Methods for Observing Microbial Biofilms Directly on Leaf Surfaces and Recovering Them for Isolation of Culturable Microorganisms

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Epifluorescence microscopy, scanning electron microscopy, and confocal laser scanning microscopy were used to observe microbial biofilms directly on leaf surfaces. Biofilms were observed on leaves of all species sampled (spinach, lettuce, Chinese cabbage, celery, leeks, basil, parsley, and broad-leaved endive), although the epifluorescent images were clearest when pale green tissue or cuticle pieces were used. With these techniques, biofilms were observed that were about 20 μm in depth and up to 1 mm in length and that contained copious exopolymeric matrices, diverse morphotypes of microorganisms, and debris. The epifluorescence techniques described here can be used to rapidly determine the abundance and localization of biofilms on leaves. An additional technique was developed to recover individual biofilms or portions of single biofilms from leaves and to disintegrate them for isolation of the culturable microorganisms they contained. Nineteen biofilms from broad-leaved endive, spinach, parsley, and olive leaves were thus isolated and characterized to illustrate the applications of this technique.

It is well documented that leaves of herbaceous and woody plants are colonized by numerous species of bacteria, yeasts, and filamentous fungi (11, 19), but little is known about the relative spatial relationships of these microorganisms at a scale corresponding to their size. Knowledge of the spatial relationships among microorganisms on leaf surfaces is essential to understand their ecology. The mechanisms of antagonism and synergy possible between epiphytic microorganisms and the probability of genetic exchange may depend, in part, on their close physical proximity on the leaf surface. Any behavior depending on autoinduction of gene expression (quorum sensing) would also require close physical proximity of individuals and a matrix for transport of autoinducer molecules among individuals.

In many environments, it is common to find assemblages of microorganisms adherent to each other and/or to a surface and embedded in a matrix of exopolymers (8). These assemblages are referred to as biofilms (6, 8). Scanning electron microscopy (SEM) images of matrix-enclosed microbial assemblages on leaf surfaces (12, 15, 32, 33) have led some authors to suggest that biofilms occur in the phyllosphere (3, 15). In other environments, such as aquatic milieu, where biofilms have been studied extensively, the microenvironment and physiology of biofilm bacteria are often markedly different from those of their planktonic counterparts because of some basic properties of many biofilms. (i) Biofilms are composed of an exopolymeric matrix and multiple layers of microbial cells leading to the creation of physical barriers and the establishment of chemical gradients. (ii) They generally contain multiple species of microorganisms, fostering metabolic and genetic exchange. (iii) Many biofilm cells are attached to a surface (biotic or abiotic, including the surface of other microorganisms or de-

bris in the biofilm), which is requisite for the expression of certain genes. Examples of these properties and their consequences are illustrated in several reviews of biofilm biology and ecology (7, 8, 37). Hence, biofilms in the phyllosphere could have a significant impact on epiphytic microorganisms. However, questions concerning their role in the ecology and physiology of epiphytic microorganisms have not yet been addressed.

No techniques other than SEM have been described for studying biofilms on leaf surfaces. Although SEM has proven useful for studying microbial colonization of leaf surfaces, it can provide only limited information about the nature of phyllosphere biofilms. The fixation and drying steps used in preparation of samples for electron microscopy may destroy or significantly dehydrate the exopolymer matrix of biofilms (9). This may give the impression that microbial assemblages in the phyllosphere consist of a monolayer of cells. Furthermore, SEM gives no information about cell viability and very limited information about identity.

Techniques are needed to help us address some fundamental questions about the nature of microbial biofilms in the phyllosphere: are they common on leaves colonized by naturally occurring microorganisms? Do they have a significant depth, or are they simply a monolayer of cells? Which microorganisms do they harbor, and which microorganisms are associated in the same biofilm? What proportion of the total microbial population on a leaf is assembled in biofilm? To be able to address these questions would allow ecologists to begin determining if microbial biofilms in the phyllosphere have the same properties as biofilms in other environments.

In this paper, we present techniques that can be used to address the first three questions listed above. To observe biofilms with hydrated exopolymer matrices, we used epifluorescence light microscopy and confocal laser microscopy. These images were compared to SEM images by using glutaraldehyde and acetone fixation and dehydration. To isolate and characterize biofilms from leaves, we have developed a technique which permits recovery and disintegration of individual biofilms or portions of single biofilms. In this study, we have

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applied these techniques to vegetables and herbs that may be soft rotted during refrigerated storage, because our preliminary results indicated that cells of *Pseudomonas fluorescens*, a postharvest pathogen, may be present in biofilms in the phyllosphere (20, 25).

MATERIALS AND METHODS

Plant material. Leaves were sampled from commercially produced spinach (*Spinacia oleracea*), Battavian lettuce (*Lactuca sativa*), Chinese cabbage (*Brassica campestris* subsp. *pekinensis*), celery (*Apium graveolens*), leeks (*Allium porrum*), basil (*Ocimum basilicum*), and parsley (*Petroselinum crispum*) purchased at a local supermarket. Leaves of broad-leaved endive (*Cichorium endivia* var. *latifolia*, cultivar *Samy*) were collected from mature plants in experimental field plots cultivated as described previously (18) in Montfavet, France, or purchased at a supermarket. At the time of purchase, all plant materials were fresh and had not been processed (e.g., chopped, packaged, etc.). The supermarket did not employ misting in the produce section. Olive (*Olea europaea*) leaves were collected from trees in a local residential zone. All leaves sampled were apparently healthy. Leaves were transported to the laboratory in clean plastic bags and stored at 4°C until analyzed (within 12 h).

Preparation of plant material for microscopic observation. Leaves of all plants except parsley were cut into squares of approximately 1 by 1 cm. For some leaves of broad-leaved endive, the cuticle was also peeled off. Parsley leaflets were cut off the petioles but were not cut into pieces. For epifluorescence microscopy, leaflets, leaf pieces, or cuticle pieces were soaked in a solution of acridine orange (0.01% in acetate buffer [pH 4]) for 2 min, rinsed twice in distilled water, set on microscope slides, and observed with an Olympus BHS microscope (Olympus Optical, Co., Tokyo, Japan; light source model BH.RFL.W) under UV light filtered through a blue filter. For all plants except broad-leaved endive, two leaf squares (or whole leaflets in the case of parsley) were observed per species. For broad-leaved endive, leaf pieces from about 20 different leaves were observed.

For confocal laser scanning microscopy, cuticles were peeled off leaves of broad-leaved endive, fixed for 10 min in 2.5% paraformaldehyde, stained for 10 min with propidium iodide (5 µg of propidium iodide per ml diluted in 1% phosphate-buffered saline–Triton X-100), set on microscope slides, and covered with coverslips that were fixed in place with nail polish. Observations were made with a Zeiss confocal laser scanning microscope (Plan-Neofluar and Plan-Apochromat oil objective lenses) equipped with two lasers (helium-neon and argon) and controlled by internal software (Zeiss LSM software).

For SEM, leaf pieces were degassed (1 h at 1 bar), fixed in 4% glutaraldehyde sodium cacodylate buffer (0.07 M) for 4 h at 4°C, rinsed three times in buffer (0.1 M sodium cacodylate, 3% glucose, pH 7.3), postfixed in 1% OsO₄ (2% OsO₄–0.1 M sodium cacodylate [1:1]) for 3 h at 22°C, and rinsed five times in double-distilled water. Samples were dehydrated in graded acetone baths (30, 50, 70, and 4 × 100%) followed by graded hexamethyldisilazane baths (25:75, 50:50, 72:25, and 3 × 100:0%, hexamethyldisilazane–acetone) of 15 min each. Samples were fixed on metal supports and sputter coated with gold (10 nm) (MED 010; Balzers Union Limited, Liechtenstein). Leaf surfaces were observed with a Philips XL30 scanning electron microscope (Philips Electronics N.V., Eindhoven, The Netherlands).

Isolation of microorganisms from biofilms. Epiphytic microorganisms were transferred to the surface of sterile agar media by one of two methods. For the first method, a direct impression of the upper surface of the leaf was made on an agar medium in a petri dish. For the second method, individual leaves were gently washed in 100 ml of buffer (per liter: 6.75 g of KH₂PO₄, 8.75 g of K₂HPO₄ [pH 7]). Flasks containing the buffer and leaf were gently rotated manually for 3 min. To eliminate small debris and the majority of isolated microbial cells, the washing solution was filtered across a polypropylene filter with pores 25 µm in diameter (Millipore, Saint-Quentin-en-Yvelines, France). The bacteria collected on the filter were gently resuspended in 5 ml of buffer. One- to 2-ml aliquots of serial dilutions of this suspension were deposited on a sterile agar surface (10-fold-diluted tryptic soy agar [TSA]; tryptone, 1.7 g; Bacto Soytone, 0.3 g; glucose, 0.25 g; NaCl, 0.5 g; K₂HPO₄, 0.25 g; agar, 15 g/liter). Observations of the plates were made after the liquid was absorbed by the agar (after 1 to 3 h).

The surface of the agar was observed under phase-contrast microscopy with an Olympus BHS light microscope. Biofilm-like particles were collected with a Pasteur pipette sampler by a method similar to single-spore isolation of fungi. Amorphous particles that projected above the plane of the agar surface were considered to be biofilms. They had no sharp angles and had a granular texture; numerous particles the size of bacterial and yeast cells could often be observed within the biofilm. Prior to collection of the biofilm, we verified that the field of view containing the biofilm and the zone within a 1.5-mm radius of the biofilm were free of other particles. If these conditions were not met, the biofilm was not collected. To recover the agar in the desired field of view, the stage was lowered and the light was focused to a pinpoint. The sampler, a bent sterile Pasteur pipette (orifice diameter, 1.5 mm), was pierced through the agar at the site of the light, and the agar was recovered by gentle aspiration. The agar plug was then transferred to a capped sterile 1.5-ml microtube. Plugs of agar from particle-free zones of agar were collected as controls to demonstrate that the microorganisms

recovered from biofilm-like particles were not contaminants of one of the isolation steps.

To disintegrate biofilms, the agar plugs were ground in 100 µl of sterile buffer with a sterile plastic pestle adapted to the microtubes. This suspension was transferred to a Duall tissue grinder (Konté Scientific Glassware, Vineland, N.J.) for additional grinding. An aliquot of 2 ml of buffer, used to rinse the plastic and glass pestles and the microtube, was added to the suspension in the tube of the Duall grinder after grinding. After mixing, all of the suspension in the grinder was plated out on 10 to 20 plates of diluted TSA.

Characterization of microbial components of biofilms. Total microbial colonies were counted on plates after 7 to 10 days of incubation at 25°C. Because bacterial and yeast colonies could not be distinguished on the basis of colony morphology, cells from bacterium- and yeast-like colonies were observed microscopically. Yeasts were not characterized further, but bacterial colonies were purified for further analysis. For biofilms having less than 300 bacterial CFU, all bacterial colonies were collected and purified. For larger biofilms, all bacterial colonies in randomly selected sectors of several plates were collected and purified until a minimum number (50, or 2 to 3% of the total, whichever was larger) of isolates was obtained. Purified bacterial isolates were characterized for their Gram reaction by the KOH technique (31), oxidase reaction (14), and production of fluorescent pigments on King's medium B (21). Filamentous fungal colonies were observed for spore production and identified to the genus level (1).

RESULTS

In situ observation. Aggregates of microorganisms embedded in an exopolymeric matrix were observed on all leaf material stained with acridine orange and examined by epifluorescence light microscopy, including broad-leaved endive (Fig. 1), spinach, lettuce, Chinese cabbage, celery, leeks, basil, and parsley. Epifluorescence microscopic images of biofilms on broad-leaved endive were of the best resolution either when the cuticle was peeled from the leaf surface and mounted under a coverslip (Fig. 1A) or when biofilms were located on flat, etiolated portions of the leaf. For leaves of the other plants observed, the cuticle could not easily be removed, and there was often bright background orange or red coloration of the leaf tissue, which made it more difficult to observe and photograph the biofilms compared to those on broad-leaved endive. Biofilms were observed on all parts of the leaves (margin, base, and upper and lower surfaces). In the case of broad-leaved endive, we observed about 100 biofilms. These biofilms were found most often at the leaf base and were rarely on the margin of green leaf tissue. About 90% of them were attached to trichomes (Fig. 1A and C).

Pieces of cuticle peeled from leaves of broad-leaved endive and mounted under glass coverslips were observed with confocal laser scanning microscopy. False-color images of biofilms stained with propidium iodide were created to indicate the location of microbial cells as a function of the depth of the biofilm (Fig. 3). Zones of biofilms containing bacteria in the same vertical plane were as deep as 25 µm for the biofilms on broad-leaved endive observed in these samples.

SEM examination of leaf surfaces confirmed that biofilms could be readily observed on naturally contaminated leaf surfaces. Biofilms observed by SEM were as large as 1 mm in length (Fig. 2) or were in networks extending for several millimeters and were composed of a copious exopolymer matrix and numerous microbial morphotypes resembling bacteria, filamentous fungi, and yeasts as well as debris-like particles.

Isolation and characterization. Transfer of microorganisms to agar surfaces by direct impression of leaves on agar media resulted in dense zones of particles with no fields of view containing biofilms well separated from other particles. In contrast, biofilms of epiphytic microorganisms were successfully isolated from leaf washings. Leaf washings contained numerous biofilms as revealed by acridine orange staining of aliquots of the washings (Fig. 1B and C). After plating of filtered and diluted leaf washings, biofilms were isolated from broad-leaved endive, parsley, spinach, and olive leaves. Here, we report the

FIG. 1. Epifluorescence micrographs of naturally occurring biofilms stained with acridine orange on excised cuticle strips (A) or in leaf washings (B and C) of broad-leaved endive (magnification, $\times 500$). A mucilaginous matrix enrobing the microbial cells is apparent in panel B. Biofilms on broad-leaved endive were often attached to trichomes (arrows in panels A and C).

FIG. 2. Scanning electron micrograph of a naturally occurring biofilm on broad-leaved endive. Bar, 200 μm .

characteristics of biofilms from which more than 50 CFU were isolated (Table 1). A total of 30 biofilm-like particles were isolated from leaf washings. From 19 (63%) of these, over 50 CFU were recovered; from the other 11 particles, less than 25 CFU were recovered. Grinding and plating of five agar plugs collected from apparently particle-free zones of the agar plates yielded 0, 1 fungal, and 2, 3, and 11 yeast- or bacterium-like culturable CFU, respectively.

The biofilms isolated had a length of between 30 and 445 μm and harbored 66 to over 4,000 culturable CFU (Table 1), but there was no relationship between their apparent size and the number of CFU recovered. For seven of the biofilms, filamentous fungi (*Penicillium* spp. and *Cladosporium* spp.) accounted for over 70% of the total CFU isolated. These biofilms were given the code "X" (Table 1) and were not characterized further. The other 11 biofilms contained principally bacteria and were given the code "MC" (Table 1). Additional details about the composition of these latter biofilms are presented below.

All MC biofilms characterized contained both gram-negative and gram-positive bacteria (Fig. 4). Gram-positive bacteria constituted 10 to 66% of the total culturable CFU (Fig. 4) and from 11 to 78% of the bacterial CFU of the biofilms characterized. With the exception of biofilms MC10 and MC11, which apparently contained a single type of gram-negative bacterium, all other biofilms contained diverse gram-negative bacteria based on cell and colony morphology. These bacteria included oxidase-positive fluorescent pseudomonads for seven of the biofilms (MC01, MC02, MC06, MC08, MC09, MC13, and MC14), which accounted for 4 to 32% of the total bacterial component of these biofilms. All of the fluorescent pseudomonads isolated from biofilms were oxidase positive. Six of the biofilms characterized contained yeasts accounting for 1 to 20% of the total culturable CFU, and six contained filamentous fungi (*Penicillium* spp. and *Cladosporium* spp.) comprising 0.5 to 50% of the total CFU (Fig. 4). Some of the biofilms also contained bacteria that were difficult to maintain in culture, including one nocardioform-like organism.

DISCUSSION

The microscopic techniques presented here allowed us to readily observe biofilms on leaves of numerous plant species.

The use of light microscopy to observe microorganisms in their native state on leaf surfaces has been reported previously. Attempts have been made to bleach leaves (10) or to cast epiphytic microorganisms in varnish and then peel them from the leaf surface (13) to permit transmission of light for bright-field microscopy. However, these techniques have not been widely used and have not led to reports of biofilms on leaf surfaces. Light microscopy and confocal laser scanning microscopy in conjunction with fluorochromes are frequently used for the study of biofilms in other environments (5, 29, 36). However, they are most commonly used to examine biofilms on

FIG. 3. False-color image from confocal laser scanning microscopy of a naturally occurring biofilm on broad-leaved endive stained with propidium iodide and observed with a Plan-Apochromat 63 $\times$ /1.25 oil objective lens. The color of the microbial cells indicates their depth in the field of view (bar, 10 μm). This biofilm has a zone about 18 μm thick.

TABLE 1. Size and composition of biofilm-like particles isolated from leaf surfaces

Code ^a	Mo of isolation	Plant	Size (μm) ^b	No. of CFU		
				Total	Fungal	Bacterium- and yeast-like ^c
X01	January	Broad-leaved endive	40	83	59	24
MC06	January		40	131	53	78
X02	July		80	324	281	43
MC02	April		100	245	1	244
X04	January		180	198	191	7
MC01	May		445	150	1	144
MC07	February	Spinach	64	148	74	74
X05	February		68	99	99	0
X06	February		68	86	83	3
MC08	February		80	66	21	45
X08	February		128	71	71	0
MC09	February		190	66	15	51
X09	January	Parsley	30	205	203	2
MC14	March		60	4,358	0	4,358
MC13	March		100	1,763	0	1,763
MC12	March		160	3,146	0	3,146
MC11	March	Olive	44	3,206	0	3,206
MC10	March		48	856	0	856
X11	March		60	126	0	126

^a Codes preceded by MC were given to biofilms whose components were characterized further than for the traits presented in this table. The results of this characterization are presented in Fig. 4. Codes preceded by X were given to biofilms that were not characterized further.

^b Maximum dimension of the biofilm captured from the surface of agar media.

^c These values are based on plate counts of CFU before the microscopic observation of cells, which was necessary to distinguish bacterial and yeast colonies.

inorganic (e.g., stainless steel) or abiotic (e.g., plastics, membrane filters) surfaces. In a previous report about the use of fluorescent stains to observe bacteria on leaf surfaces, Hossel and Baker (16) found that all the stains tested were unsatisfactory for use on leaves of water plants such as *Ranunculus* spp. and *Lemna* spp., due, in part, to fluorescence of residual and absorbed stain and to autofluorescence of the leaf. The method we report here gave the best results for light green or white leaves or leaf parts, i.e., when chlorophyll content was apparently low or when the cuticle was peeled from the leaf surface.

The techniques reported here permitted us to illustrate that biofilms in the phyllosphere may be tens of micrometers thick, may have a copious exopolymeric matrix, and may form extensive networks several millimeters long. Bacterial aggregates in the phyllosphere have been observed previously with SEM (2, 11, 15, 27, 30, 32, 33), but most have been very small (less than 20 μm long) or have lacked an obvious exopolymeric matrix (2, 27, 30, 32, 33). Although sample preparation for SEM may lead to the dehydration of biofilm matrices (9), we have, nevertheless, succeeded in using SEM to observe large biofilms with an abundant exopolymeric matrix. We suggest that this is due, in part, to our use of naturally contaminated plant material. The majority of SEM images of bacteria on leaf surfaces depict single isolates that have been cultured in the laboratory and inoculated on greenhouse- or growth chamber-grown plants (2, 24, 27, 30, 32, 33). These conditions might not be optimal for biofilm formation or may lead to the formation of an exopolymeric matrix that is not copious or complex enough to resist fixation and drying. It should be noted that the largest biofilms (60 to 80 μm long) with the most extensive exopoly-

meric matrix in previously published SEM images are on naturally contaminated plant material (12, 15). Here, we used SEM only as a basis of comparison with previously reported images and with our fluorescence microscopy images. The recently developed environmental scanning electron microscope may eventually replace conventional SEM for studies of microbial colonization of the phyllosphere. However, our experience indicates that the origin and type of leaf material examined may have an important effect on the extent to which biofilms can be observed in the phyllosphere.

The isolation technique described in this study allows for collection of a wide range of bacteria and other microorganisms coexisting in close physical proximity. The most widely used techniques for isolating bacteria, for example, from leaf surfaces—washing, grinding, or stomaching of leaves (19)—result in the loss of information about relative localization of the bacteria isolated. Using the technique presented here, we have illustrated that diverse microorganisms, including both gram-positive and gram-negative bacteria as well as yeasts and filamentous fungi, may inhabit the same biofilm in the phyllosphere as has been observed in other environments (4, 22, 28). This preliminary characterization of the strains collected with this technique could be complemented with tests for phenotypic properties related to presumed metabolic and catabolic activities in the environment (20a) or clonal relatedness and plasmid profiles (20). This type of analysis will lead to an understanding of microbial functions and interactions in biofilms in the phyllosphere and the factors resulting in the community composition observed within these biofilms. Such questions have not yet been addressed for phyllosphere microorganisms.

Several important points should be considered when using the isolation technique described here. First, although we observed individual biofilms up to 1 mm long and networks of biofilms extending for several millimeters, the biofilms captured on agar were frequently only 50 to 100 μm long. Liberation of biofilms from leaf surfaces by washing probably has limited efficiency as a means of isolation and likely leads to breakage of large biofilms into smaller pieces. However, isolation of biofilms by direct impression of leaves seems impractical, because biofilms were often in zones of dense debris and other particles, including fungal spores, etc. Second, in this study, we collected strains only from biofilm-like particles that

FIG. 4. Taxonomic composition of naturally occurring biofilms isolated from broad-leaved endive (MC01, -02, and -06), spinach (MC07 to -09), olive (MC10 and -11), and parsley (MC12 to -14). Each biofilm was ground in buffer to liberate individual microbial propagules and the proportions of gram-positive bacteria (▨), nonfluorescent gram-negative bacteria (□), fluorescent pseudomonads (▤), filamentous fungi (▥), and yeasts (■) were determined for the total culturable CFU recovered.

contained several times more CFU than were obtained from apparently particle-free zones on the agar plates. Although it is possible that the CFU collected from particle-free zones originated from biofilm fragments not visible at the magnification used here, we felt that it was safest to assume that these CFU represented contaminants or nonaggregated microorganisms. Third, biofilms may contain microorganisms that are not culturable or grow poorly on synthetic media (23) or whose viability is destroyed upon disintegration of the biofilm. Some of the bacteria isolated in this study did not survive successive transfers and storage. Hence, the diversity of organisms within single biofilms described with this technique gives only a partial picture of the actual diversity within naturally occurring biofilms on leaf surfaces. For other environments, use of culture media for isolation and detection of biofilm components resulted in diversity profiles that were different from those determined with probes used *in situ*. For example, the relative proportions of five taxonomic groups of bacteria found in biofilms in wastewater treatment plants determined with rRNA-targeted probes *in situ* differed markedly from the proportions determined with the same probes after the bacteria in these biofilms were isolated on culture media (23). It would be possible to modify our isolation technique by using molecular probes just after the disintegration step, hence avoiding culture of the microorganisms on synthetic media.

The techniques we describe for observing and isolating biofilms from leaf surfaces provide tools for microbiologists to pose new questions about the ecology of phyllosphere microorganisms. First, with these techniques it can be rapidly determined if biofilms readily form on leaves of other plants and at what sites they are apparently most abundant. This will lead to questions concerning the environmental factors that favor biofilm establishment in the phyllosphere. Also, staining of epiphytic microorganisms directly on leaf surfaces with acridine orange or other fluorescent indicators of metabolic or respiratory activity may permit, for example, studies of the role of biofilms in the resistance of epiphytic bacteria to pesticides. This is one of many important questions concerning the ecology of epiphytic plant pathogenic bacteria. Similar techniques have revealed the role of biofilms in the response of bacteria to disinfectants used in other environments (17, 35). The isolation technique we describe here will allow phyllosphere ecologists to determine which microorganisms are frequently associated in biofilms. This may lead to the identification of saprophytic organisms that play a role in plant disease processes incited by phytopathogenic organisms with which they are associated or may help to explain mechanisms of genetic exchange observed on leaf surfaces. Use of the tools described here may lead us to reconsider some of the current models of the function and interaction of microorganisms in the phyllosphere. The models that have been proposed for nutrient competition among epiphytic bacteria (26, 34), for example, are probably too simplistic in light of the potential metabolic exchanges and recycling of dead microbial cells that could occur within biofilms. Characterization of the microbial components of individual biofilms in the phyllosphere may lead to new hypotheses about trophic interactions or chemical communication among these organisms.

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