Microbial Life in the Underworld: Biogenicity in Secondary Mineral Formations

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One unresolved issue in geomicrobiology is the involvement of microbial activity in the formation of secondary mineral deposits, or speleothems, in caves. Although there is extensive literature demonstrating the importance of bacteria in the precipitation of calcite in noncave environments, the role that these organisms play within caves remains unclear. Evidence in support of microbial involvement in deposition of speleothems has often not been compelling. Following the "Rules for the Hunt" first proposed by Schopf and Walter to determine whether structures in rock were biogenic in origin, we propose a similar set of guidelines for evaluation of microbial association with cave features. We also illustrate methods that may help unravel the complex problem of microorganism involvement in secondary mineral deposition in caves.

The geological cave environment, beyond the cave entrance and its associated organic input, is a starved landscape. Without light, organisms necessarily extract energy for life processes from thermodynamically unstable chemical gradients. There is much evidence for rich and diverse chemoautotrophic communities in caves (Angert, Northup, Reysenbach, Peek, Goebel, and Pace 1998; Sarbu, Kane, and Kinkle 1996). It remains unclear however, what role, if any, these communities play in speleothem formation in caves [for the purposes of this article we use the term "speleothem" as defined by Hill and Forti (1997)]. There are claims for the active association of microbes with speleothem formation, such as in moonmilk and corrosion residues, based on microscopic, molecular phylogenetic, and culture-based evidence (Gradzinski, Szulc, and Smyk 1997; Northup et al. 2000; Vlasceanu, Sarbu, Engel, and Kinkle 2000). However, the question remains whether these identified organisms are actively involved in speleothem formation, or simply buried during mineral precipitation (Polyak and Cokendolpher 1992). Indeed, even the biogenicity of mineral-associated, purportedly biological features, can be questionable and extremely difficult to resolve (Schopf 1999). As we undertake to study microbial processes in caves, some of

the tools and concepts required to answer such questions are already documented through studies of questions related to the origins of life on our planet.

The hunt for early microbial life in Precambrian-era rocks has a long and tumultuous history. The first evidence of early life as microfossils was found by Walcott in the ancient sediments of the Grand Canyon, Arizona, almost 100 years ago (Schopf and Walter 1983). This find sparked the race for further evidence of Earth's earliest biosphere, a race to be the first to proclaim when life began on our planet. With limited tools and a less refined philosophy, microscopic and macroscopic artifacts were misidentified and published as fossil evidence (Barghoorn and Schopf 1966; Pflug 1978; Schopf 1975; Schopf and Walter 1980). As the field matured, investigators established methods to identify microfossils with more sustainable interpretations. Beyond the enthusiastic overinterpretation of results of a field in its infancy, another problem with misidentification of structures stemmed from a lack of collaboration between disciplines, as noted by Schopf and Walter (Schopf and Walter 1983):"[Many structures have been misidentified] by biologically unsophisticated geologists and geologically unsophisticated biologists."

It is similarly important that investigators in the young field of microbiospeleology also embrace this interdisciplinary approach. Speleologists, particularly those with a geological background, must remain wary of classifying apparently nonmineral forms as obviously biological.

Rules for the Hunt

The application of electron microscopic methods alone to examine rock samples for evidence of early life led to the misidentification of many objects on the basis of morphology (Schopf and Walter 1983). These misidentified objects can be broken down into two broad groups: those of geological origin and those that are artifacts of the electron microscopy-preparation processes (Table 1). Several structures that appeared biogenic were found to be produced either during normal geologic processes and sample preparation, or are the result of contamination. It therefore appears daunting, with so many sources of potentially misleading objects (see Table 1), to prove that a microorganism or fossil, embedded in or associated with a cave feature, is real. To overcome this problem in paleobiology, Schopf suggested a simple rationale: produce evidence that confirms a biogenic origin of the object (Schopf 1999). Neutral evidence, the simple identification of an object as appearing nonmineralic and therefore biogenic, is inference by default and is likely to generate misleading information. Microscopic evidence alone for the identification of microbes falls into this category. Figure 1 illustrates such a problem. Figure 1A reveals apparent microbial growth, however Figure 1B more clearly illustrates that these strands are extremely small, at the very limit of possible microbial size (Benner 1999). One would have to carry out extensive investigations to convince a sophisticated microbiologist that they represent life.

To accumulate positive evidence for the biogenic origin for structures within rock, five rules were developed by Schopf and Walter, which they term the "Rules for the Hunt." These are five properties that must be known in order to prove, beyond a reasonable doubt, a biogenic origin for a specimen (Schopf 1999). At first, this may seem like a daunting set of rules, but many are satisfied by careful sampling techniques and observation. We have revised the rules into guidelines more applicable to the field of microbiospeleology:

1. ***Provenance***—the source of the sample. Is the sample assuredly from the feature of interest? This ensures the sample is not from another source, such as corrosion residue falling onto a speleothem. It is therefore imperative that a geologically sophisticated investigator is responsible for collection of the sample.

TABLE 1 Objects misidentified in the search for microfossils (adapted from Schopf and Walter 1983)

Source of contamination	Reported object	Interpretation
Geological	Yeast-like microfossils (Pflug 1978)	Nonfossil produced by metamorphically produced multiphase inclusions (Bridgewater et al. 1981)
Geological	Hematitic and geothitic fossilized microorganisms (Marshall et al. 1964)	Tertiary crystallite products of weathering (Muir 1978)
Geological	Menneria, Microtaenia, Archaeosphaeroides microfossils (Lopuchin, Moralev 1973)	Irregular graphitic aggregates (Schopf, Prasad 1978)
Geological	Mineralic spheroid microfossils (LaBerge 1967)	Largely hematitic pseudofossils (Muir 1978)
Preparation	Filamentous and coccoid microfossils (Nagy et al. 1976)	Unconvincing artifacts of preparation (Muir 1978)
Preparation	Alga-like or unicell-like carbonaceous spheroids (Engle et al. 1968)	Probably nonbiogenic floccule-like globules (Schopf, Prasad 1978)
Preparation	Blue-green alga-like filaments (Engle et al. 1968)	Nonfossil contaminants (Brooks et al. 1973)
Preparation	Kakabekia umbellata-like microfossils (Dungworth, Schwartz 1972)	Desiccated, clumped organic matter (Schopf 1975)

2. The in situ *age* of the sample is appropriate. This simply reconfirms the origin of the feature. For example, in Wind Cave, South Dakota, samples of romanechite have been seeded into the system through inclusions from a much older paleocave. Without an understanding of the true source of this material, it would confuse possible interpretations of speleogenesis (Palmer and Palmer, July 1999 personal communication).

3. *Indigenousness* of the microfossil within the sample. This simply refers to whether the microbe or fossil is truly part of the feature, and not a contaminant. Evidence is usually presented by the crystal lattice forming around the object, or a characteristic isotopic signature of the crystals suggesting a biological influence.

4. *Syngenicity* of the fossil within the sample, rather than from the introduction of microbes at a later date. It is important to examine the formation for cracks and microfissures, which may have allowed secondary contamination within the speleothem.

5. *Biogenicity*—is the object associated with the speleothem assuredly biological. The rules associated with obtaining evidence for biogenicity, or the biological origin of an object, are the most pertinent for determining the association of bacteria with secondary cave features.

Biogenicity

Schopf's rules for biogenicity, although aimed at identifying microfossils, are pertinent to organisms found incorporated within speleothems and secondary deposits. Of the nine tests,
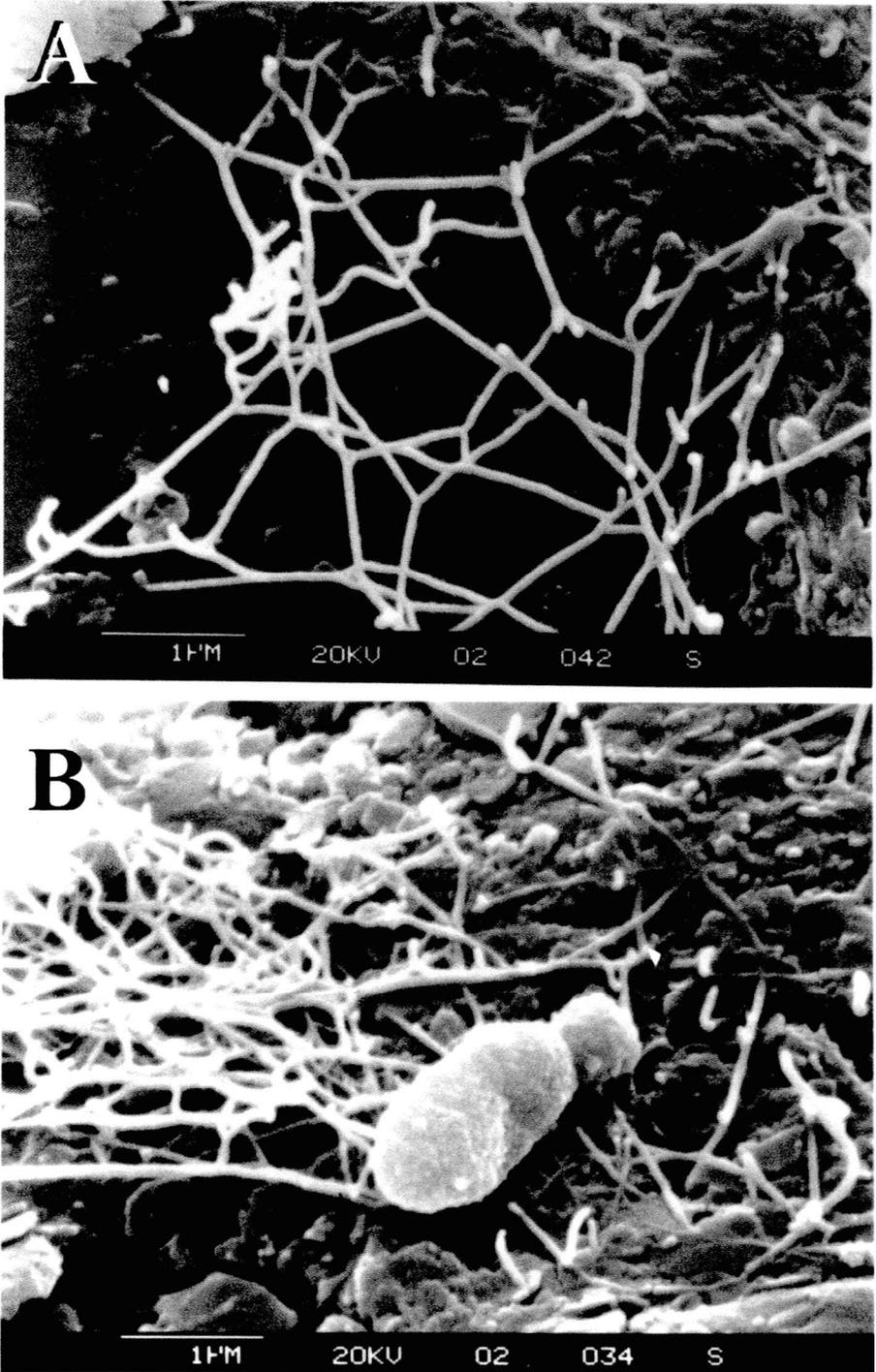
FIGURE 1 Scanning electron micrographs from a deep-sea hydrothermal-vent community. (A) demonstrates how an image can apparently reveal microbial growth. Upon further examination (B), the extremely small size of the strands implies poor evidence of microbial life.

the first can be satisfied by context of the speleothem within the cave environment. Tests two through seven can be satisfied by careful electron microscopic observations, whereas tests eight and nine necessitate additional chemical (mass spectroscopy) tests that augment those of the electron microscopy results. Again, although these tests may seem daunting they are relatively simple to satisfy. It behooves speleologists to incorporate the talents of a number of disciplines, particularly a microbiologically trained eye, to help confirm biogenicity. These criteria ideally must be satisfied to constitute evidence that an object is assuredly biogenic.

1. Found in a livable environment—are there appropriate conditions and sufficient energy sources available that allow biological growth? When answering this question, investigators must take into account the varied environments in which microorganisms are able to survive (Pace 1997).
2. Complex form—do the structures observed contain assuredly cellular structures, such as complex surfaces, septa between cells or motility structures? Figure 2A clearly shows a bacterial cell with a complex surface and distinct septa (indicated by an arrow).
3. Representations by numerous specimens—if one putative organism is identified, then similar forms are expected within the same environment. Rarely does life exist in a solo fashion. Figure 2B shows such multiple cell bodies.
4. Members of a multicomponent assemblage—ecosystems are usually complex, containing more than one species of organism, with the occurrence of multiple and symbiotic interactions possible, such as those seen in Figure 2C. Ecosystems rarely contain mono-morphological populations.
5. Morphological (gene-based) variability—the same species of organism will rarely grow with an exactly identical structure or appearance (Figure 2B).
6. Reproduction by biological means—evidence is present that cell division is occurring, such as through binary fission. Various stages of cell division should be evident in the sample, as seen in Figures 2A and 2D.
7. Exhibition of a range of degradation—consistent with the length of time of incorporation within the speleothem, and thus deterioration of the organism. Deterioration from sample preparation may also be observed, as indicated by the arrow in Figure 2E.
8. Contains organic matter—when an organism becomes trapped within a mineral substrate, death and decay follow. This leaves behind a measurable organic residue (Chafetz 1986).
9. Exhibition of biogenic isotopic features—microscopic organisms obtain inorganic carbon by fixing it in a process that favors the use of $\delta^{12}C$, depleting this isotope from the carbon pool (Burne and Moore 1987). In calcite, this would result in a mineral residue enriched in $\delta^{13}C$ (Sumner 2001).

Whereas biogenic-like structures can form in the absence of life, the same is true for organic molecules. Therefore, the presence of organic molecules alone does not provide evidence of life. It is crucial that as many tests for biogenicity as possible be applied to confirm, without a doubt, a biogenic origin for the sample (Schopf 1999).

Biological-Based Techniques

Schopf and his colleagues developed the Rules for the Hunt to test for the biogenic origin of an object incased in rock more than 3 billion years ago. Speleologists have an advantage over these investigators; they may enter caves and directly examine active microbial involvement in certain speleothem formations, such as dripstone formations, corrosion residues, and moonmilk. Therefore, these investigators may employ a number of biological techniques to augment geologically based methods.
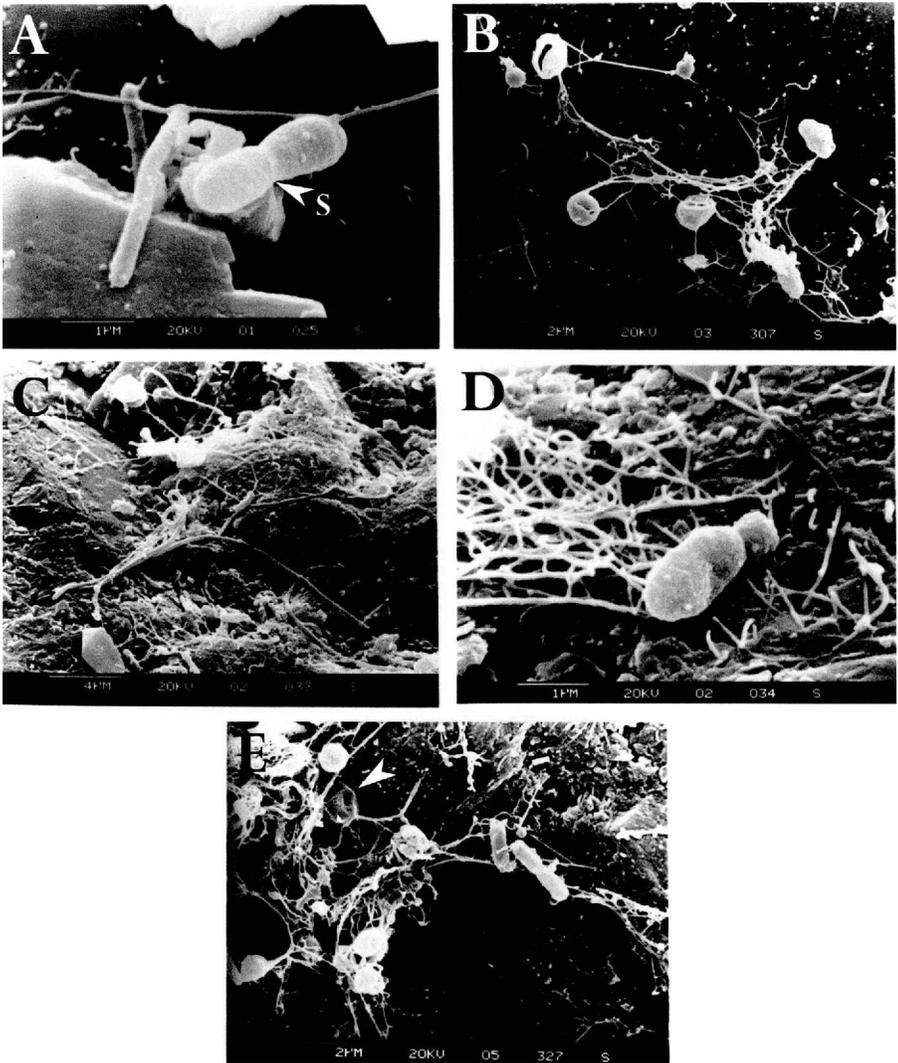
FIGURE 2 Scanning electron micrographs from a deep-sea hydrothermal-vent community illustrating how the "guidelines for biogenicity" can be applied to samples. A demonstrates an organism with "complex form." B shows how multiple specimens of the same organism can be represented and also indicates morphological (gene-based) variability. C displays a multicomponent assemblage within this environment, with rods, cocci, and filamentous organisms present. D, along with A, illustrates different stages of cell division within the sample. E reveals differing states of sample preservation. Degradation of one organism within the group is indicated.

The simplest technique that may be employed is the direct staining of samples from the cave with biological stains. These stains, such as 3,6-bis [dimethylamino] acridinium chloride (acridine orange) and 4′,6-diamidino-2-phenylindole (DAPI) DNA stains (Weinbauer, Beckmann, and Hofle 1998) allow for in situ identification of the number of viable organisms associated with the deposit. Alternatively, direct viable count methods allow a more selective identification of actively growing bacteria (Kogure, Simidu, and Taga 1979). This method relies on the uptake of nalidixic acid in situ by metabolizing organisms, preventing

cell division. Thus, bacteria actively growing in the environment can be identified as they form elongated cells.

Staining techniques are severely limited in that they only demonstrate the presence of viable microbes, without differentiating whether the organism is actively involved in speleothem formation or simply a passive bystander, deposited by air, water, or visitors to the cave. To establish the potential involvement of a microbe in secondary deposit formation, we need to understand the physiology of the organism and determine that this fits the chemistry through which the cave feature is forming; for example, are sulfur-oxidizing bacteria present at the site of gypsum ($CaSO_4$) deposition? There are two techniques presently available to speleologists; cultivation of microorganisms and molecular phylogenetic analysis.

Cultivation techniques remain very selective for the identification of organisms in the environment due to the fastidious nature of the diet of most microorganisms. It is presently estimated that less than 1% of all organisms in an environment can be cultivated using standard techniques, and thus identified by this method (Amman, Ludwig, and Schleifer 1995; Pace 1997). However, best-guess cultivation experiments have allowed for the identification of organisms from the cave environment (Olson and Thompson 1988). Indeed, some investigators have used this approach to demonstrate an active process of bacterial calcite precipitation (Chafetz and Buczynski 1992). It is unlikely, however, that cultivars (cultivated representatives) obtained from a complex environment represent a cross-section of the community present. Maintenance in a "pure" culture, or minimally communal state, is not representative of the natural ecosystem from which the culture was isolated. To better identify these organisms requires the use of molecular phylogenetic techniques.

Molecular phylogenetic studies, based on PCR amplification of genetic signatures from the environment, followed by cloning and DNA sequencing, have allowed organisms from complex systems in the environment to be identified (Barns, Fundyga, Jefferies, and Pace 1994; Dojka, Hugenholtz, Haack, and Pace 1998). Such methods have also been used to identify organisms in cave environments (Angert et al. 1998; Harris, Kelley, and Pace 2001). It has been argued that although PCR amplification allows for the identification of many different organisms within an environment, the inability of these organisms to be cultivated and hence their physiologies to be understood limits the usefulness of this procedure. This is far from the case.

Parker Cave, Kentucky, contains five streams, one of which, Sulphur River is fed by sulfurous-water from the "Phantom Waterfall." Olson and Thompson (1988) examined the sulfur deposits at Phantom Waterfall and determined that these deposits contained elemental sulfur. These investigators further identified the presence of bacteria associated with these deposits, suggesting that sulfur-metabolizing bacteria were involved in this deposition process. Although these investigators made a significant contribution to the field with the first association of bacteria within cave sulfur deposits, their results were limited in demonstrating an active involvement of these organisms in the process of secondary mineral deposition at Phantom Waterfall.

Further studies using molecular phylogenetic techniques by Angert et al. (1998) have expanded on the information obtained by Olson and Thompson (1988). These investigators isolated environmental DNA from Sulphur River, and used the 16s rRNA gene as a genetic marker for the organisms in that environment. By amplifying this gene as a genetic signature, organisms can either be directly identified by their actual DNA sequence, or identified by their similarity to other known sequences in the 16s rRNA gene database [http://www.ncbi.nlm.nih.gov/Taxonomy/tax.html]. Using this technique, Angert et al. were able to demonstrate that the organisms present at Sulphur River were similar to the known species *Thiomicrospira and Thiothrix* spp., which are sulfur-oxidizing bacteria.

The sequences obtained from this environment also demonstrated the presence of chemoautotrophs able to fix carbon dioxide, thus adding another level of understanding to the initial observations of Olsen and Thompson (Angert et al. 1998).

Further studies in Parker Cave by Harris et al. (this laboratory) have taken this process one step further to selectively examine this environment for members of the putative new bacterial division (Kingdom), OP11 (Harris et al. 2001). Even though there are no cultivated representatives of this new division, comparing the environments in which they exist can at least provide an approximation as to their lifestyle. The majority of OP11 clones are found in anaerobic environments, with at least one sulfur compound present, suggesting that members of this group may be involved in sulfur cycling within the environment. At Sulphur River, 58 different representatives of this division were identified, representing a significant proportion of the community present. Along with the work of Angert et al. (1998), this strongly suggests that the microbial communities at Sulphur River primarily utilize a sulfide–sulfate reduction–oxidation cycle as their chemical energy source.

Molecular phylogenetics are, therefore, a powerful tool, allowing identification either directly, or through comparison, of the organisms in a complex community. Speleologists may then use this information to better understand the biochemical processes that are taking place in the formation of secondary cave features; they may then use this knowledge in future attempts to directly cultivate organisms from a particular cave environment. Additionally, fluorescent in situ hybridization (FISH) may be carried out using the gene sequences identified in the PCR amplification (Delong, Wicknam, and Pace 1989). FISH uses short segments of DNA, oligonucleotides <34 nucleotides long, which are labeled with a fluorescent dye such as fluorescein or rhodamine. Fluorescence microscopy then allows for the specific identification, distribution, and morphology of the organism in the environment in question. Using these advanced techniques will allow speleologists to unravel the potentially complex biochemical processes involved in secondary cave features.

Conclusions

At this point in time, we have had only a small glimpse of the complex biological processes that occur in caves (Sarbu et al. 1996). We have no clear idea as to the significance of biological involvement in secondary mineral or speleothem formation, although there are clues. Athough there is no known role for calcium carbonate in bacterial metabolism, certain organisms precipitate calcite during their growth (Buczynski and Chafetz 1990). Organisms such as *Achromatium okaliferum* contain internal calcite inclusions during growth (Head, Gray, Clarke, Pickup, and Jones 1996). There also is an established role for bacteria in the nucleation of calcium carbonate precipitation for stromatalite formation (Ehrlich 1996; Laval et al. 2000). However, although microfossils have been identified in carbonate speleothems, no direct connection with active precipitation processes in the formation of these features has been demonstrated (Polyak and Cokendolpher 1992).

It is likely that the search for the processes that lead to speleothem formation will remain a contested field for several years to come. Progress in the field will depend on cross-disciplinary studies; the abilities of biologists to recognize assuredly biological structures and measure these processes within the cave environment; and geologists, who can apply the complex tools of chemistry and geology to the problem. The continuous history of microfossil research alerts us that it is vitally important that we continue to evaluate results critically, across multiple disciplines, and avoid placing a biological tag on anything that does not appear assuredly mineral.

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