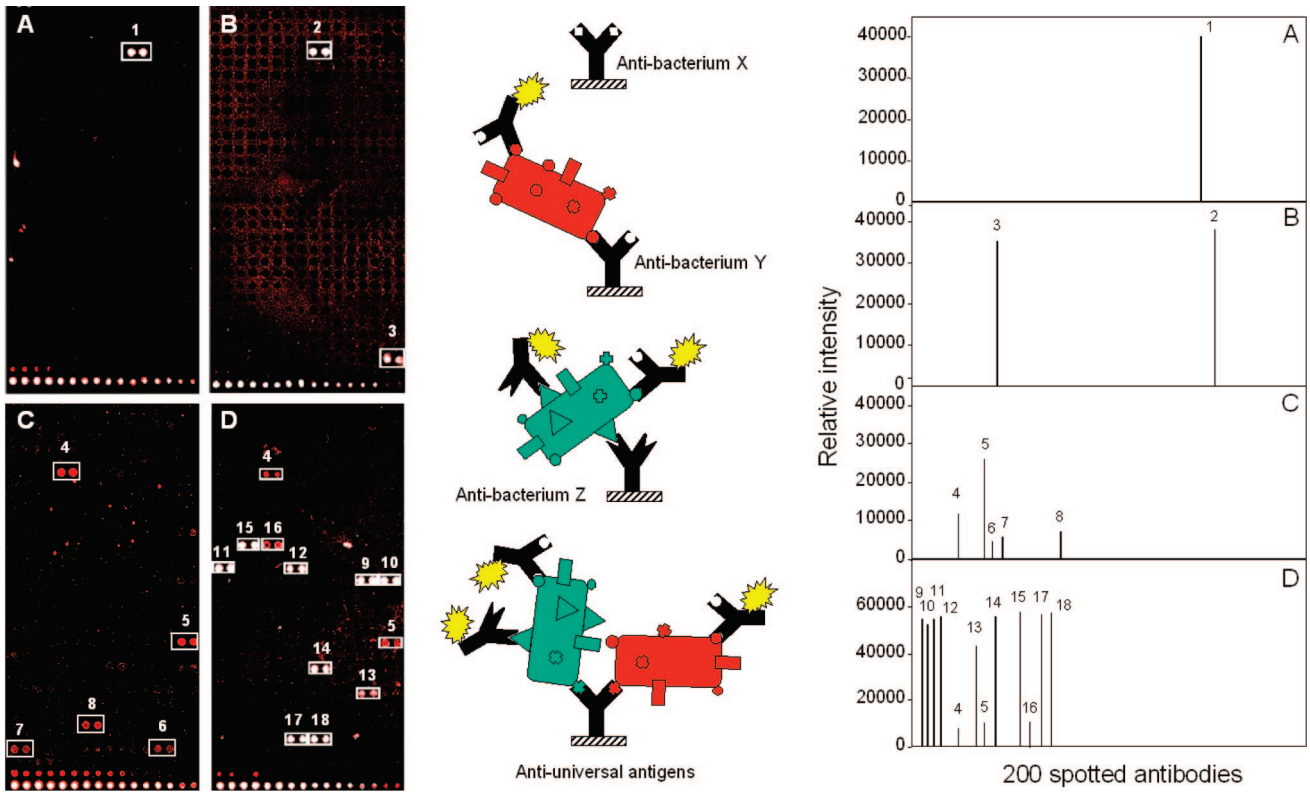

Figure 3. Examples of EMCHIP200 sandwich microarray immunoassay. Each array was incubated with an antigenic sample consisting of 20 μL of 10^6 cells mL^{-1} (for bacterial cultures) or 20 μL (30 μg of total dry weight) of a complex multianalyte containing environmental extract: (A) a bacterial whole cell lysate from *T. maritima*, (B) a whole cell lysate from *G. metallireducens*, (C) a cellular fraction from a filamentous Biofilm sampled at Rio Tinto stream (extract IA2C1), and (D) a whole cell lysate from *A. ferrooxidans*. After 1 h of incubation and washing, the arrays were revealed for 1 h with 4 μg mL^{-1} fluorescent antibodies: (A) IVI13C1, anti-*T. maritima*; (B) IVI12C1, anti-*G. metallireducens*; (C) IA2C1, anti-cellular fraction of a Río Tinto filamentous Biofilm extract; and (D) A183, anti-*A. ferrooxidans*. The signal intensity of the spots from each array was quantified, corrected by the values obtained in the negative control arrays (not shown), and plotted in an immunogram (right). Numbers indicate the capturing antibodies showing a positive reaction: (1) IVI13C1, anti-*T. maritima*; (2) IVI12C1, anti-*G. Metallireducens*; (3) ID4S2, from Peña de Hierro, a 162-m-deep sample (MARTE project); (4) IA2C1, anti-cellular fraction of Río Tinto filamentous biofilm; (5) IC4C1, cellular fraction from a dry brown filaments from Río Tinto side; (6) IC1S1, anti-supernatant fraction from Río Tinto sediments; (7) ID5S2, from Peña de Hierro, a 151-m-deep sample (MARTE project); (8) IVE3S1, *A. ferrooxidans* culture supernatant; (9) A152, anti-a pink superficial layer sediment from Río Tinto (whole Gu/ClH extract); (10) A182, anti-*A. ferrooxidans* whole cells; (11) A183, anti-*At. ferrooxidans* whole cell lysate (sonicated); (12) A186, anti-*L. ferrooxidans* whole cells (partial lysate by sonication); (13) IC3C1, anti-cellular fraction from brown wet filaments from Rio Tinto's main spring (pH 1.8); (14) IC7C1, anticellular fraction from Río Tinto (3.2 water dam) dried wall sediments; (15) IVE3C1, anti-*At. ferrooxidans* whole cells; (16) IVE4C1, anti-*A. thiooxidans* whole cells; (17) IVE3C2, anti-*At. ferrooxidans* cell pellet after S100 extraction; and (18) IVE4C2, anti-*A. thiooxidans* cell pellet after S100. The positive reactions can be localized on the EMCHIP200 antibody microarray scheme (SI, Figure S6 and Table S1). The spot series at the bottom of the arrays correspond to serial dilutions of a fluorescent labeled preimmune antibody. Cartoons (middle) represent the different immunoreactions that are taking place on the EMCHIP200 microarray: no antigen captured (top), a sandwich with a specific anti-bacterium *Y* antibody (case of A), and different degrees of specificities for an antibacterium *Z* or a more universal antibody (cases of B−D). Signal amplification phenomena may be taking place when multiplex fluorescent cocktails are used (two bottom cartoons). Big rectangles represent whole bacterial cells or high molecular weight complexes, and small figures (triangles, crosses, etc.) represent antigenic determinants recognized by the antibodies (black *Y*s).

cently labeled antibodies (see Supporting Experimental Procedures in SI). The results indicated that it is possible to detect by a SMI less than 0.2 ng mL^{-1} (200 parts per trillion, ppt) 10-kDa protein thioredoxin, up to 2 ng mL^{-1} 60-kDa protein GroEL, up to 10^4−10^5 cells mL^{-1} *B. fungorum* and *Salmonella bongorii* (in spite of the antibody raised against another *Salmonella* strain. SI, Table S1), or 10^3−10^4 of *B. subtilis* spores and *P. putida* cells per mL (Figure 2)

The specificity and cross-reactivity of all the antibodies (except those commercial ones against small compounds) were tested one by one by SMI, using the same antigen that was used to produce the antibodies, and the corresponding fluorescently labeled antibodies as tracers (Figure 3). The range of specificity was variable so that some antibodies showed a high specific reaction against their immunogen or closely related antigens, while other antibodies cross-reacted with a broader antigen spectrum. For example, the antibody raised against *Thermotoga maritima* (IVI13C1; Figure 3A) was in the first case, while that against *A. ferrooxidans* (A183) showed a broader cross-reactivity against *A. ferrooxidans*, *A. thiooxidans*, and *L. ferrooxidans* (Figure 3D). In agreement with the results showed in Figure 1, the antibody ID4S2, raised against a natural extract obtained from the Río Tinto subsurface (162-m depth), not only showed a positive signal against its own antigen (spots 3 in Figure 3B), but also reacted against *G. metallireducens* (spots 2 in Figure 3B) and *B. fungorum* (not shown) in an SMI revealed with the fluorescent antibodies raised individually against these bacteria. This result indicated that either these bacteria are present in the Rio Tinto subsurface

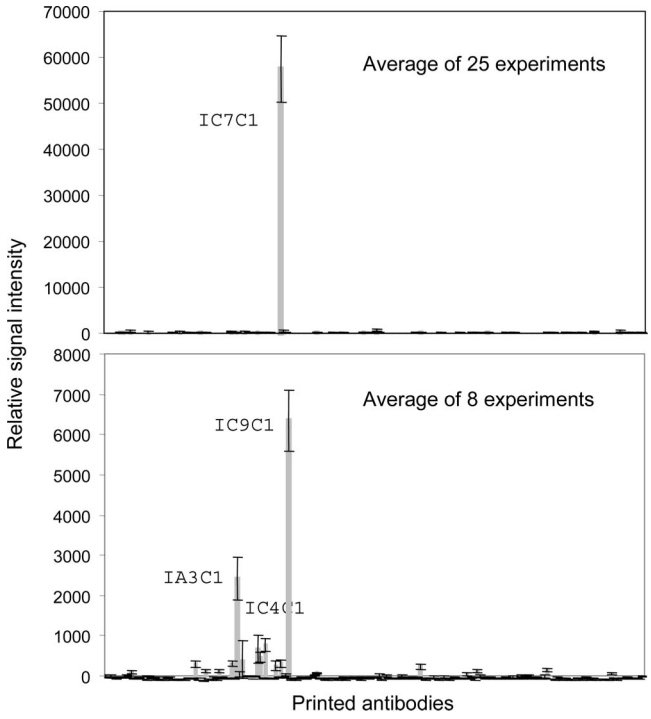

Figure 4. Reproducibility of sandwich microarray immunoassays with natural extracts as multianalyte containing samples and their own antibodies as tracers. It is shown the average results from 25 and 8 different replica experiments with 25 μg (dry weight) of extracts IC7C1 (upper plot) and IC9C1 (bottom) revealed with their corresponding antibodies (SI, Table S1). The signal from IC7C1 was highly specific while that of IC9C1 showed cross-reactions with other antibodies raised against natural samples.

or the native ones are closely related or are sharing antigenic determinants and, consequently biomarkers.

The fluorescent signal intensity of all the microarrays was quantified using commercial software and the resulting relative signal intensity was orderly clustered for a cross-reactivity analysis (SI, Figure S7). The results showed that the big majority of the antibodies gave a clear positive reaction against their own antigens (diagonal line) and that some of them, mainly those against natural extracts, presented certain cross-reaction with other related extracts, as expected.

All fluorescent tracer antibodies were used at the same working concentration (4 μg mL^{-1}). Therefore, a more accurate titration will be necessary to calculate the optimal working dilution for each one. In fact, some antibodies have already been titrated, with an optimal concentration of 0.5−1.0 μg mL^{-1} (a 1/4000−1/2000 dilution with respect to the fluorescent-labeled stock at 2 mg mL^{-1}).

The sandwich microarray immunoassay using EMCHIP200 is highly reproducible. This was tested with two environmental antigenic extracts and their corresponding antibodies by 25 and 8 replica experiments. All replicates produced the same results with a standard deviation of the specific signals no higher than 10% among all the experiments (Figure 4)

Multiplexing Fluorescent Sandwich Microarray Immunoassay with 200 Antibodies. Using one or a few fluorescent antibodies for a SMI is straightforward, but as the number increases, the unwanted background is also higher and it can lead to false positive signals or to a reduction in the dynamic range of the assay.[28] In order to check the reliability of using a fluorescent cocktail containing a large number of antibodies as tracers, several antigens tested by SMI were revealed with their own fluorescent antibody or with a 200-fluorescent antibodies cocktail (200FAC). Good signal-to-noise ratios were obtained for 10^6 *B. subtilis* spores (Figure 5B) or natural extracts (Figure 5D) when the 200FAC was used at 100 μg mL^{-1} compared to the individual antibodies at 0.5 μg mL^{-1} working concentration. The results indicated that the specificity was the same, although the signal intensities and the dynamic range were affected: the sensitivity was 1 order of magnitude lower for *B. subtilis* spores (from 10^4 to 10^5 spores mL^{-1}) when using the 200FAC instead of its own antibody alone (Figure 5A). Similar results were obtained when an extract from an iron oxide-rich rock crust from Río Tinto was analyzed with EMCHIP200 and then revealed either with anti-*A. ferrooxidans* (IVE3C1) antibody or with the 200FAC mixture (Figure 5C,D). The same positive reactions were obtained either with IVE3C1 alone or with the 200FAC, with the additional advantage that 200FAC allowed the detection of extra specific reactions, like that for an anti-Gram-positive bacterial antigen and A139 (anti-*L. ferrooxidans*). This result indicates the presence on the rock surface of polymeric compounds having structures the same or highly similar to others in *A. ferrooxidans* antigens, apart from other polymers found in Gram-positive bacteria. This is extremely important for life detection experiments, because it validates our EMCHIP as an analytical tool for the detection of high molecular weight environmental biomarkers.

Immunoprofiling Environmental Samples with EMCHIP200. We hypothesized that it would be possible to obtain a kind of environmental immunoprofile, which can help in the characterization of natural samples as well as in the rapid estimation of the types of dominating microorganisms and biomarkers. An immunoprofile can be defined by just one specific immunogram after one sample analysis by EMCHIP200 or by a series of immunograms obtained from different samples. To check whether EMCHIP200 is valid to detect compounds in samples from very different environments, extracts from different parts of the world have been tested with the EMCHIP200 by SMI using the 200FAC as tracer (Experimental Section; Figure 6). The extracts came from the following places: a biofilm from a 3-km-deep South African gold mine, a rock from the Geyser locality (Iceland), a rock from the Mauritania desert, microbial mats and sediments from Yellowstone National Park (USA), an iron-rich sediment from Patagonia (Argentina), cyanobacterial mats from the McMurdo Dry Valley lakes (Antarctica), rock crusts, sediments, water, and dried red mud from Río Tinto banks (SI, Table S1), or a lichen-rich granite rock from the Sierra de Gredos (middle of Spain). Immunoprofiling studies were carried out by ordering and analyzing the resulting immunograms by hierarchical clustering, using the same commercial software as for DNA microarrays. Samples sharing similar immonoprofiles group together, independently if they are in a small group (Figure 6) or as part of a higher number of samples (SI, Figure S8). Consequently, similar samples show similar immunoprofiles, like those corresponding to a *Cyanidium* spp.-rich mat and sediments from Yelowstone, both taken at the same place (Nymph Creek[12]); the

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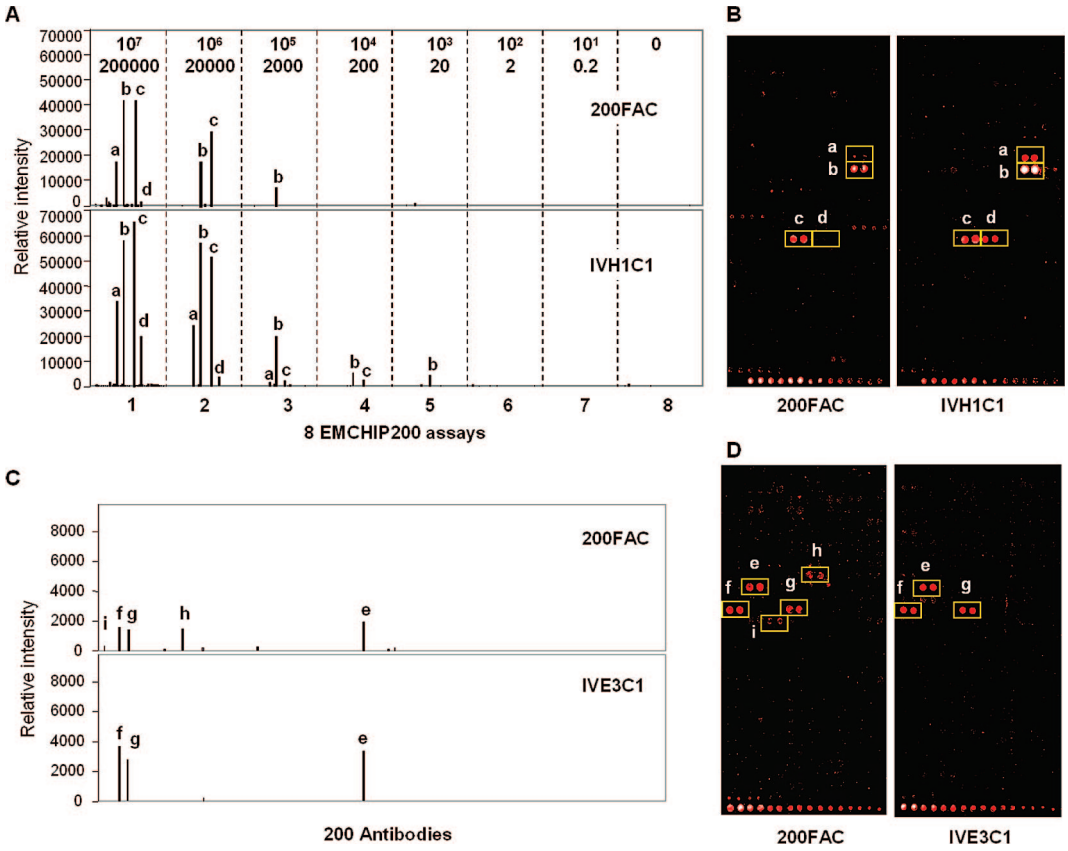

Figure 5. Multiplexing sandwich microarray Immunoassays: single versus multiple antibodies. (A−B) Determination of the sensitivity of EMCHIP200 with *B. subtilis* spores either with a single antibody (IVH1C1) or with a 200 fluorescent antibody cocktail (200FAC). (A) Sandwich microarray immunoassays (1−8) were carried out using 20 μL of serial dilutions of *B. subtilis* spores as antigenic samples and either a 200FAC (1/750 dilution, top immunograms) or the anti-*B.subtilis* spores antibody (IVH1C1) (1/500, bottom) as tracers. The images were treated and analyzed as indicated in the SI, Supporting Experimental Section and, once corrected by the background and negative control (incubation without antigens), positive signals were identified in those spots corresponding to antibodies raised against *B. subtilis* spores (b), a *Bacillus* spp. isolated from Río Tinto (a), cells from a *B. subtilis* biofilm (c), or a culture medium from the biofilm forming *B. subtilis* (d). The limit of detection was $\sim 10^5$ spores mL^{-1} with the 200FAC and 10^4 spores mL^{-1} with the anti-*B.subtilis* spores alone, corresponding to 2000 and 200 spores per assay, respectively. (B) Example of EMCHIP200 after SMI assay with 20 000 *B. subtilis* spores revealed with its own antibody (IVH1C1) or with 200FAC. (C−D) An extract from 1 g of an iron oxide-rich rock crust was incubated with LDCHIP200 and then revealed either with IVE3C1 antibody (anti-*A. ferrooxidans*) or with 200FAC mixture. The images were quantified, and plotted (C) as above. The same positive reactions obtained with IVE3C1 alone (bottom immunogram) were also obtained with 200FAC (upper one), plus two additional ones (h and i). (e) IVE3C1, ant-*A. ferrooxidans*; (f) A183, anti-*A. ferrooxidans*; (g) A186, anti-*L.ferrooxidans*; (h) anti-a polymer from Gram-positive bacteria; (i) A139, anti-*L. ferrooxidans*.

two taxa of cyanobacteria (*Nostoc* spp. and *Phormidium* spp.) taken from the streams of McMurdo Dry Valleys in Antarctica and, surprisingly, the environmentally different and distantly faraway rock samples from Sierra de Gredos (middle Spain) and Geyser (Iceland). What these two samples had in common was the rocky substrate, the exposition to atmosphere and sun, and that both had visible cyanobacterial mats. Interestingly, several positive reactions were obtained in the capture antibodies raised against natural extracts from Río Tinto and against cultured bacterial strains. Especially reactive were those against *A. albertensis* (IVE5C1), *A. thiooxidans* (A184, IVE4,. .., series), and a cellular extract from bacterial rich filaments from Río Tinto (IC4C1), apart of other extracts of the IC series (SI, Table S1), indicating that they were recognizing highly reactive universal epitopes (Figure 6). This universality is evident in the profile obtained with the sample from the deep South African mine, showing positives with anti-Río Tinto sediments and filaments (IC1C1, IC3C1, and IC4C1) antibodies, as well as with others like anti- *Methanobacterium formicicum* (IVJ4C1), or the potato lectin (whose binding specificity is for polymers of GlcNAc, *N*-acetylglucosamine). Also interesting is the high signal obtained with the anti-amino acids Asp and Glu in the two cyanobacterial-rich Antarctic samples, probably due to the presence of poly-Asp and poly-Glu known to be produced by cyanobacteria and other bacterial strains.[29,30] In addition, the antibodies raised against peptides from proteins of nitrogen fixation metabolism (under *Nif* in Figure 6) gave positive reaction mainly with *Nostoc* spp., and a Río Tinto rhizosphere extract (not shown). This reaction was confirmed by dot blot hybridization with the anti-NifH peptides antibody A1484 (SI, Figure S9 and Table S1). Other extracts showed only a few positives and even none, like that from one iron-rich sediment from Patagonia or from Mauritania desert (not shown).

Western and immuno-TLC analysis with anti-IC4C1 and IVE5C1 antibodies (two of the most reactive ones by SMI in Figure 6) confirmed the following: (1) the presence of high molecular weight

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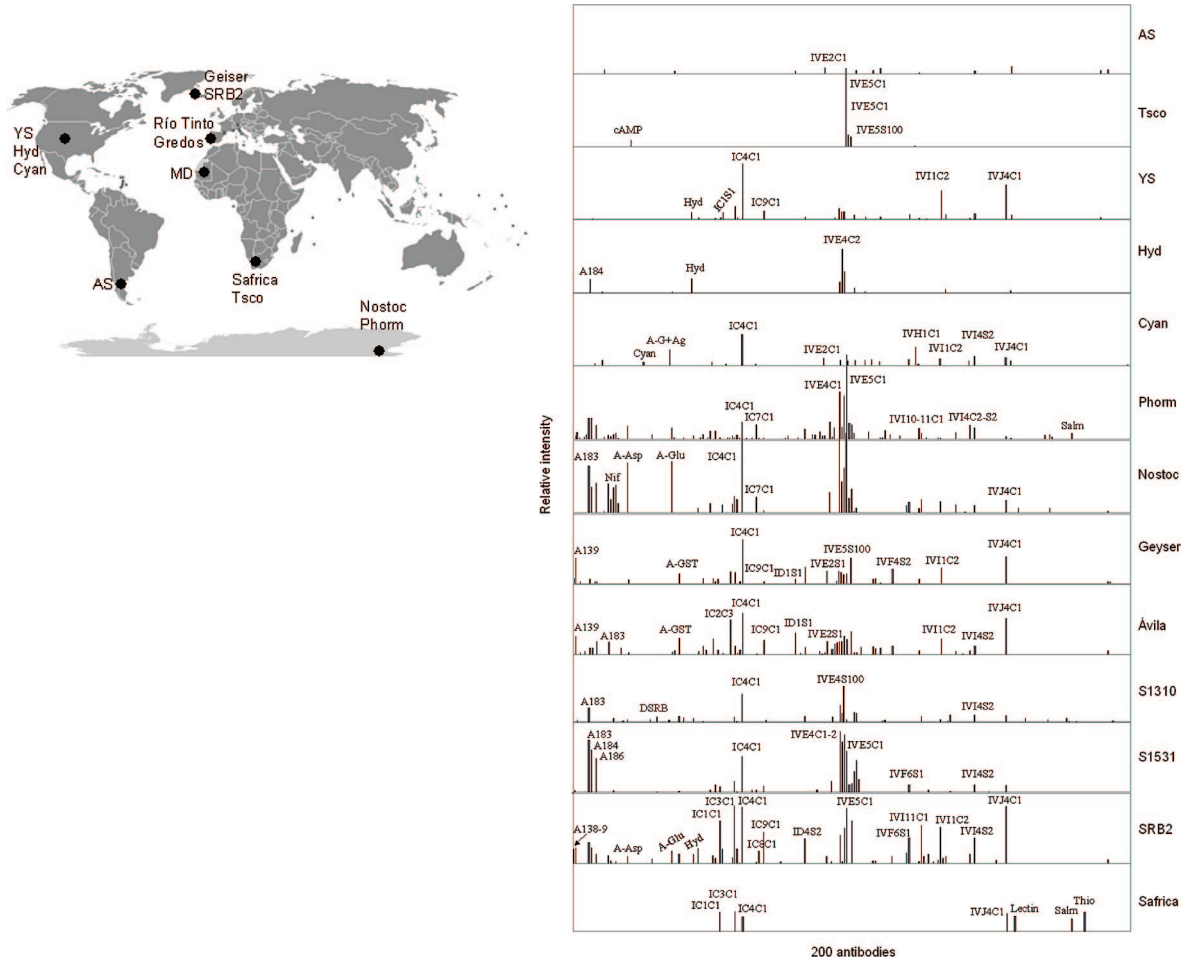

Figure 6. Immunoprofiling worldwide environmental samples with EMCHIP200. The validation of EMCHIP200 for immunoprofiling was proved by analyzing samples from different environments around the world (map). Each sample has a characteristic immunogram whose pattern can be compared with those obtained with the rest of samples. The multianalyte-containing sample extracts corresponded to the following: *AS*, an iron-rich sediment from Argentine; *Tsco*, whole cells from *Thermus scotoductus*, a strain isolated from a deep subsurface, South Africa; *YS*, a Yellowstone sediment; *Hyd*, *Hydrogenbacter*-rich filaments from Yellowstone National Park; *Cyan*, a *Cyanidium* spp.-rich sample from Yellowstone; *Phorm*, a *Phormidium spp*. containing sample from McMurdo Dry Valleys, Antarctica; *Nostoc*, a *Nostoc* spp.-containing sample from McMurdo Dry Valleys, Antarctica; *Geyser*, a cyanobacteria-containing rock from Geyser, Iceland; *Avila*, a lichen-rich granite rock from Sierra de Gredos, Spain; *S1310*, an hydrothermal sample, Iceland; *S1531*, hydrothermal sample, Iceland; *SBR2*, a 62 °C hydrothermal mud from Iceland; *Safrica*, South African deep mine hydrothermal biofilm. All these samples and the extract preparation are described in SI, Experimental Procedures. The sandwich microarray immunoassays were done as indicated in Figure 3 and SI, Experimental Procedures. The most relevant capturing antibodies showing positive results are indicated in each immunogram (see the code translation in SI, Table S1). Some or then are as follows: IVE4... and IVE5..., corresponding to anti-*A. thiooxidans* and *A. albertensis*, respectively; IC4C1, anti-dry brown filaments from Río Tinto; IVI1C2, anti-*P. putida*; Hyd, anti-*Hydrogenbacter*-rich extract from Yellowstone; A-Asp and A-Glu, anti-aspartic and anti-glutamic amino acids; IVI11C1, anti-*Geobacter sulforreducens*; IVI4S2, anti-*B. fungorum*; IC1..., IC2..., IC3..., IC7C1, IC8C1, and IC9C1, corresponding to different anti-Río Tinto sediment extracts (SI, Table S1); A183, anti-*A. ferrooxidans*; 184, anti-*A. thiooxidans*; 186, *L. ferrooxidans*; the lectin potato, specific for NAG-NAM; Thio, anti-thioredoxin; etc.

biomarkers in these worldwide environmental extracts, and (2) the universality of the epitopes recognized by the antibodies (Figure 7). Western results also showed how both antibodies share antigenic specificities against the *Phormidium* spp. extract (Figure 7 top, lines 3). We did not detect high molecular weight compounds in the Gredos and Geyser samples by SDS-PAGE nor by Western blotting, but TLC analysis showed a similar pattern and the same positive immunoreactive spots when tested against IC4C1 antibody (Figure 7 bottom).

In conclusion, EMCHIP200 can be used for the detection of universal high molecular weight biomarkers and to classify environmental samples as a function of the obtained immunoprofile.

Antibody Microarray-Based Life Detector System for Planetary Exploration. We have demonstrated in this work the feasibility of a sandwich microarray immunoassay with more than 200 antibodies. Our antibody collection is increasing and we are investigating which is the maximal number we can use for sandwich immunoassay without compromising sensitivity and specificity. The universality of our EMCHIP200 is supported by the fact that good reactions were obtained against natural extracts from samples around the world (Figures 6 and 7). These results further support the use of polyreactive polyclonal antibodies for the detection of biomarkers, either for characterizing natural ecosystems as a function of their immunoprofile or for the search for life remains in planetary exploration.[3,12,20] In this sense, it is

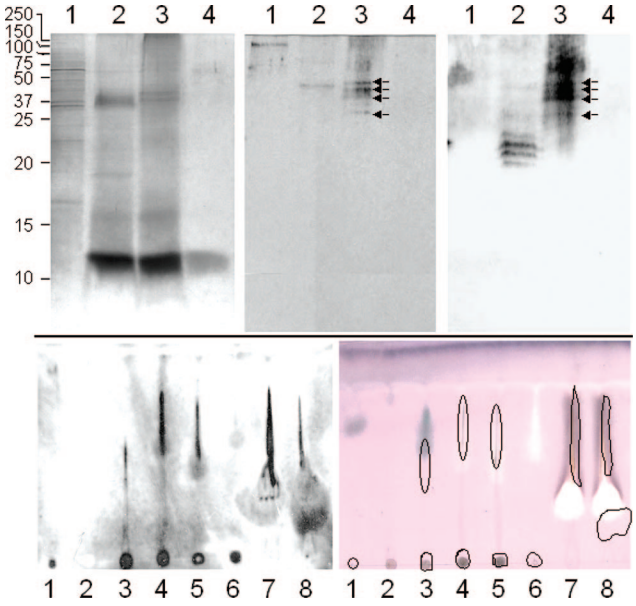

Figure 7. Antibody-detected high molecular weight polymeric biomarkers in different and far away environmental extracts. (Upper figures) Silver-stained 15% SDS-PAGE (left) and Western blots revealed with anti-Río Tinto filaments antibody IC4C1 (middle) and anti-*A. albertensis* IVE5C1 (right) showing high molecular weight compounds in some of the environmental samples from different parts of the world assayed in Figure 6: (1) *SRB2*, (2) *Nostoc*, (3) *Phorm*, and (4) *YS*. Relative molecular mass markers are indicated (from 10 to 250 kDa). Arrows indicate common biomarkers recognized by both antibodies. (Bottom figures) Immuno-TLCs show positive reactions with fractionated compounds from different environmental extracts around the world. The TLC was run using isobutiric:ammonium hidroxide (1 N): water (5:3:2, by volume). Three plates were run in parallel, one was assayed with preinmune P-IC4C1 (not shown), another was incubated with IC4C1 antibody (left), and the third was stained with vainillin (right). The samples corresponded to extracts from the following: *A. albertensis* cells (lanes 1); cellular extract fraction from *T. scotoductus* culture (lanes 2); *SBR2* (lanes 3); *Nostoc* (lanes 4); *Phorm* (lanes 5); *YS* (lanes 6); *Geyser* (lanes 7); *Ávila* (lanes 8). The specific immunoreactive spots are indicated (irregular circles and ovals).

highly relevant how antibodies against natural extracts from an extremely acidic environment recognize antigens from very different environments such as hydrothermal, deep subsurface, or extremely cold ones (see above). Even more, antibodies against isolated strains, like anti-*A. albertensis* (IVE5C1), showed a clear cross-reaction with bacteria from different genera, like the thermophilic bacterium *T. scotoductus* (Figure 6). Also unexpected was the neat reaction of A183, A184, and A186 (anti-*A. ferrooxidans*, *A. thiooxidans*, and *L. ferrooxidans*, respectively) with extracts from hydrothermal environments S1310, S1531, and SRB2 (Figure 6). These results in fact indicate that the antibodies recognize universal structural components common not only between species of the same genus but also between different genera. Therefore, we consider these antibodies extremely useful for life detection experiments in planetary exploration. In the same line, the positive reaction obtained with *G. metallireducens* and *B. fungorum* extracts with a capture antibody obtained from a 162-m-deep core sample at Río Tinto is highly relevant for subsurface life detection (Figures 1C and 3B), indicating the presence of related bacteria, or that they are sharing biomarkers with native microbiota.

Consequently, our strategy for life detection in astrobiology is based on the identification of biomarkers with different degrees of universality, the production of a set of antibodies against them, and development of microarray-based biosensors. Polysaccharides or their multiple combinations with proteins and lipids are excellent targets for searching for extant life in planetary exploration. The production of polysaccharides like EPS, lypopolysaccharides, or anionic polymers (teichoic and teichuronic acids), for example, is a universal response of living organisms to environmental variables like temperature, water availability, or interaction with surface minerals either to obtain energy or for biofilm formation.[31−33] Polysaccharides may constitute up to 30% of the cell dry weight under certain circumstances.[34] Also, high molecular weight pigments (melanin and carotenoid derivatives) are produced by microorganisms to protect themselves against solar radiation and oxidative stresses. These polymeric organic pigments may also serve as biomarkers of an extant microbial community.[35] For further discussion about biomarkers for astrobiology, see refs 20 and 36.

Our EMCHIP200 has an antibody redundancy with overlapping specificities (see Figures 3−7); that is, different antibodies recognize similar structural components in addition to their own antigens (e.g., IC4C1 and IVE5C1, Figure 7 top, lines 3). This property favors the detection of biomarkers through a signal amplification effect in the sandwich assay due to the multiple fluorescent tracer antibodies that can bind to different epitopes of the same captured biomarker (Figure 3, middle). In addition, a sandwich immunoassay has by itself two degrees of specificity, that exhibited by the capturing antibody and that of the fluorescent antibody tracers.

How to use our EMCHIP for astrobiology? We have previously reported a method and an apparatus (SOLID) for automatic analysis of solid (soil or powdered rock) samples.[3,37] The current field prototype, SOLID V2.0 (Figure 8), was successfully tested during several field campaigns, especially that for a Mars drilling simulation held jointly by Centro de Astrobiología and NASA-Ames.[21,22] The instrument can analyze from 0.1 (or less) to 0.5 g of a solid sample and from a few microliters to 2 mL of a liquid one. As expected, the quality of the results depends on the concentration of the analytes in the sample. SOLID2 was fed with 0.5 g of drill core sample by means of a robotic arm, and the analysis was run automatically by following the next command series:[21] buffer injection (up to 2 mL), sample homogenization (by ultrasonication), sample filtering and injection to a flow cell with the antibody microarray, incubation, washing, dissolution and addition of a fluorescent antibody cocktail from accessory chambers, incubation, washing, fluorescent excitation with a laser beam, and a final image capture with a CCD camera. For either an

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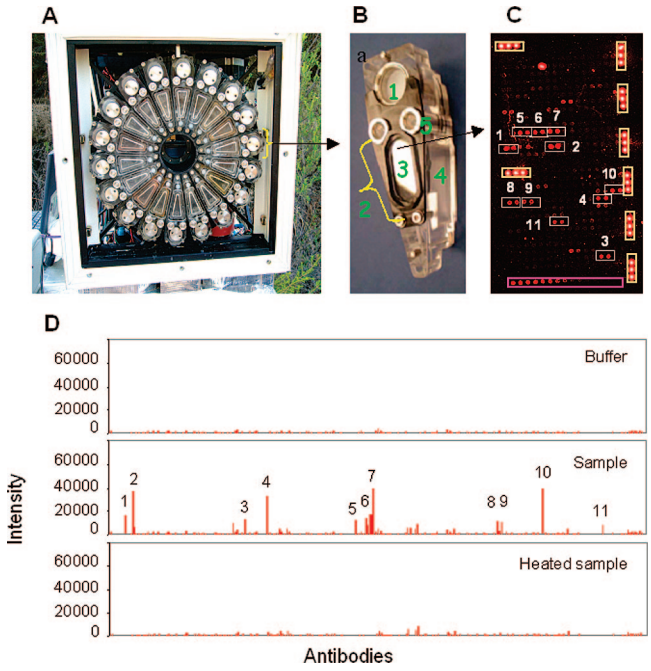

Figure 8. Antibody microarray-based life detector instrument for automatic online analysis and planetary exploration. Our EMCHIP200 is being implemented for use as the biosensor element in the SOLID instrument.[3,20,21,37] (A) SOLID V2.0 field-tested prototype showing its 18 independent analysis modules each of them loaded with one EMCHIP200. (B) One analysis module with the following: (1) the homogenizing chamber, where up to 0.5 g of solid material or up to 2 mL of liquid sample can be loaded; (2) the reaction or flow cell chamber with (3) the EMCHIP200 antibody microarray; (4) the waste deposit; (5) additional reaction chambers for lyophilized fluorescent antibody cocktails or other reactants. (C) Image obtained with SOLID 2 and processed with GenePix Pro (Genomic Solutions) software. In this experiment, 0.5 g of a phyllosilicates-rich sediment from of Río Tinto was loaded into SOLID 2 instrument and subjected to a complete and automatic EMCHIP200 sandwich immunoassay process, including the following: (i) liquid buffer addition (up to 2 mL); (ii) homogenization by ultrasonication; (iii) filtering through 15 μm; (iv) incubation with EMCHIP200 microarray; (v) washing; (vi) dissolution and addition of a 200 fluorescently labeled antibody mixture and incubation; (vii) washing; and (viii) image capturing. The signal intensity was quantified and plotted (middle immunogram in D). The most relevant positive spots were as follows: (1) A183, anti-*A. ferrooxidans*; (2) A186, anti-*L. ferrooxidans*; (3) IC1S1, anti-Río Tinto sediment extract; (4) IC4C1, antidry brown filaments from Río Tinto; (5) IVE3C1, anti-*A. ferrooxidans*; (6) IVE4C1, anti-*A. thiooxidans*; (7) IVE5C1, anti-*A. albertensis*; (8) IVI4S2, anti-*B. fungorum*; (9) IVF4S2, anti-*Psychroserpens burtonensis* supernatant extract, grown at 4 °C; (10) IVE4S100, anti-*A. thiooxidans* S100 extract; (11) IC7C1, anti-Río Tinto wet brown-green filaments. A frame of fluorescent spots is routinely printed (yellow rectangles enclosing triplicate spots) for an easy location of the microarray, together with a dilution fluorescent curve (purple rectangle). Two negative controls are usually run: one using just buffer as sample (upper immunogram), and another one consisting of subjecting the sample to a heat treatment at 300 °C for 12 h to destroy the organic material (bottom) and then analyzed in parallel with the nontreated sample (middle).

environmental or an astrobiological mission, we propose a routine protocol including three analyses with EMCHIP200: (1) a negative control with no sample, just buffer to check the contribution of fluorescent antibodies to the background; (2) the sample analysis (from 0.1 to 0.5 g of grinded material); and (3) one extra control to check whether the positive reactions are in fact a consequence of the binding of high molecular weight compounds and not due to unspecific interaction with minerals present in the samples. For this control, one aliquot of the same sample used for the analysis was previously incubated at 300 °C for 12 h and then analyzed with EMCHIP200. Under such conditions, most of the biological polymers are expected to be destroyed, at least their three-dimensional structures. Consequently, the antigen−antibody interaction does not take place and no signal is detected (Figure 8). Interestingly, when we analyze the protein and sugar content, they still are there, confirming that what has really been destroyed is the structure of the antigenic determinant and what we were detecting really corresponded to complex molecular structures and not to unspecific interaction with minerals (not shown).

CONCLUSION

Assuming that similar or identical molecular mechanisms allow microorganisms to thrive under similar physicochemical parameters, it is possible to obtain environment- or microenvironment-specific immunoprofiles or immunopatterns. Our results demonstrate that it is possible to use antibody microarrays for the detection of environmental biomarkers with different degrees of universality. Similar immunopatterns mean common biomarkers or shared structural features and, consequently, either similar organisms or, at least, common biochemical responses to environmental parameters. In this work, we have validated the universality of EMCHIP200 as a real-life detector chip for both astrobiological and environmental monitoring purposes. Other interesting applications of our EMCHIP200, as well as new implemented versions, could be online biomarker detection in industrial (biomining), bioremediation, pollution, or even climate change monitoring.

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SUPPORTING INFORMATION AVAILABLE

Tables S1 and S2; Figures S1−S9; detailed experimental procedures; and supporting references. This material is available free of charge via the Internet at http://pubs.acs.org.

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