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Immunoanalytical Approach for Detecting and Identifying Ancestral Peptide Biomarkers in Early Earth Analogue Environments

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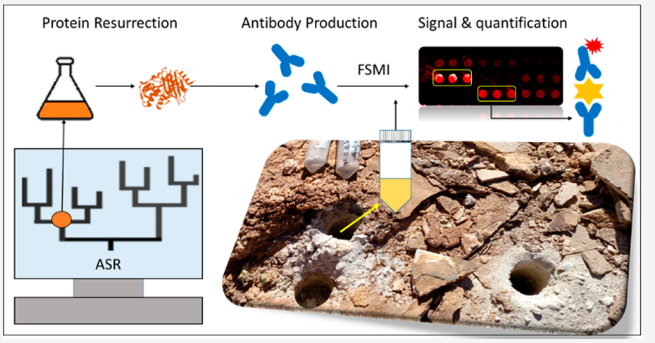
ABSTRACT: Several mass spectrometry and spectroscopic techniques have been used in the search for molecular biomarkers on Mars. A major constraint is their capability to detect and identify large and complex compounds such as peptides or other biopolymers. Multiplex immunoassays can detect these compounds, but antibodies must be produced for a large number of sequence-dependent molecular targets. Ancestral Sequence Reconstruction (ASR) followed by protein "resurrection" in the lab can help to narrow the selection of targets. Herein, we propose an immunoanalytical method to identify ancient and universally conserved protein/peptide sequences as targets for identifying ancestral biomarkers in nature. We have developed, tested, and validated this approach by producing antibodies to eight previously described ancestral resurrected proteins (three β-lactamases, three thioredoxins, one Elongation Factor Tu, and one RuBisCO, all of them theoretically dated as Precambrian), and used them as a proxy to search for any potential feature of them that could be present in current natural environments. By fluorescent sandwich microarray immunoassays (FSMI), we have detected positive immunoreactions with antibodies to the oldest β-lactamase and thioredoxin proteins (ca. 4 Ga) in samples from a hydrothermal environment. Fine epitope mapping and inhibitory immunoassays allowed the identification of well-conserved epitope peptide sequences that resulted from ASR and were present in the sample. We corroborated these results by metagenomic sequencing and found several genes encoding analogue proteins with significant matches to the peptide epitopes identified with the antibodies. The results demonstrated that peptides inferred from ASR studies have true counterpart analogues in Nature, which validates and strengthens the well-known ASR/protein resurrection technique and our immunoanalytical approach for investigating ancient environments and metabolisms on Earth and elsewhere.



Understanding the presence and distribution of life in the universe is the major goal of astrobiology,[1] and the discovery of any form of it on another planet would be a refutation of the universality of life. The planetary exploration community has already deployed, or plans to deploy, a set of analytical techniques for searching for molecular signatures of life on Mars or the Ocean Worlds Enceladus and Europa,[2−5] as well as the return of samples from Mars for comprehensive laboratory analysis.[6] These approaches are mostly based on the assumption that life uses similar (bio)chemistry and molecular strategies and patterns as it does in the only example of life we know.[7]

The search for in situ molecular fingerprints of life requires robust, sensitive, reliable, and space-suitable analytical techniques. So far, and due to mass and other space-related constraints, only gas chromatography coupled to mass spectrometry (GC-MS) together with several spectroscopic techniques have been used in planetary exploration (for a review, see ref 8). Other technologies based on the analysis of liquid solutions/suspensions, such as capillary electrophoresis,[9] antibody-based biosensor chips (reviewed in ref 8), or nanopore-based molecular analysis,[10] are being developed to increase the variety and complexity of life-predictive target molecules to be detected. Antibody-based biosensor chips complement the techniques discussed above, with benefits such as the feasibility of multiple internal calibration curves, multiple negative and positive controls, a wide range of molecular size targets (from amino acids to cells), and easy readable results (reviewed in ref 11).

Our group[11,12] and others[13] have proposed the use of antibody microarrays to search for molecular biomarkers on the surface of Mars and Ocean Worlds such as Enceladus or

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Europa (reviewed in ref 14). To that end, we have developed the Life Detector Chip (LDChip), an antibody microarray containing over 200 antibodies,[15,16] directed at a similar number of targets selected among the most universal biomolecules (aromatic amino acids, nucleotides, DNA, peptidoglycan, lipopolysaccharides), microorganisms (members of Bacteria and Archaea) isolated from terrestrial analogues (e.g., Atacama, Antarctica, Rio Tinto), and other organic compounds (e.g., mellitic acid, polycyclic aromatic hydrocarbons [PAHs]).

We have also developed the SOLID (Signs of Life Detector) instrument, capable of obtaining, remotely and automatically, a liquid extract from 0.5 to 1 g of soil, sediments, or ground rock and searching for molecular biomarkers by fluorescence sandwich microarray immunoassay (FSMI) with the LDChip.[17,18] The LDChip performance has been demonstrated in many field campaigns,[16,19−21] as well as the suitability of antibodies for Mars exploration,[17,22,23] and the SOLID-LDChip system has been proposed as a payload instrument for searching for life on Mars in response to ESA and NASA calls.[3]

Life detection by immunosensors is limited by the fact that they cannot contain antibodies against the high number of potential target biomarkers. One way to mitigate this apparent weakness is by resorting to bioinformatics and molecular techniques and searching for common well-conserved molecular biomarkers that Earth and Mars could have shared, in both the past and currently.

Ancestral sequence reconstruction (ASR) and protein "resurrection" techniques allow the reconstruction of ancestral protein sequences, theoretically dating back to 4 Ga, and their expression and characterization in the laboratory.[24] To date, more than 200 ancestral proteins and 89 crystallized structures have been described.[25] Their study has provided insights into the properties that true ancestral proteins could have had during the early stages of life on Earth,[26−29] contributing to the understanding of ancient biogeochemistry and providing a wider integration of the evolution of life into the geologic records of our planet.[30]

Assuming that Mars and Earth were habitable and exchanged material between ca. 4 to 3.5 Ga,[31] after which Mars became frozen, the evolution of any potential martian microbiota must have halted or severely slowed down. Thus, there is the possibility that proteins obtained through ASR and protein resurrection would assist in understanding ancient Earth and, possibly, Mars microbial metabolisms. Even current harsh terrestrial analogue ecosystems, particularly in hydrothermal settings, may maintain many of their ancient traits.[32] We hypothesize that comparing ancestral proteins and their modern counterparts enables the identification of conserved residues or short peptide sequences, providing a framework for biomarker discovery.[33]

Therefore, we propose an immunoanalytical technique for detecting and identifying specific yet broadly distributed peptides in natural environments. This is done by producing antibodies against Precambrian resurrected proteins, fine epitope mapping, and fluorescent immunoassays. By focusing on well-conserved peptide sequences (epitopes), we can narrow the peptide biomarker space of sequences to look for while increasing the likelihood of finding them in natural environments, on Earth, or beyond. We show that protein resurrection and epitope immuno-detection techniques can identify evolutionarily conserved peptides in current early-Earth-like settings, which may be present in multiple proteins, increasing the likelihood of detection by an antibody microarray like the LDChip.

EXPERIMENTAL SECTION

Antibody Production, Purification, and Fluorescent Labeling. Polyclonal rabbit antisera were produced against eight previously reported ancestral proteins, namely, three thioredoxins (LBCA, LPBCA, and LGPCA), three β-lactamases (PNCA, GNCA, GPBCA), a RuBisCO, and an Elongation Factor Tu.[26−29] The IgG fraction of each antiserum was purified and fluorescently labeled (Alexa 647^{R}, Molecular Probes), as described in the SI, Experimental Section.

Antibody Microarrays Production. Microarrays containing anti-ancestral protein antibodies (APChip) or ancestral peptides (PEChip) were printed by triplicate or duplicate spot pattern, respectively, on epoxy-activated glass slides (Cel Associates Inc., Pearland, TX, U.S.A.). For fine epitope mapping,[34] sequences from the ancestral thioredoxins and ancestral β-lactamases[27,29] were translated into 14 amino acid-overlapping 15mer peptides and printed as a custom-designed commercial high density peptide chip (HDPChip) by PEPperPRINT GmbH (Heidelberg, Germany). For a detailed description, see SI, Experimental Section.

Fluorescent Microarray Immunoassays and Analysis. Fluorescent sandwich microarray immunoassays (FSMI), direct, and inhibitory immunoassays were performed as described.[15] For a detailed description, image processing, and data treatment, see SI, Experimental Section.

Sample Collection and Processing. Eleven natural samples were tested in this work, corresponding to different environments (hydrothermal, arctic, and mediterranean) and different types (silica precipitates, biofilm, salt crust, sediment, and soil). Most samples have been described elsewhere,[22,35−38] and their main features are gathered in Table S1. Crude liquid sample extracts to be analyzed by FSMI were prepared by ultrasonication and filtered (see SI, Experimental Section).

Metagenomics Sequencing and Data Analysis. Total DNA from a sample collected at the hydrothermal area of El Tatio (Chile) was extracted and randomly sequenced by Illumina Novaseq (Illumina NovaSeq 6000 Sequencing System, RRID:SCR_016387). For a detailed description on the procedure and metagenome analysis, see SI, Experimental Section.

Metaproteomics Sequencing and Data Analysis. We extracted total proteins from a sample from the same pool as above and, after trypsin digestion, peptides were ionized and analyzed on a Q-Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific). For a detailed description on the procedure, see SI, Experimental Section.

Statistical Analysis. The statistical significance of the differences observed between samples and controls in the inhibition immunoassays (Figure 3) was assessed with a GLM model analysis (Factor: Treatment, Subject: Array, Repeated measures: Spot). Besides, an independent-sample Mann−Whitney U test[39] was used to confirm the differences with a nonparametric approach, significance level of 0.05, carried out in IBM SPSS Statistics V28.

RESULTS

Antibodies Distinguish between Different Ancestral Resurrected Versions of the Same Protein. To investigate

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Anal. Chem. 2023, 95, 5323−5330

the viability of using ancestral resurrected proteins as a source of antibody probes for detecting ancestral protein analogues in modern paleoenvironments, we first produced polyclonal antibodies against eight previously published proteins obtained through ASR, theoretically from the Precambrian period, ranging in age from 4 to 2 Ga (Table 1).[26−29] We purified the IgG fraction of each antiserum with protein A, and we labeled them with fluorochrome Alexa Fluor 647 (see Experimental Section). Antibody microarrays were produced by printing the unlabeled IgG-purified antibodies on epoxy-activated glass slides. Labeled tracer antibodies (TAbs) and proteins were set up for FSMI. Using polyclonal antibodies diminishes the risk of obtaining false negatives since they can target different epitopes in the same protein.

Table 1. Ancestral Proteins Used in This Study

name	description	ref
RuBisCO_Anc	RuBisCO forms 1 A/B last common ancestor (Anc)	26
β-lactamase_PNCA	β-lactamase (β-lact) class A gram-positive and gram-negative bacteria last common ancestor (PNCA)	27
β-lactamase_GNCA	β-lactamase (β-lact) class A gram-negative bacteria last common ancestor (GNCA)	
β-lactamase_GPBCA	β-lactamase (β-lact) class A gammaproteobacteria last common ancestor (GPBCA)	
EF-Tu_Anc	elongation Factor Tu (EF-Tu) N168 bacterial ancestor (Anc)	28
Trx_LBCA	thioredoxin (Trx) last bacterial common ancestor (LBCA)	29
Trx_LPBCA	thioredoxin (Trx) last common ancestor of the cyanobacterial, deinococcus, and thermus groups (LPBCA)	
Trx_LGPCA	thioredoxin (Trx) last gammaproteobacteria common ancestor (LGPCA)	

Optimal antibody concentration, assessed by FSMI using total affinity-purified IgG, ranged between 0.5 and 1 $\mu g \cdot mL^{-1}$ (see SI, Experimental Section). The limits of detection (LoD), defined as the minimal concentration of analyte that produced a reproducible fluorescent signal (see SI, Experimental Section, Figure S1a, Table S2) at the optimal antibody concentration, ranged from 0.2 to 50 $ng \cdot mL^{-1}$, demonstrating the sensitivity of the assay, as observed for other biomolecules.[15] With minor cross-reactivity, the antibodies exhibited the highest signal with their own protein version and the lowest reactivity with the most divergent ones (Figure S1b and Table S3). A certain level of cross-reaction between the anti-thioredoxin_LBCA antibody and β-lactamase_PNCA was observed, suggesting shared epitope(s) between Trx_LBCA and β-lactamase_PNCA proteins, not inferred from sequence comparison alone. Polyclonal antibodies produced against current bacterial *Leptospirillum ferrooxidans* (Lfe) RuBisCO did not recognize the ancestral RuBisCO, which could be explained by low sequence homology (BAM08137.1, 20% sequence identity).[19]

Strong Immuno-Detection Signals Detected in Natural Hydrothermal Environments. To investigate whether the antibodies to ancestral proteins could detect specific epitopes in natural samples, crude liquid extracts from various environments (Figure S7 and Table S1) were tested by FSMI using the APChip, a microarray chip containing the anti-ancestral protein antibodies as capturing probes (CAb) and each individual fluorescent antibody as tracer (TAb). As negative capturing control probes, we used the corresponding preimmune IgG fraction of each antibody. As a blank control sample to rule out any artifact due to the presence of minerals in the extracts, we used the same samples but preheated at 500 °C before extraction to denaturalize or burn the organic (peptide/protein) matter (pyrolyzed samples). We obtained positive signals in extracts from a paleo-sinter mound and three biofilms, for antibodies against β-lactamase PNCA, thioredoxin LBCA, and EF-Tu (Figure S8). Additionally, positive signals for the first two antibodies above-mentioned were observed during a campaign in the Atacama Desert, with SOLID-LDChip[40] (Moreno-Paz et al., in press). We focused on the paleosinter mound sample from the hydrothermal field of El Tatio (Chile) and repetitively (n = 5) obtained strong immuno-detection signals on capturing antibodies to the most ancient (ca. 4 Ga) proteins of β-lactamase (PNCA) and thioredoxin (LBCA; Figure 1). Signals were lost after testing the same, but pyrolyzed, sample, indicating that the above immuno-detections were truly due to organic, mostly proteinaceous, material (Figure S2). A cross-reaction between these two antibodies suggested that the captured antigens exhibited epitopes recognized by both antibodies, as previously observed (Figure S1b).

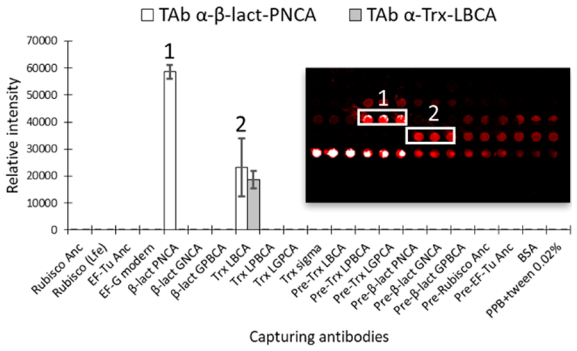

Figure 1. Immunological detection of ancestral peptide analogues from El Tatio hydrothermal environment (Figure S7 and Table S1). Crude extracts from a silica paleosinter mound sample (*ET1*) were analyzed with an ancestral-protein antibody microarray (APChip) by FSMI. (Inset) An APChip fluorescence microarray image with capturing antibodies spots in a triplicate pattern, where 1 and 2 correspond to capturing anti-β-lactamase_PNCA and anti-Trx_LBCA antibodies, respectively. (Plot) Merged plots of relative fluorescence units after quantification. FSMI performed with each antibody (error bars represent median standard deviation, n = 5). White series shows the results after revealing with anti-β-lactamase_PNCA and the gray one with anti-Trx_LBCA as tracers.

Identification of Peptide Targets by Fine Epitope Mapping. The strong immunological signals obtained with the silica paleosinter mound sample with the anti-Trx_LBCA and anti-β-lactamase_PNCA antibodies (Figure 1) suggested the presence of proteins that could be sharing common structural features (epitopes) with the ancestral reconstructed proteins. To determine the peptide epitopes that were being recognized by the antibodies, we carried out fine linear epitope mapping. For that, we used high-density 14 amino acids-overlapping 15-mer peptide microarrays[34] from the ancestral thioredoxins (LBCA, LPBCA, LGPCA) and β-lactamase (PNCA, GNCA, GPBCA) sequences. After direct immunoassays with each labeled TAb, we identified three peptide epitopes showing a strong fluorescent signal (Figure 2 and Table S4). The anti-Trx_LBCA antibody recognized a linear

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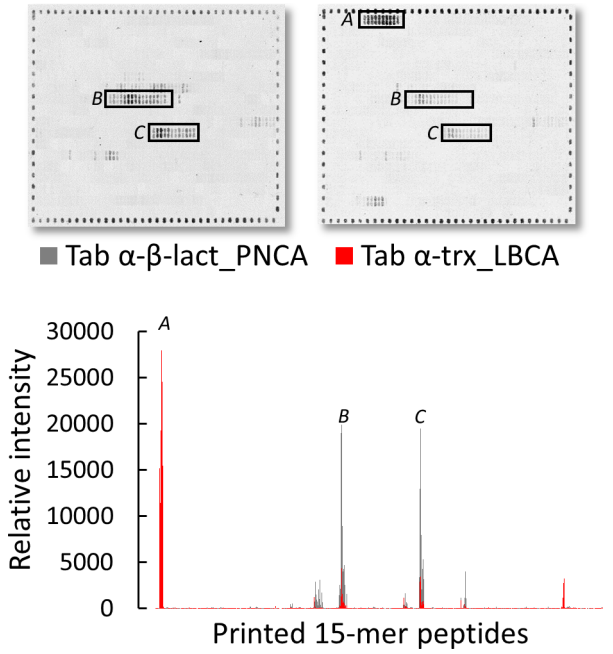

Figure 2. Fine mapping of linear epitopes recognized by anti-ancestral thioredoxin-LBCA and anti-ancestral β-lactamase PNCA antibodies. A custom-made high density peptide microarray containing 15-mer peptides (x-axis) overlapping by 14 amino acids of several thioredoxin and β-lactamase sequences (see Experimental Section), was incubated with anti-thioredoxin LBCA and anti-β-lactamase PNCA fluorescent (tracer) antibodies (TAb), and scanned for fluorescence. (Top) Scanned fluorescence of each peptide microarray showing the tiling effect of fluorescence intensity with a maximum at the peptide zones *A*, *B*, and *C* that are best recognized by the antibodies. The differences in fluorescence intensity at each peptide zone corresponded to different binding efficiencies of the antibody to the one-amino acid difference peptide in each spot, being maximum at the best-fit peptide epitope. (Bottom) Merged plot showing relative fluorescence units counts from anti-thioredoxin LBCA (red bars) and anti-β-lactamase PNCA (gray bars) with a maximum at the best match peptides *A*, *B*, and *C*.

epitope deduced from the Trx_LBCA sequence (peptide *A*, IEINDENFEEEVLKS), while another two linear peptides from β-lactamase_PNCA (peptide *B*, WEPELNEALPGDPRD), and β-lactamases_GPBCA/GNCA (peptide *C*, WEPELNEAAPGDPRD) sequences, were strongly recognized by anti-β-lactamase_PNCA and, to a lower extent, by anti-Trx_LBCA antibodies, in agreement with the cross-reaction observed previously (Figures 1 and S1b and Table S3).

Confirmation of Peptide Targets by Inhibition Immunoassays. We used inhibition immunoassays to (i) validate the peptides identified by fine peptide mapping (see above) as targets for anti-β-lactamase_PNCA and anti-Trx_LBCA antibodies, and (ii) validate inhibition immunoassays for the identification of specific peptides as antibodies targets. Then, two separate microarray chips were printed either with peptide *A*, or with peptides *B* and *C*, and shorter versions of these peptides, namely, *B1* (PELNEALPGD) and *C1* (PELNEAAPGD; Table S4), and set up for an inhibition assay of the corresponding TAb in the presence of an inhibitory/competing peptide.

Results (Figure S3) showed that preincubation with peptide *B* inhibited the recognition of all the peptides by both tracer antibodies. Preincubation with peptide *C* decreased peptide *B* recognition by both antibodies and inhibited the recognition of peptide *C*. Preincubation with peptide *B1* decreased the recognition of peptides *B* and *C* and completely inhibited the recognition of peptide *B1*. Preincubation with peptide *C1* did not alter the antibody recognition of any of the other peptides, meaning that none of the antibodies recognized the specific sequence of peptide *C1*. This could be due to a protrusion created by the leucine residue in peptides *B*/*B1*, absent in peptides *C*/*C1* (Figure S4), which would hinder the antibody binding. Preincubation with peptide *A* completely inhibited the recognition of peptide *A* by anti-Trx_LBCA antibody, as expected. Peptide *A* was not tested with the anti-β-lactamase_PNCA antibody, because previous results showed no recognition between this pair (Figure 2).

Identification of ASR Inferred Peptides from El Tatio Hydrothermal System Sample. We performed highly specific inhibitory immunoassays to check whether analogues of peptides *A* and *B* were indeed present in the El Tatio sample (Figure 3) and if they were responsible (at least partially) for

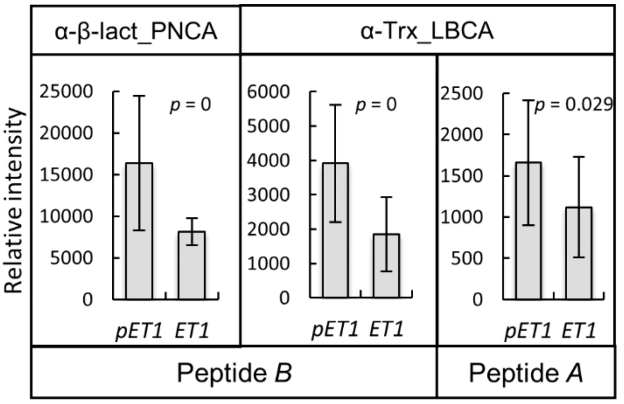

Figure 3. Detection of ancestral peptides *A* and *B* in nature. Fluorescence tracer antibodies were preincubated in separate tubes with crude extracts from a silica paleo-sinter mound sample from El Tatio (*ET1*, competing sample), and with an extract from the same, but previously pyrolyzed, sample (*pET1*, control). After preincubation, they were incubated with peptide microarrays (PEChip) containing printed peptides *A* or *B*, for binding of the still free tracer antibody molecules. After scanning for fluorescence, bars showed the average percentage of inhibition in relation with the noninhibition control (*pET1*). Error bars represent standard deviation (n = 6 for peptide *B* assays, n = 3 for peptide *A* assay; Table S8).

the fluorescence signal in the FSMI with crude extracts (Figure 1). For that, crude extracts were preincubated with tracer antibodies to let potential peptide analogues bind to anti-β-lactamase_PNCA and/or anti-Trx_LBCA TAbs and, in doing so, sequester TAbs and inhibit their binding to immobilized peptides on a low-density peptide microarray. A pyrolyzed sample extract was used as a blank control for inhibition (no inhibition). In this type of inhibitory assay, a loss of fluorescence signal on the microarray spot reveals the presence of the same epitope in the extract. As predicted, fluorescence signals on the printed peptide spots were significantly lower after preincubation with sample extracts, compared to the pyrolyzed controls (independent-samples Mann–Whitney U test, significance 0.05; Figure 3 and Table S8), indicating that epitopes in the sample extract were competing with the peptides for binding the antibodies. On average, antigens/epitopes in crude extract samples inhibited the binding of anti-

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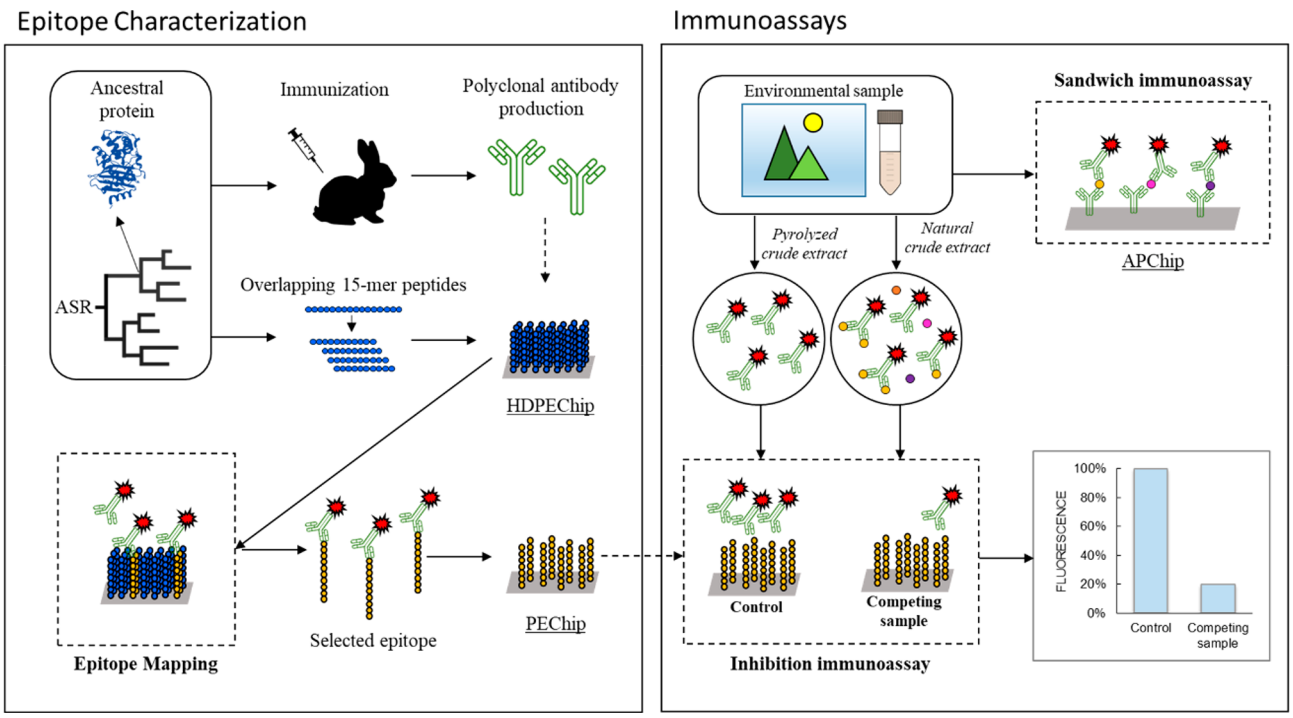

Figure 4. An immunoanalytical approach for detecting ancestral-like and well evolutively conserved peptides in nature. See text for a detailed description. ASR, ancestral sequence reconstruction; HDPEChip, high density peptide chip; PEChip, peptide chip; APChip, ancestral-protein antibodies chip.

β-lactamase_PNCA and anti-Trx_LBCA antibody tracers to peptide *B* by about 50%, while the inhibition of peptide *A* binding by anti-Trx_LBCA was on average 32%, compared to noninhibition controls.

Confirmation of Analogue Peptide Sequences by Metagenomics. Metagenomic analysis from the El Tatio sinter mound sample allowed us to reconstruct and analyze several thioredoxin and *β*-lactamase encoding genes, mostly assigned to Actinobacteria, among others (Figure S5). The thioredoxin genes with higher copy numbers per genome were annotated to the general thioredoxin domain (PF00085). Most *β*-lactamase genes were mapped into the metallo-*β*-lactamase superfamily (PF00753) or identified as serine *β*-lactamases (PF00144). After translating into amino acids, significant matches to peptides *A*, *B*, or *C* were observed (Figure S5). Partial matches between peptides *B* and *C* contained leucine (L) and arginine (R), among others, as the most relevant epitope residues. The PELNEAL sequence, the core of peptide *B*, was the most frequent. Partial matches to peptide *B* were also identified in proteins detected by metaproteomics (Figure S6).

■ DISCUSSION

Immunoanalytical Strategy for the Identification and Validation of Ancestral Peptide Biomarkers. Multiplex fluorescent antibody microarray immunoassay for detecting microbial markers and xenobiotics has been proposed for environmental monitoring and planetary exploration.[11] For life detection, we can think of a high number of potential molecular targets and their corresponding antibodies to ensure a high probability of success. However, the number of antibodies that can be handled in antibody microarray sensors is subjected to technical limitations since cross-reactivity, unspecific reactions, and general background noise increase with the number of antibodies used.[41] One strategy to mitigate this drawback is to increase the likelihood of detecting biomarkers. This is done by focusing on and selecting universal and well-evolutionarily conserved target molecules along the tree of life. Such molecules may be specific to an environment, *taxa*, or as a component of a particular family of functional molecules such as lipids or proteins.[15,17] Life detection in astrobiology requires such biomarkers displaying some level of complexity and information that make them distinguishable from abiotic targets.[42]

We take advantage of ASR and protein resurrection to travel back in time and search for well-conserved epitope peptides that could be present in multiple proteins and multiple *taxa*. Thus, we constrain the target range while increasing the likelihood of detecting biomarkers in a positive and informative manner with a limited number of antibody probes. For that, we have developed an immunoanalytical pipeline (Figure 4) where protein sequences obtained through ASR and expressed as recombinant proteins are used as immunogens for antibody production. Epitope mapping using a high-density 15 amino acids in length peptide array (HDPEChip) from the same proteins allows the identification of the exact recognized peptides. The selected peptides are used in a second low-density peptide microarray (PEChip) to probe their presence in complex natural samples by inhibition immunoassays, including pyrolyzed samples as blank controls (no inhibition, 100% signal). A significant loss of antibody binding to the PEChip compared to the control indicates that something in the sample competes with the printed peptides for antibody binding.

Since the antibodies are highly specific to their targets, and one amino acid change can hinder antibody recognition, as shown for peptide *C1* (Figures S3 and S4), the presence of the peptide or at least a large part of it is validated as present in the

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Anal. Chem. 2023, 95, 5323−5330

studied sample. Through this approach, we validate the use of antibodies against resurrected ancestral proteins as a capturing and detection system for well-conserved peptide features in nature by FSMI-type immunoassay.

Universal Peptides in Ancestral β-Lactamases and Thioredoxins in an Early Earth Hydrothermal Analogue Environment. We validated the approach depicted above (Figure 4) by screening several natural samples with a set of antibodies raised against ancestral resurrected proteins: one biofilm and three samples from different silica sinters from the El Tatio geyser field in Chile, one biofilm and one fumarole regolith sample from two hydrothermal areas in Iceland, one biofilm and two sediment samples from a hydrothermal field in New Zealand, one salt crust sample from a hypersaline perennial spring in the Canadian High Arctic, and one soil sample from a Mediterranean forest near Madrid (Spain; Table S1 and Figure S7).[22,35−38] Out of them, sample *ET1*, from El Tatio geothermal field, showed positive immunodetection with anti-β-lactamase_PNCA and anti-Trx_LBCA antibodies (Figure 1), the antibodies against the theoretically oldest proteins as inferred by ASR (Table 1). Later, we demonstrated that analogues to peptides *A* and *B* were indeed present in the sample (Figure 3). Other samples were tested, in particular, from other hydrothermal environments as well, but with negative results. Moreover, three samples were directly comparable, since they originated from nearby sites at El Tatio, but again, only one of the samples produced positive immunodetection against these two antibodies. Apparently, microbial community composition did not seem to be the cause of the different immunological behavior between samples since Actinobacteria and Proteobacteria members were also present in another El Tatio dry mound sample[35] or in the hydrothermal Icelandic sample as well.[36]

Significant matches to peptides *A*, *B*, and *C* were identified by metagenomics (Figure S5), however, no exact matches were found, which strengthens our immunological approach, since otherwise, these conserved epitopes would not have been recognized and identified. On the other hand, perhaps exact matches could have been present, but lost during DNA extraction or due to bad DNA reads. Alternatively, given the three-dimensional nature of peptide epitopes, many times they cannot be inferred from the sequence alone. In fact, partial matches to peptide *B* were observed in the abundant protein family of chaperonins identified by metaproteomics in sample *ET1* (silica sinter from El Tatio, Figure S6). However, whether chaperonins in this particular sample were, at least partially, responsible for the immunodetection obtained, is unknown at this time and requires further investigation. Whatever the case, the results showed that these epitopes, obtained from artificial ASR proteins, were abundant in the sinter sediment sample *ET1* and readily identified using our proposed immunoanalytical approach, besides the fact that thioredoxins[43] and β-lactamases[44] are old and ubiquitous proteins. Interestingly, the dominant amino acid type in peptides *A* and *B* are common in thermophilic proteins (charged and hydrophobic residues), which include leucine and glutamic acid, previously shown to be preferential in ancestral thioredoxins and β-lactamases,[45,46] but further development on this matter is beyond the scope of this study.

CONCLUSIONS

We devised an immunoanalytical approach for identifying and validating peptide biomarkers in natural environments, which we validated by detecting peptide epitopes from ancestral, theoretically 4 Ga, thioredoxin, and β-lactamases. The findings demonstrated that epitopes derived from ASR and resurrected proteins could be identified by this immunoanalytical approach.

Given the intensity and reproducibility of the immunosignals obtained, our results strengthen the utilization of ASR and artificial ancestral proteins as proxies for identifying well-conserved peptides in early Earth analogous environments.[47] This is of astrobiological relevance since hydrothermal areas as El Tatio are among the best known Mars analogous environments.[32,48] Overall, our methodology and findings support the use of ASR and protein resurrection as proxies for biomarkers discovery in astrobiology, and in the characterization of paleo-environment analogues[33] on Earth and possibly Mars. As the NASA and ESA Mars Sample Return program[6] is on the way, and martian samples can be on Earth by mid-2030s, having well-focused immunoassays for detecting potentially conserved peptides and other target biomarkers could contribute to the search for past or current life on our neighbor planet.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c05386.

Detailed description of experimental procedures. Illustration of antibodies characterization, other FSMI results, inhibition immunoassays, target epitopes, samples, and metagenomics and metaproteomics results. Tables with sample descriptions, antibody characterization (limits of detection and coefficient of variance), and statistics results (PDF)

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R.S. and V.P. designed the work and wrote the manuscript. R.S. performed the immunoassays and metaproteomic analysis. M.M.-P. supervised the immunoassays. F.-P.S. did the metagenomics analysis. V.A.R. and J.M.S.-R. produced the ancestral β-lactamases and thioredoxins. N.C. and L.S.-G. performed fieldwork.

Notes

The authors declare no competing financial interest.

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