Chemical and structural characterization of exopolymers produced by *Pseudomonas* sp. NCIMB 2021 in continuous culture

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The growth of marine *Pseudomonas* sp. NCIMB 2021 as continuous cultures in the presence of surfaces of AISI 316 stainless steel allowed the isolation and partial chemical characterization of exopolymers released into the culture medium (free exopolymers), as well as capsular and biofilm exopolymers. Fourier-transform infrared (FTIR) spectroscopy demonstrated the presence of O- and N-acetylation within the carbohydrate moieties and a predominant 3_{10} -helical structure of the protein component, highly resistant to hydrogen/deuterium exchange. Differences between the exopolymers were apparent. Relatively less uronic acid residues were detected in the capsular exopolymers compared to either the biofilm or free exopolymers. O- and N-acetylation were greatest in the biofilm exopolymer. SDS-PAGE protein profiles confirmed differences between exopolymers. The secondary structures of proteins determined using FTIR spectroscopy indicated that the capsular exopolymer had reduced helical content and an increased aggregated strand content compared to the biofilm exopolymer. However, the free exopolymer had an increased β -sheet component and a reduced unordered component when compared to the biofilm and capsular exopolymers. These data suggest that exopolymer chemistry varies with cellular mode of growth.

Keywords: *Pseudomonas* sp. NCIMB 2021, extracellular polymeric substances, capsule, biofilm, FTIR

INTRODUCTION

Bacteria produce a wide range of extracellular polymeric substances (EPS) composed of polysaccharides, proteins, nucleic acids and lipids. EPS are often classified as capsules, sheaths or slimes dependent upon their proximity or attachment to the cell wall (Beveridge & Graham, 1991). Such classifications take no regard of the chemistry of the EPS, despite the fact that they may have markedly different functions. A polymer responsible for irreversible cell adhesion to a surface is likely to contrast with EPS which forms a protective gel-like matrix around attached cells, and the respective structures may reflect these differences (Allison & Sutherland, 1987; Neu & Marshall, 1991; Allison *et al.*, 1991).

It is becoming clear that planktonic (suspended) and biofilm (sessile) bacterial cells may be phenotypically distinct (Dagostino *et al.*, 1991; Davies *et al.*, 1993; Hoyle *et al.*, 1993; Vandevivere & Kirchman, 1993). The change upon adhesion to surfaces is precipitated by a σ -factor acting to derepress inactive genes (Deretic *et al.*, 1994; Martin *et al.*, 1994). Phenotypic differences in *Pseudomonas aeruginosa* are particularly evident, with the up-regulation of alginate production following adhesion (Davies *et al.*, 1993; Hoyle *et al.*, 1993). In any system with sufficient nutrients for bacterial growth, biofilms will predominate (Geesey *et al.*, 1978), with all the attendant advantages and disadvantages to man. It is therefore important to understand the biochemical transformation that cells undergo following adhesion since assumptions based upon planktonic cells may be misleading.

Whilst the chemical characterization of EPS is not novel, the exopolymers in question are invariably either

Abbreviations: EPS, extracellular polymeric substances; FTIR, Fourier-transform infrared.

released into the culture medium ('free' exopolymer), or associated with cells attached to a substratum (e.g. Williams & Wimpenny, 1977; Christensen *et al.*, 1985; Abu *et al.*, 1991; Robijn *et al.*, 1995). During a programme of research investigating the influence of the biofilm formed by marine *Pseudomonas* sp. NCIMB 2021 on the deterioration of AISI 316 stainless steel and the possible role of exopolymers in this process, the use of a continuous-flow bioreactor, with a large surface area potentially available for colonization (1600 cm²), allowed the study of the three types of EPS produced: capsular exopolymers, those constituting the biofilm matrix (biofilm) and those released into the culture medium (free). Here we report on the chemical and structural differences between the three types as established by SDS-PAGE protein profiles and Fourier-transform infrared (FTIR) spectroscopy.

METHODS

Organism. A strain of the marine isolate *Pseudomonas* sp. NCIMB 2021 was grown and maintained as batch cultures at 25 °C in an artificial seawater-based laboratory-designed medium (l⁻¹: 1.54 g CaCl₂, 11.1 g MgCl₂, 24.53 g NaCl, 0.1 g KBr, 0.017 g SrCl₂, 0.003 g NaF, 0.7 g KCl, 0.03 g H₃BO₃, 4.09 g Na₂SO₄ and 0.2 g NaHCO₃ in double-distilled water) containing (l⁻¹): 2 g tryptone, 2 g Casamino acids, 0.1 g glucose and 2 ml vitamin solution (l⁻¹: 0.5 mg nicotinic acid, 0.6 mg B₁, 0.05 mg B₁₂, 0.2 mg B₂, 0.6 mg B₅, 0.6 mg B₆, 2 mg C and 0.001 mg H) and 1 ml trace element solution [1.5 g nitrilotriacetic acid l⁻¹, adjusted to pH 6.5 with 1 M KOH, plus (l⁻¹): 3 g MgSO₄, 0.5 g MnSO₄, 1 g NaCl, 0.1 g FeSO₄, 0.1 g CoSO₄, 0.1 g NiCl₂, 0.1 g CuCl₂, 0.1 g ZnSO₄, 0.01 g CuSO₄, 0.01 g AlK(SO₄)₂, 0.01 g H₃BO₃, 0.01 g Na₂MoO₄ and 0.001 g Na₂SeO₃, adjusted to pH 6.5–7.0, filter-sterilized (0.2 µm pore size filters; Millipore) and stored at 4 °C]. A 4.5 l continuous-flow bioreactor was designed to study exopolymer production by *Pseudomonas* sp. NCIMB 2021 in the presence of AISI 316 cold-rolled 1600 grade stainless steel surfaces (Rightons). The computer-controlled bioreactor accommodated a sterile air purger, temperature probe and pH and O₂ electrodes. Sterile stainless steel coupons (total area 1600 cm²) were aseptically introduced into the bioreactor and held vertically in PTFE holders. The bioreactor was run supplied with sterile growth medium (1 ml min⁻¹) for 24 h in order to confirm the maintenance of sterile conditions, after which it was inoculated with 40 ml of a 5-d-old batch culture of *Pseudomonas* sp. NCIMB 2021 grown as described above. A magnetic propeller provided slow agitation of the medium and sterile air was supplied via an in-line 0.2 µm membrane filter (Millipore).

Recovery of EPS. After 36 d operation, the bulk phase was removed from the bioreactor and the planktonic cells harvested using centrifugation (10000 g for 30 min, 4 °C). The resultant supernatant was dialysed for 24 h against running tap water using 10 kDa molecular mass cut-off dialysis membrane (Fisher Scientific) at 4 °C, followed by 48 h extensive dialysis against double-distilled water, again at 4 °C. The exopolymers (termed 'free' EPS) were lyophilized at -60 °C and stored at -20 °C. The cells removed by centrifugation from the bulk phase were gently suspended in 200 ml isotonic TE buffer (10 mM Tris/HCl pH 8, 10 mM EDTA) containing 2.5% NaCl and incubated overnight at 4 °C, after which the cells were removed from the capsular

EPS by centrifugation as described previously. Stainless steel coupons were removed from the bioreactor aseptically and placed in TE buffer as before. Biofilm material was recovered from the coupons by scraping with a sterile, blunt-tipped poly(propylene) automatic pipette tip (Gilson). The biofilm suspension was centrifuged and dialysed as stated above to obtain biofilm EPS.

Partial purification of EPS. To eliminate confusion of carbohydrates associated with polysaccharides from those present in nucleic acids (i.e. ribose and deoxyribose), 6 mg of each lyophilized EPS sample was resuspended in sterile distilled water and incubated for 24 h at 37 °C with RNase and DNase (Boehringer Mannheim) at a final enzyme concentration of 20 mg ml⁻¹, buffered with 10 mM NaCl, 10 mM Tris/HCl pH 8. Following digestion, the samples were extensively dialysed against double-distilled water at 4 °C. All samples were lyophilized and stored at -20 °C until use.

Characterization of EPS. Colorimetric assays were employed to determine the soluble neutral carbohydrate (Chaplin & Kennedy, 1986) and protein (Bradford, 1976) contents of the EPS samples.

SDS-PAGE of EPS was performed on 10% (w/v) acrylamide slab gels with a discontinuous buffer system (Laemmli, 1970). A constant current of 40 mA was applied until the bromophenol blue tracking dye front reached the bottom of the gel. Low-molecular-mass markers (Bio-Rad) were employed as protein standards. Coomassie brilliant blue R-250 staining procedures were conducted as described by Dzandu *et al.* (1984).

For FTIR spectroscopy, samples were prepared as 15 µg ml⁻¹ solutions in distilled, deionized water (Elgastat UHQ II, 18 MΩ cm resistivity at 25 °C). A 7.5 µl aliquot of each sample was then layered onto the surface of a gold-coated glass slide (1000 Å Au over 20 Å Cr) with a glass spreader under a stream of dry N₂ until a dry film was formed. Prior to sample application, slides were degreased by sonication with Decon90 (Decon Laboratory) followed by sonication in concentrated HNO₃ and then 5 mM NaOH, resulting in a hydroxyl-rich surface. Measurement of spectra of dried samples layered onto such surfaces has been shown to conserve polymer secondary structure whilst avoiding the problems inherent in studying aqueous solutions (Kossovsky *et al.*, 1994). For the hydrogen/deuterium (H/D) exchange, samples were dissolved in deuterium oxide (D₂O > 99.9%; Goss Scientific Instruments) for 12 h, lyophilized and the cycle repeated three times.

Single-channel reflectance-absorbance FTIR spectra were obtained at an angle of incidence of 70 ° using a Bruker IFS 113v FTIR spectrometer (Bruker) equipped with a narrow-range, liquid-N₂-cooled, Hg-Cd-Te (MCT) detector. The spectra were recorded at a resolution of 4 cm⁻¹. The optics chamber, which housed the grazing-angle attachment (Graseby Specac), was evacuated for 3 h prior to the collection of spectra. This procedure ensured minimal spectral interference from atmospheric CO₂ and water vapour. This, coupled with the use of D₂O, enabled a better understanding of the spectral features associated with the amide I band, which would otherwise have been obscured by the absorbance (around wavenumber 1650 cm⁻¹) associated with the bending mode of H₂O (Hernandez & Kalaji, 1996). A total of 1000 interferograms were averaged for each sample, apodized using a triangular function, zero-filled with a factor of 2 and Fourier-transformed. For curve fitting in the amide I region, derivative spectroscopy [second derivative, 9-point Savitzky-Golay-smoothed (Savitzky & Golay, 1964)] and Fourier self-

deconvolution (Rahmelow & Hübner, 1996) were employed. Curves were fitted using a Levenberg-Marquardt algorithm employing linear combinations of Lorentzian and Gaussian line shapes.

RESULTS

All samples used for the isolation of EPS material were inspected to determine whether the cells remained intact throughout the procedure. No lysis of cells was observed, indicating a lack of EPS contamination with cellular material. To determine whether cell lysis was taking place, two independent methods were employed. The first approach involved light microscopy observations using total enumeration technique (counting chamber) prior to and following EDTA extraction. There was no significant difference in cell number between treatments, indicating that no bursting or dissolution of cell walls occurred. Following enumeration, aliquots of the samples were deposited on glass and 10 randomly chosen areas on each sample were viewed using atomic force microscopy (AFM), as described by Tapper (1998). No apparent distortions in

Fig. 1. SDS-PAGE profiles of proteins present in exopolymers produced by *Pseudomonas* sp. NCIMB 2021 under continuous growth conditions. Lanes: 1, free EPS; 2, biofilm EPS; 3, capsular EPS; 4, molecular-mass markers.

Fig. 2. Reflectance-absorbance FTIR spectra of exopolymers produced by *Pseudomonas* sp. NCIMB 2021 under continuous growth conditions. The spectra have been vertically displaced for the sake of clarity. AU, absorbance units.

cell size and shape were noted, confirming that the level of contamination of EPS samples due to possible cell lysis would have been insignificant. All harvested EPS were also examined under a light microscope ($\times 100$ objective) and the absence of bacterial cells was confirmed.

Neutral carbohydrate and protein contents of each sample were measured in triplicate (pseudoreplication) and expressed in μg (mg EPS material) $^{-1}$. Statistical analysis were carried out using a two tailed *t*-test ($P = 0.025$) and analysis of variance (ANOVA) employing MINITAB version 11. A significantly lower neutral sugar content ($P = 0.025$) was recorded in biofilm EPS ($11.46 \pm 0.06 \mu\text{g mg}^{-1}$) compared to the capsular and free EPS (13.32 ± 0.05 and $13.35 \pm 0.07 \mu\text{g mg}^{-1}$, respectively). There was also a significant difference between the protein content of the capsular EPS ($14.14 \pm 0.06 \mu\text{g mg}^{-1}$) and the biofilm EPS ($9.55 \pm 0.08 \mu\text{g mg}^{-1}$) as well as the free EPS ($9.31 \pm 0.08 \mu\text{g mg}^{-1}$). Although SDS-PAGE showed that the biofilm and capsular EPS shared several common bands, each polymer had its own specific protein profile. Interestingly, fewer distinct protein bands (100, 60 and 35 kDa) were visible against the background in the free EPS (Fig. 1) compared to either the capsule or biofilm EPS.

FTIR spectra (Fig. 2) revealed extensive homology between the samples. Replicate spectra of individual

Fig. 3. Reflectance-absorbance FTIR spectrum of the capsular exopolymer of *Pseudomonas* sp. NCIMB 2021 showing the amide I and amide II regions. The original spectrum (dashed line) is shown together with the deconvoluted (continuous line). The second derivative spectrum is displaced for clarity. AU, absorbance units.

EPS samples demonstrated that differences in absorption were well within the resolution employed. Common to all spectra were vibrations due to the carbohydrate backbone. Strong complex absorptions centred between 1064 and 1078 cm^{-1} for the three exopolymers are ascribed to complex vibrations of ring structures (including bending, stretching and coupling between these

modes; Malfait *et al.*, 1987). Bands between 1200 and 1100 cm^{-1} are assigned to $\nu\text{C}-\text{C}$ and $\nu\text{C}-\text{O}$ modes, including a shoulder due to the $\nu_{\text{as}}\text{C}-\text{O}-\text{C}$ of the glycosidic linkage ($\sim 1124 \text{ cm}^{-1}$) whilst the main absorption is due to $\nu\text{C}-\text{O}$ weakly coupled to $\delta\text{C}-\text{O}-\text{H}$ (Malfait *et al.*, 1989). Bands at 1728–1724 cm^{-1} are due to $\nu\text{C}=\text{O}$ associated with O-acetyl ester bonds. Complex absorptions (not shown for the sake of clarity) at $\sim 2930 \text{ cm}^{-1}$ are ascribed to the asymmetric stretch ($\nu_{\text{as}}\text{C}-\text{H}$) of $-\text{CH}_2$ groups combined with that of $-\text{CH}_3$ groups. The corresponding symmetric stretches are found at $\sim 2853 \text{ cm}^{-1}$ and $\sim 2877 \text{ cm}^{-1}$, respectively. Absorption bands between 1319 and 1326 cm^{-1} , assigned to the $\nu\text{C}-\text{O}$ of carboxylic acids, suggested that all three exopolymer were acidic.

Protein-related bands, the $\nu\text{C}=\text{O}$ of the amide I and $\delta\text{NH}/\nu\text{C}=\text{O}$ combination of the amide II bands, were also observable for all three EPS samples. The principal amide I absorption between 1661 and 1665 cm^{-1} for all three exopolymers suggested a 3_{10} -helical secondary structure (Rothschild & Clark, 1979; Jackson & Mantsch, 1995). However, the presence of amino sugars, which is manifested by an absorbance band in the same region due to *N*-acetyl or even glucuronamide ($-\text{CONH}_2$) groups, cannot be discounted.

Amide II bands were detected at 1547 cm^{-1} . Fourier self-deconvolution and second derivative spectroscopy of the amide I and II regions, however, indicated the presence of additional bands.

Fig. 3 shows the FTIR spectrum of the capsular exopolymer together with the deconvoluted and smoothed derivative spectra. Band positions obtained from the derivative spectrum were similar for the three exopolymers. However, as will be shown later, the relative 'band intensities' were different for the three exopolymers. A band at 1742 cm^{-1} is assigned to the

Table 1. Protein secondary structures of exopolymers produced by *Pseudomonas* sp. NCIMB 2021 in continuous culture as determined by curve-fitting analysis of FTIR spectra

Secondary structure	Biofilm		Capsule		Free	
	Wavenumber (cm^{-1})	% *	Wavenumber (cm^{-1})	% *	Wavenumber (cm^{-1})	% *
Tyrosine side chain	—	—	1602	1	1602	1
Aggregated strands	—	—	1615	10	—	—
β -Sheet	1631	12	1631	7	1627 + 1632	43
Unordered	1642 + 1648	24	1642	27	1641	4
3_{10} -helix	1661 + 1668	42	1662	29	1664 + 1667	47
Antiparallel β -sheet/ aggregated strands	1687 + 1691	22	1677 + 1695	26	1682 + 1695	5

—, None detected.

* Percentage contribution of the absorbance at that wavenumber to the peaks identified in the amide I region. The percentage was calculated using linear combinations of Lorentzian and Gaussian line shapes, fitted using a Levenberg–Marquardt algorithm following baseline correction.

Fig. 4. Reflectance-absorbance FTIR spectrum of the capsular exopolymer of *Pseudomonas* sp. NCIMB 2021 following H/D exchange. AU, absorbance units.

$\nu\text{C}=\text{O}$ of O-acetyl ester bonds and is accompanied by a doublet at 1262–1251 cm^{-1} (Fig. 2), including a $\nu\text{C}-\text{O}$ absorption at 1251 cm^{-1} (Sherbrock-Cox *et al.*, 1984). However, the doublet may arise from the amide III ($\nu\text{C}-\text{N}$ and $\delta_{\text{ip}}\text{C}-\text{N}-\text{H}$) of proteins. N-Acetyl ester bonds were also present; the unusually high amide II absorption at 1565 cm^{-1} has been variously ascribed to either N-acetyl (in hyaluronate; Parker, 1983) or N-methyl (in N-methylacetamide; Miyazawa *et al.*, 1958) groups. Protein secondary structure was determined by fitting curves to peaks identified in the amide I region (see Jackson & Mantsch, 1995) and revealed distinct differences between the three exopolymers (Table 1). The principal structure in all three samples was the (3_{10} -helix/N-acetyl or $-\text{CONH}_2$) peak representing 42% of the total amide I region of the biofilm EPS, 47% of the free EPS but only 29% in the capsular EPS. Whilst the biofilm and capsular EPS contained substantial amounts of unordered structure (24% and 27%, respectively), the free EPS contained considerably less (4%). In contrast, parallel β -sheet structures represented 43% in the free EPS but only 12% and 7% in the biofilm and capsular EPS, respectively.

The FTIR spectrum after H/D exchange confirmed the presence of absorption bands at 1742, 1726 and 1567 cm^{-1} due to unexchanged O- and N-acetyl groups (Fig. 4). The principal amide I peak at 1661 cm^{-1} remained (further emphasizing the possibility of $-\text{CONH}_2$ groups being present), together with some

residual amide II absorption at 1550 and 1532 cm^{-1} . Amide I' and II' absorptions were identified at 1643–1630 cm^{-1} and 1464–1447 cm^{-1} , respectively. It is interesting to note that the doublet associated with either the $\nu\text{C}-\text{O}$ of O-acetyl ester bonds or the $\nu\text{C}-\text{N}/\delta\text{C}-\text{N}-\text{H}$ modes at 1261 and 1251 cm^{-1} for the H spectra (prior to H/D exchange, Fig. 2) was replaced by a single peak in the D spectra (after H/D exchange, Fig. 4). Hence, the 1261 cm^{-1} absorption was due to amide III, whilst the 1251 cm^{-1} was indeed due to the acetyl group. However, no new absorption at 1040–940 cm^{-1} due to $\delta\text{N}-\text{D}$ of amide III' was observed in the D spectra.

The relative uronic acid content, together with the extent of acylation, was estimated as a proportion of the total carbohydrate content of the EPS. This was achieved by calculating the ratios of integrated peak areas A, from the spectra of peaks at 1319–1326 cm^{-1} ($\nu\text{C}-\text{O}$, carboxylic acids, A1), 1728–1724 cm^{-1} ($\nu\text{C}=\text{O}$, O-acetyl ester bonds, A2), and 1565 cm^{-1} (N-acetyl ester bonds, A3), with the peak centred between 1064 and 1077 cm^{-1} due to the carbohydrate backbone (A4). The biofilm and free EPS contained roughly equivalent levels of uronic acid residues (relative to carbohydrate content) with A1:A4 ratios of 0.102 and 0.084, respectively, whilst the capsular EPS A1:A4 ratio was only 0.044. The greatest degree of O- and N-acylation was found in the biofilm EPS with A2:A4 and A3:A4 ratios of 0.011 and 0.014, respectively, compared to 0.006 and 0.003, respectively, for the capsular EPS, and 0.003 and 0.000, respectively, for the free EPS.

DISCUSSION

The distinction between capsular and slime exopolymers is an imprecise one, the latter often attributed either to overproduction of a tightly bound (to the cell wall) capsular polymer or to insufficient binding of the polymers to the wall (Beveridge & Graham, 1991; Fletcher & Floodgate, 1973). Although the assumption is that the two polymers are chemically identical and that it is only in their position relative to the cell that differences are observed, chemical differences between capsular and 'slime' exopolymers have been reported, for example, in *Rhodopseudomonas capsulata* Sp11 (Omar *et al.*, 1983).

By collecting exopolymers from the culture medium (free EPS), planktonic phase (capsular EPS) and adhered cells (EPS associated with a biofilm; i.e. slime), we were able to qualitatively characterize these exopolymers using SDS-PAGE and FTIR spectroscopy. The data showed that the three polymers shared certain features, in agreement with published data. For example, all were carbohydrate/protein co-polymers, with the carbohydrate moieties being both acidic (Fletcher & Floodgate, 1973) and O-acetylated (Christensen, 1989).

Reported protein contents of bacterial exopolymers range between 1 and 18% (Abu *et al.*, 1991; Robijn *et al.*, 1995; Guezennec *et al.*, 1994; Vincent *et al.*, 1994)

and for *Pseudomonas* sp. NCIMB 2021 varies depending upon the type of EPS. FTIR spectroscopic analysis demonstrated that the predominant protein secondary structure of all three EPS types is a 3_{10} -helix (although there could be some confusion with amino sugars; see above), which, moreover, is resistant to H/D exchange. SDS-PAGE profiles demonstrated differences in protein composition between biofilm, capsular and free EPS (Fig. 1) while FTIR spectroscopy revealed differences in overall protein secondary structure (Table 1). The capsular EPS protein moiety displayed a considerably reduced 3_{10} -helical component and an increased aggregated strand component compared to the biofilm EPS, whilst the free EPS protein displayed a marked increase in β -sheeting and a reduced unordered component. Since an equal amount of protein, as estimated using the Bradford (1976) assay, was present in each EPS sample loaded onto the gel, it is apparent that each polymer has its own specific profile. This strongly indicates differences in the protein composition between EPS samples. The most obvious difference was noted for the free exopolymer released into the bulk phase of the *Pseudomonas* NCIMB 2021 culture. The smeary appearance of the gel and the presence of only few distinct bands in the free EPS indicated that proteins in this sample were degraded to produce different length polypeptides. Indeed, the free EPS protein profile (lane 1) extended beyond the dye front. The observed degradation is most likely due to the action of the non-specific proteases which would be present in the bulk phase.

Exopolymer production by *Pseudomonas* sp. NCIMB 2021 is therefore similar to exopolymer production by *P. aeruginosa*, if somewhat more subtle. Attachment of *P. aeruginosa* is associated with up-regulation of *algC* and *algD* promoters (Davies *et al.*, 1993; Hoyle *et al.*, 1993) (similar to the up-regulation of the *lacZ* marker gene in attachment studies using *Pseudomonas* S9; Dagostino *et al.*, 1991), resulting in alginate production. Christensen *et al.* (1985) demonstrated the presence of N-acetylglucosamine and 6-deoxyhexoses in exopolymers produced in the late stationary phase in batch culture which were not present in exopolymers produced by cells in growth phase. Protein profiles also appear to differ between biofilm and planktonic cells (Costerton *et al.*, 1995). Phenotypic differences arising from cell attachment in *Pseudomonas* sp. NCIMB 2021, did not result in the production of completely novel EPS. This is in contrast to *P. aeruginosa*, which was characterized by low exopolymer production (Christensen *et al.*, 1985). However, we have demonstrated that ratios between carbohydrate residues, the degree of polysaccharide acetylation and the profiles and secondary structure of the protein moiety were dependent upon the cellular mode of growth.

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