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The role of biotechnology in art preservation

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Biotechnology has played a key role in medicine, agriculture and industry for over 30 years and has advanced our understanding of the biological sciences. Furthermore, the tools of biotechnology have a great and largely untapped potential for the preservation and restoration of our cultural heritage. It is possible that these tools are not often applied in this context because of the inherent separation of the worlds of art and science; however, it is encouraging to see that during the past six years important biotechnological applications to artwork preservation have emerged and advances in biotechnology predict further innovation. In this article we describe and reflect upon a unique example of a group of scientists and art restoration technicians working together to study and treat of a piece of colonial art, and review some of the new applications in biotechnology for the preservation of mankind's cultural heritage. We predict an expansion in this field and the further development of biotechnological techniques, which will open up new opportunities to both biologists and artwork preservers

Introduction – La Inmaculada

In the nineteenth century, Alexander von Humboldt, in his account of trips to equinoctial regions of the New Continent, marveled at the high level of culture attained by the settlers in the Province of Venezuela [1]. One of the few remaining artworks of this culture is a woodcarving of the young Immaculate Creole Virgin Mary (La Inmaculada) (Figure 1). This piece was colonized by wood-eating insects and this infestation inspired a joint effort by local scientists to apply biotechnological techniques, normally used in forensics and agriculture, to identify the infectious agent and tailor biological measures to rid the artwork of it [2].

Identifying the biological agents that cause artwork destruction

Apart from pollution, the most common agents causing artwork deterioration are fungi, bacteria and insects. Identification of the agent responsible is a crucial step in developing a treatment strategy for an infected artwork. The genome of an organism contains unique variable polymorphic regions, which differ between individuals and species. Using molecular DNA fingerprinting techniques, such as ribotyping (Box 1), these regions can be used to identify an infectious agent even without previous culturing: a useful property when identifying fastidiously growing anaerobic bacteria or slow-growing fungi. DNA typing, however, cannot tell us whether the sample corresponds to a live or a dead organism; therefore it has to be combined with other technologies, such as detecting ATP consumption [3] or the use of biosensors.

One example of a combined approach, although a previous culturing step was performed, is the molecular identification of *Halobacillus* populations isolated from a biodegraded wall painting from the 14th century Catherin Chapel in the castle of Herberstein, Austria [4]. Other salt-tolerant bacteria, known as halophiles, responsible for the biodegradation of wall paintings have been identified using such molecular techniques [5–7].

In tropical climates many insect species prey on wood and paper and the most common pests are termites of the taxonomical order Isoptera, although other groups, such as Coleoptera (beetles) and Lepidoptera (carpenter worms) are also prevalent. When termites infest an artwork, their rapid growth makes it relatively easy to spot the infestation. However, members of the Anobiidae family (Coleoptera) have long larval periods with associated low metabolic activity and very often, infestations pass unnoticed until, in the words of a conservationist, 'the sculpture crumbles like a saltine cracker.'

Identification of wood-eating insects

Traditional taxonomy requires the morphological analysis of the insect, but procedures to retrieve it can be detrimental to the artwork. In the case of La Inmaculada, we were able to locate the excavated tunnels by using computerized tomography (Figure 2) and recover the insects, which were identified by traditional taxonomy as *Calymmaderus punctulatus* (a member of the Annobiidae family with more than 1100 species described worldwide). Adult *Calymmaderus* are ~3 mm long and 1.5 mm wide and lay their eggs in the cracks in susceptible timbers. A few weeks later, the larvae hatch. The larval stage is

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Figure 1. La Inmaculada: an 18th Century wood sculpture of the young Immaculate Creole Virgin Mary, originating in the Province de Venezuela.

the only time the insect is responsible for destruction of the wood. After a long developmental period (three years on average), the larvae move closer to the surface and construct pupal chambers. Mature larvae pupate and adults emerge, leaving behind a tiny circular hole at the surface. After a few weeks, the adult beetles mate and during this time, the artwork is prone to reinfestation:

Box 1. Identification of organisms by ribotyping techniques

Ribotyping is a common and widely used technique to generate molecular fingerprints. It analyzes the DNA sequences coding for the ribosomal RNA (rRNA) genes (and their flanking sequences), crucial components of the cellular protein-synthesizing machinery. These sequences are used because of their particular evolutionary trend among species. Although minor variations (or ribotypes) do accumulate among species, mutations in a ribosomal gene would have lethal effects and therefore sequence variation is limited. Other regions of the DNA that do not code for particular functions (so called junk DNA) are randomly variable and cannot be used to reliably identify species.

Traditional ribotyping is based on restriction fragment length polymorphism (RFLP) analysis and is typically used for identifying bacteria, fungi and even higher organisms. It uses universal probes, targeting specific conserved domains of the rRNA coding sequences. Following nuclease digestion, the resulting band pattern can be compared with those from known organisms to determine the genetic and evolutionary relationships, if any. Standard ribotyping protocols have been adapted for PCR amplification where the products can be analyzed by RFLP analysis or direct DNA sequencing.

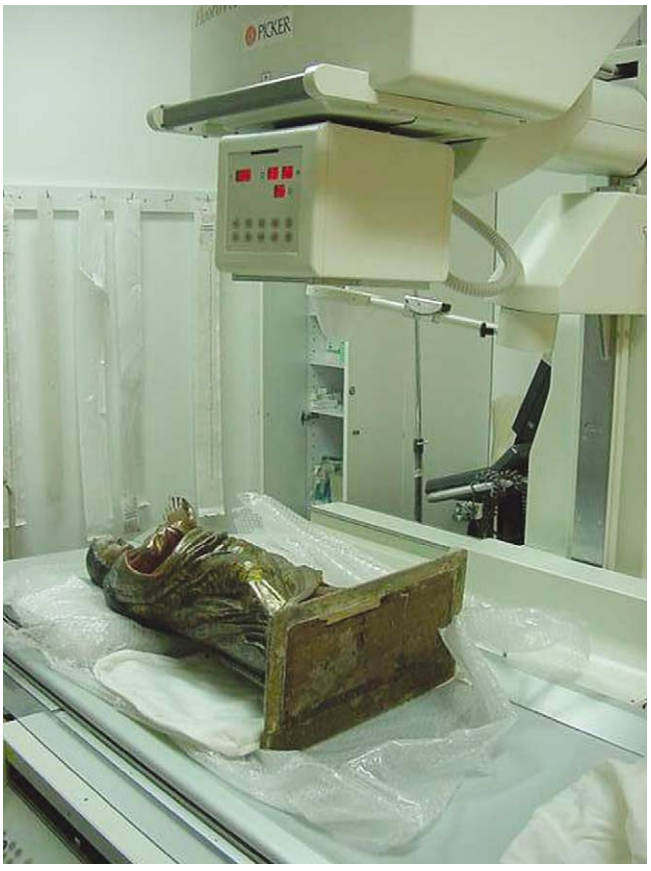

Figure 2. La Inmaculada undergoing tomographic analysis as part of the biorestoration process.

a cycle that can go on until the piece is completely honeycombed.

In parallel to the traditional taxonomy, we were also able to isolate DNA from the beetle, and we sequenced part of its 18S ribosomal genes (GenBank accession no. AY948418). A GenBank search, using this sequence, matched a homologous sequence (96% similarity) from *Ptinus* sp. (GenBank accession no. AF423772), a genus of the Annobiidae family. At the time of writing, no other *Calymmaderus* sequences are available in public databases. Currently, we are creating a ribotype database of wood-feeding (xylophagic) insects that, along with traditional taxonomy, will give us a comprehensive catalogue of the insects commonly affecting artworks. The database can be used to identify these insects using DNA recovered from the epithelial cells present in their droppings or faecal pellets obtained from sawdust samples. This approach is particularly important in cases where the recovery of the insect can damage artwork or when an adult form of the specimen would be needed to complete the identification.

Options to eradicate the infestation

The eradication of woodborer infestations is not a simple task especially in the tropics, where repeated treatments and perseverance are needed. In treating an affected artwork, Hippocrates's medical rule '*primum non nocere*' – 'first, do not harm'– should be followed.

In enclosed museum environments, control of fungal infections is generally accomplished by reducing air humidity; however this approach is not easy to apply in open environments. Because of their high toxicity and the potential to cause harm to the artwork, the use of biocides is not recommended [8]. Furthermore, physical treatments, such as ultrasonic microwaves, electromagnetic shocks of low current, high frequency and high voltage, freezing or heating are not recommended because most paint materials and wood textures can be altered by such treatments.

Two innocuous alternatives to the above are fungicides and bioactive peptides, which are both easily degraded and do not threaten the artwork. Antibiotics and peptides can also be used on bacterial infections.

Although less effective for fungal control, oxygen depletion has proven to be a successful approach for controlling insect infestations in artworks, where appropriate [8]. Despite being harmless, this technique, in its original version, requires cumbersome and costly equipment and long treatment periods. Recently, cheaper soft-case chambers have considerably reduced the cost of this technology [9]. One disadvantage of using oxygen depletion is that some insects (and their eggs) can live for long periods under hypoxic conditions by reverting to anaerobic metabolism. In addition, this treatment does not prevent reinfestations by the same bug.

An alternative to physical or chemical methods is biological control, which is widely used in agriculture to control pests. This method involves the use of either parasitic insects that prey on other insects, entomopathogenic fungi, or bacteria and viruses that kill other bugs. The most commonly used bacterium is *Bacillus thuringiensis* (*Bt*), which produces a repertoire of toxins (Box 2) able to kill more than a thousand insect species [10] and some *Bt* subspecies have been described as effective against termites [11].

Organic agriculture has long benefited from the use of *Bt* spores and the crystal (Cry) proteins that are present inside the sporulating bacteria. Recently, genes coding for *Bt* toxins have been spliced into the genome of a handful of crops, such as soybean, rice, corn, canola and cotton. One disadvantage of this method is that Cry proteins are order-specific toxins, for example Cry1 is specific for Lepidoptera, an order that includes butterflies and other important agriculture pests, but is not effective against other insect groups.

Box 2. How *Bt* toxins kill bugs

The Cry proteins present in *Bacillus thuringiensis* spore cells are aggregates of a large-protoxin protein (~130–140 kDa).The protoxin is highly insoluble in normal conditions making it entirely safe to humans, higher animals and most insects. However, in the reducing conditions and high pH (>9.5) found in the mid-gut of lepidopteran larvae, it is solubilized. Once solubilized, the protoxin is cleaved by a gut protease to produce the active form of the toxin (~60 kD). This toxin, called delta-endotoxin, binds to the mid-gut epithelial cells of susceptible insects and opens cellular membrane pores, which causes a disruption of the ion balance and consequential paralysis of the digestive system. The infected insect stops feeding and the change in the gut integrity allows the bacteria to invade; eventually, the insect dies as a result of starvation and septicaemia.

Application of biological control in biorestoration

Most insects preying on artwork materials belong to the orders Coleoptera and Isoptera. For *Calymmaderus* and other coleopterans, the process of identifying the most effective *Bt* toxins is ongoing, although Cry3 has been used successfully [12]. Two important properties make the application of *Bt* desirable in artwork treatment. First, it produces no detrimental side-effects in the material and is easy to degrade. Second, the use of spores gives residual protection to the artwork, preventing reinfections by the same insect. Spores are resistant to harsh environments and will only germinate when they contact insect larvae. *Bt* spores need a very rich milieu, such as the insect hemolymph, to grow; therefore, it is unlikely that they will proliferate in paper or wood. Although the possibility of toxin resistance cannot be ruled out, the relatively small-scale application and the small population size of insects present in artworks make this possibility remote.

In temperate climates the number of species preying on wood is small, and the application of *Bt* spores can immunize the artwork from future reinfestations by the same group of bugs. In tropical environments, however, insect biodiversity requires constant monitoring. Once the infectious agent has been identified and the most effective *Bt* toxin is chosen, a treatment protocol can be designed. Insects can be inaccessible and traumatic ways of delivering the *Bt* spores and Crys must be avoided. As mentioned above, Anobiidae insects undergo complete metamorphosis following a long larval period, after which they move to the surface to metamorphose into the adult organism. Application of the *Bt* spores in and around the bore holes on the surface will ensure that when the adult bug lays her eggs they will be contaminated with these spores.

Unlike beetles, termites undergo gradual metamorphosis and have a complex social life with nymphs and adults sharing the same habitat and feeding on the same foods. In a similar way to the chemical strategy, termites can be attacked with baits impregnated with *Bt* spores or pathogenic funguses like *Metarhizium anisopliae* [13] and allowed to carry the infection back to the colony.

Until a more permanent eradication program is established, and as a preventive measure, La Inmaculada was treated by passing fumes of Ciflutrine/Diclofluanid (0.10%/0.55%) through the excavated tunnels, previously mapped by tomography, killing some of the *Calymmaderus*. However, before any novel intervention is applied to a precious artwork, models are required. Therefore, pieces of wood infested with different insects are currently the subject of experimental treatment with *Bt* spores to determine how easily bacterial infection spreads, and how efficiently the bugs are killed, compared with other chemical or physical methods.

Biotechnological tools in artwork restoration

Once an artwork or document has been cured of pests, there are two approaches to restoration: the first is restoring the affected parts with materials close to the original ones; and the second is restoring with easy-to-remove materials, which can be taken away later without

compromising the artwork. Alternatively, a final option is to make no intervention and wait for future developments.

Identifying art materials

Throughout history, artists have experimented with new techniques and materials, the origins and identity of which are often unknown to modern restorers. Today artistic expression even includes transgenic animals as an aesthetic proposition (www.ekac.org) [14].

DNA typing techniques can also play an important role in the identification of materials used by the restorer and to customise the preservation treatment. The material sources, such as animal or vegetable glues, fibres, woods and natural pigments can be identified by amplifying minute amounts of DNA using PCR techniques [15]. To illustrate the problem, in the study of materials used in La Inmaculada, three kinds of wood were identified using morphological methods and tomography (Figure 2). One corresponded to *Swietenia macrophyla* (mahogany), an American wood frequently taken to Spain and shipped back as sculptures. The other two were softwoods characteristic of South America's low tropical forests. The presence of these softwoods suggests that this piece was crafted locally, but the exact species of the softwoods have not yet been identified. In combination with traditional wood taxonomy, DNA fingerprinting techniques can help to answer questions, such as what is the species of wood and what is the geographical origin of a particular piece of wood? Despite the limitations of working with dead cells, partially degraded DNA has been successfully isolated from dry wood, artworks, historical objects and plant tissue fossils [16,17]; however, more work needs to be done to improve this technology. The high copy number and stability of chloroplast and mitochondrial DNA make them promising targets for genetic analysis [17]17]. Recently, based on data from chloroplast DNA haplotype studies and molecular and statistical analyses, the geographical origins of oak wood in Europe have been traced [18,19].

The challenges posed during the restoration of the Inmaculada have prompted us to create a molecular database of woods commonly used in tropical regions. This database, in combination with traditional xylographic analysis, can be used in restoration studies, as well as in forensics and archaeology [20].

Other applications of biorestoration

Biorestoration of stone sculptures and monuments

Time and pollution erode the protective skin from stones, thus accelerating the deterioration caused by chemicals in the environment or microorganisms. To reverse the damage, a biomineralization process has been perfected by European art restorers (http://www.ub.es/rpat/bioreinforce/bioreinforce.htm), which is suitable for use on historical monuments. Some non-pathogenic bacteria, such as *Bacillus cereus*, have the natural ability to produce calcium accumulations. These bacteria are cultured and then inoculated onto the affected stone, along with liquid nutrients, and as the bacteria multiply across the surface area of the monument, they accumulate calcium. When the food supply runs out, they die and leave behind a coating of the mineral. This approach was first used to restore the Sain-Médard de Thouras church in France and six years later, the stones are less permeable and the deterioration has stopped. This biomineralization calcite process, patented by the Curie University in 1989, is being used successfully in the biorestoration of many European monuments.

Biorestoration of frescoes

In the restoration of artworks exposed to open environments, such as frescoes, it was common practice to apply organic resins, which were incompletely removed afterwards. These organic residues can serve as a substrate for microorganisms and accelerate the deterioration process. Recently, Ranalli et al. [21] have used soft-biotechnology techniques to eliminate organic material from the frescoe *Coversione di S. Efisio e battaglia*, painted by Spinello Arentino in the 14th century. The biorestoration procedure involves the use of *Pseudomonas stuzeri* to start the degradation of the organic residues, followed by treatment with a cocktail of degrading enzymes (proteases and collagenases) to complete the cleaning process. In a more recent work, the same authors have applied proteomics techniques to determine the enzymatic mechanisms that led to the recovery of the frescoe [22].

Biosensors in artwork preservation

The natural fluorescence of bacteria, fireflies and jellyfish has been applied in the development of light-emitting biosensors with a wide range of applications in medicine, industry, ecology and in the environment [23]. The genes encoding the fluorescent products have been inserted next to promoter regions responsive to either traces of pollutants or the presence of live organisms. These biosensors are sensitive, specific and are widely used to detect environmental contamination, an application that can be extended to artworks. Biosensors can also be used, without prior culturing, to assess whether a given microorganism is alive: a disadvantage of tests that are based on DNA detection alone.

Implications and future directions

The experience with La Inmaculada and the other artworks quoted here, illustrates the potential use of biotechnology in the preservation of our cultural heritage. Biotechnological techniques are powerful, environmentally friendly, low-cost and present low-risk to human health. The successes described here also highlight the need to strengthen the dialogue between the art world and the science world so that biotechnology, in conjunction with other techniques based in physics and/or chemistry, can be routinely applied by practitioners of art restoration. By combining the expertise of each field, we can develop novel techniques to preserve our historical artworks: an important part of the human legacy. This is particularly pertinent in tropical countries where heat, humidity and the diversity of organisms that prey on artworks pose special challenges: a tour through museums in tropical countries can give us an idea of the lack of preservation efforts. The techniques described can be implemented easily in most countries and these applications will open

new horizons in art preservation and provide novel art-oriented careers for young biologists.

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