Extraction of extracellular polymeric substances from the acidophilic bacterium Acidiphilium 3.2Sup(5)
J. M. Tapia, J. A. Muñoz, F. González, M. L. Blázquez, M. Malki and A. Ballester

ABSTRACT

Extraction of extracellular polymeric substances (EPS) from Acidiphilium 3.2Sup(5) was investigated using five methods: EDTA, NaOH, ion exchange resin, heating and centrifugation. The bacterium studied presents promising application in microbial fuel cells (MFCs). The degree of cellular lysis provoked by each method was determined by UV-visible spectroscopy of cultures before and after EPS extraction. In addition, two electron microscopy techniques (TEM and SEM) were employed to determine the degree of attachment and the growth of the biofilm overtime on two solid supports: carbon fibre cloth and graphite rods. The main constituents of the EPS extracted by all methods were proteins and carbohydrates, as confirmed by FT-IR analysis, showing the major presence of carboxylic, hydroxylic and amino groups. The greater extractions of EPS were obtained using EDTA. This method also produced a less degree of cellular lysis. Furthermore, both the amount and the chemical composition of EPS strongly depended on the extraction method used.

Key words | Acidiphilium, EPS, extracellular polymeric substances, extraction, MFC

INTRODUCTION

Extracellular polymeric substances (EPS) are produced by biological processes and participate in the formation of microbial consortia. Its origin is related to processes involving active secretion, shedding of cell surface material, cell lysis and adsorption from the environment. Its composition mainly depends on the type of ecosystem where microorganisms grow, with the major constituents being carbohydrates and proteins, and the minor constituents nucleic acids, uronic acids and humic substances (Wingender et al. 1999).

A difficult task in the analysis of EPS is that related to its extraction from microbial consortia. There is not a standard extraction protocol for EPS but a wide variety of methods based on physical or chemical principles or a combination of both. The extraction methods include among others: ion exchange resins (Frölund et al. 1996), EDTA (Sheng et al. 2005), centrifugation (Liu & Fang 2002), heating (Comte et al. 2006) and NaOH (Brown & Lester 1980).

Unfortunately, the results published are sometimes contradictory regarding the base units given. For that reason, replication of results is difficult and there is not an established protocol able to assure a high extraction and purity of the EPS obtained (Liu & Fang 2002; Comte et al. 2006). In fact, the amount and chemical composition of EPS extracted are different as a function of the extraction method used, even for similar bacterial cultures and experimental conditions. Differences up to 100 times in the amount of EPS obtained have been reported (Brown & Lester 1980). In addition, the extraction methods largely affect the chemical composition of EPS, resulting in
significant differences in the amount of the different polymeric substances determined. Furthermore, those methods also affect both structure and functional groups of EPS (Schmitt & Flemming 1998).

In the present work, the heterotrophic bacteria *Acidiphilium 3.2Sup(5)* was used [A. 3.2Sup(5)]. The strain was provided by Dr. M. Malki of the Molecular Biology Centre (CBM-CSIC, Madrid, Spain) and its origin was an ecosystem of Rio Tinto (Huelva, Spain). It shows 99% genetic similarities with the specie *Acidiphilium cryptum* (López-de-Lacey et al. 2006). That strain has been studied in detail due to its ability to transfer electrons directly to carbon electrodes in aerobic conditions (Malki et al. 2008), unlike most microorganisms studied which are strictly anaerobic (Bond & Lovley 2003). That particular capability makes this type of bacterial strains potentially attractive for its possible use in microbial fuel cells (MFC’s) in order to obtain electric power under environmental conditions, such as from organic residues. In this way, this study is an attempt to provide insights into its potential applicability through the determination of key operational parameters in MFC’s.

The mechanism of electron transfer from the oxidant to the electrode has not been elucidated yet, but it has been attributed, among others, to the action of pili (Reguera et al. 2005) and direct surface contact (Chaudhuri & Lovley 2003). An important and scarcely studied aspect of this phenomenon is the role played by EPS during electron transfer, bacterial attachment and crystalline surface nucleation. Such study should consider in first instance the extraction of large amounts of those substances but with a low degree of contamination by intracellular substances or reagents used during extraction.

This work evaluates the efficiency of five methods to extract EPS from pure bacterial cultures of *A. 3.2Sup(5)*: centrifugation, EDTA, NaOH, heating and ion exchange resin. The amount and composition of EPS extracted are compared. The degree of cellular lysis and disruption provoked by the extraction was estimated from the protein/carbohydrate ratio of the EPS extracted and from the UV–Vis spectra of bacterial cultures before and after extraction. EPS was characterized by quantification of the content of proteins and carbohydrates, its structure by Fourier transform infrared spectroscopy (FT-IR), and its morphology and surface attachment by electron transmission microscopy (TEM) and field emission scanning electron microscopy (FE-SEM).

**MATERIALS AND METHODS**

**Bacterial growth**

The growth of bacterial cultures of *A. 3.2Sup(5)* was achieved aerobically in a liquid medium, at pH 2.5, stirred at 150 rpm and at 30°C, with or without the addition of a solid substrate as support. The nutrient medium used was similar to that employed during the isolation and characterization of the strain (González-Toril et al. 2006) (per litre): 2.0 g (NH₄)₂SO₄, 0.1 g KCl, 0.25 g MgSO₄·7H₂O, 0.25 g K₂HPO₄, 0.01 g Ca(NO₃)₂·4H₂O, 0.1 g yeast extract and 1.0 g of glucose. All chemical reagents used were of analytical grade (PA). The cell growth was performed in sterile conditions in order to obtain bacterial cultures homogeneous regarding its phenotypic characteristics.

Two different solid supports were used: carbon fibre cloth and graphite rods. The carbon fibre cloth was added to the bacterial culture as disks of 2 cm of radius and 1 cm of thickness. The characteristic of that material was its high specific surface, which increased the probability of bacterial attachment and, thus, the electron transfer taking into account the possibility of its utility as electrode of a microbial fuel cell. On the other hand, graphite was added as rods of 1 cm of diameter and 1 cm of height, presenting a significant surface rugosity. That can be a relevant factor since bacteria tend to adhere preferentially to surface imperfections (Ghauri et al. 2007).

**EPS extraction**

EPS were extracted from bacterial cultures using five different methods: EDTA, NaOH, ion exchange resin, heating and centrifugation. Centrifugation was used as control method in order to elucidate the reference amount of carbohydrates and proteins in each culture. In this case, the experimental procedure was as follows: 40 mL of bacterial culture were centrifuged at 14,000 rpm.
EDTA and NaOH extractions were performed similarly according to the following procedure: 40 mL of bacterial culture was contacted with the respective dissolution for 3 h and at 4°C. In the former case, EDTA 2% (Panreac) was used in a dose of 3.2 g de EDTA per gram of dry cell weight. In the latter, the volume of culture was treated with 8 mL of NaOH 1 N (Panreac) (Sheng et al. 2005). The ion exchange resin extraction was performed with 40 mL of bacterial culture in contact with a sodic resin Dowex Marathon C (Sigma-Aldrich), using a ratio of 70 g de resin/g dry cell weight. That resin pulp was stirred for 2 h at 600 rpm and at 4°C. Finally, a physical technique consisting in heating 40 mL of bacterial culture at 70°C and 1 bar of pressure for 1 h was tested (Frolund et al. 1996).

EPS obtained in each extraction were separated by centrifugation from solution and under experimental conditions identical to those used in the control method. The separation of free cells in solution containing EPS was performed by vacuum filtration using nitrocellulose of 0.22 µm of pore size (Millipore). The supernatant obtained was the solution of raw EPS which later was dialyzed to eliminate metabolites and salts of low molecular weight, using a dialysis membrane (Slide-a-Lyzer, Pierce) of 3500 MWCO in 1 L of deionized water for 24 h at 4°C (Comte et al. 2006). That final solution corresponded to the fine EPS fraction extracted in each case, named simply EPS. Figure 1 shows a flow scheme of the methods described above.

**EPS characterization**

Proteins and carbohydrates were determined photocolorimetrically using an UV–Vis spectrophotometer (Biochrom Libra S11). The amount of proteins in fine EPS was determined by the method of Lowry (Lowry et al. 1951), using bovine serum albumin (BSA) as standard compound (Sigma-Aldrich) and measuring absorbance at 750 nm. The amount of carbohydrates was determined by the anthrone method (Trevelyan et al. 1952), using glucose (Panreac) as standard and a wavelength of 660 nm.

**IR and UV–Vis spectroscopy**

Two milligrams of freeze-dried EPS (24 h at −56°C, Labconco SciLab) solution was mixed with 300 mg of KBr (Panreac) and compacted to form a pellet. The infrared (IR) spectra were then obtained using a spectrum Nicolet Magna 750, at 4 cm−1 resolution. The ultraviolet visible (UV–Vis) spectrum of bacterial culture samples before and after the extraction of EPS was measured between 325 and 825 nm, using a Biochrom Libra S11 spectrophotometer and aliquots of 4 mL.

**FE-SEM and TEM microscopy**

FE-SEM microscopy was performed in a JEOL JSM-6330 F microscope, using samples treated by progressive dehydration with acetone (Panreac). Then, the solid support was covered with a conductor film of graphite. TEM microscopy was carried out in a JEOL JSM 6430 T microscope on bacterial cells stained with uranyl acetate 2% per 30 min.

**RESULTS AND DISCUSSION**

**Bacterial growth**

The bacterium studied showed an exponential growth during the first three days and after that a plateau was reached. Under all culture conditions tested, the bacterium A. 3.2Sup(5) achieved maximum bacterial populations between 3 and 10 days, ranging between 1.28 ¥ 10⁹ and 1.50 ¥ 10⁹ cells/mL. In addition, the acidity of the culture was kept approximately constant around the starting pH value (pH 2.5). Similarly, bacterial cultures of the pure strain were grown in the presence of solid supports (carbon fibre cloth and graphite rods). In this case, the parameters measured were basically similar to those followed in tests without solid support.
Extraction of proteins and carbohydrates from cultures grown in absence/presence of solid supports

Figure 2 depicts the amount of proteins and carbohydrates extracted, expressed in mg/g of dry cell weight (mg/g-DW) in the absence of solid support and the corresponding protein/carbohydrate ratio. Error bars represent standard deviations obtained from measurements in triplicate.

Figure 2 shows that the total amount of EPS (carbohydrates + proteins) obtained varied significantly as a function of the extraction method used. Also shows that the chemical composition of EPS extracted differed as a function of the method used. Thus, the amount of proteins varied between a minimum value of 97 mg/g-DW, obtained with the ion exchange resin, and a maximum value of 227 mg/g-DW, when the extraction was performed by heating. Therefore, the amount of proteins was 233% higher in the latter than in the former case. However, the amount of carbohydrates was only 35% higher by heating (586 mg/g-DW) than by ion exchange resin (378 mg/g-DW). That different behaviour is expressed in the protein/carbohydrate ratio obtained for each EPS. For instance, that ratio was 0.45 for the extraction with NaOH and 0.26 for the ion exchange resin, indicating 1.7 times more proteins in the EPS of the first than in the second case.

In general, the amount and composition of EPS extracted by centrifugation was similar to that obtained by heating and EDTA. This could be attributed to the low sedimentation rate of cells and the strong action of shearing forces on individual cells resulting in a high extraction of EPS, even without a pre-treatment, but also in a certain cell lysis. Nevertheless, since EPS showed protein/carbohydrate ratios slightly higher for the centrifugation and heating methods, the extraction with EDTA could be more recommendable because of its combination of a high extraction with a low cellular lysis.

The effect of incubation time and the presence of a solid support on the production and composition of EPS were evaluated by extractions with EDTA at two different times of bacterial culture growth: at the end of the exponential growth phase (96 h), and in the stationary phase (216 h) (Figure 3). Error bars represent standard deviations obtained from measurements in triplicate.

Figure 3 shows that EPS extracted with EDTA after 96 h had a chemical composition very similar to that obtained from bacterial cultures in the absence of solid supports (the protein/carbohydrate ratio ranged between 0.4 and 0.5). However, the amount total EPS extracted was slightly lower (between 360 and 380 mg/g-DW). That low extraction could be associated with the difficulty to extract cells and EPS adhered to solid supports during the concentration process.

The amount and composition of EPS in the microbial cultures was significantly different after 216 h. The amount of EPS extracted with both solid supports was lower, especially for graphite rods. That could be related to the fact that the growth and consolidation of the biofilm interfere with the extraction of these substances. Furthermore, there was a significant change in the composition of EPS as the protein/carbohydrate ratio increased up to values of 1.1 for graphite and 1.5 for carbon fibre cloth. This change in composition can be attributed to the presence of additional proteins and intracellular substances produced by destruction of cells, which is used as an indicator of the
The degree of cell lysis during the extraction (McSwain et al. 2005; Comte et al. 2006).

Similar differences in the amount and composition of EPS as a function of extraction conditions have been reported in the literature for pure cultures. Sheng et al. (2005) used EDTA and NaOH as extractants for pure bacterial cultures of acidophilic *Rhodopseudomonas*. Their results showed a difference of 12 times in the amount of EPS extracted, 12.9 mg/g-DW by centrifugation and 159.2 mg/g-DW by NaOH. In addition, those authors mentioned EPS compositions very different, as shown by the protein/carbohydrate ratio: 1.5 and 16.4 respectively.

Those differences have also been reported when using more complex microbial consortia such as activated sludges. (Liu & Fang 2002) reported extractions of EPS ranging between 25.7 mg/g volatile solid suspended (VSS) by centrifugation and 164.9 mg/g-VSS by a chemical combination of NaOH and formaldehyde; a difference of more than 600% in the amount of EPS extracted from the same culture but with a different method.

**UV–Vis spectroscopy and density of bacterial culture**

For each method, the degree of bacterial lysis was evaluated comparing the cell concentration before and after EPS extraction by measuring the turbidity of the cultures by UV–Vis spectroscopy and active cells enumerations by optical microscopy. Figure 4 depicts the UV–Vis spectra

<table>
<thead>
<tr>
<th>Culture</th>
<th>Extraction method</th>
<th>Total EPS</th>
<th>Ratio Prot/Carboh</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture pure of <em>Rhodopseudomonas acidophila</em></td>
<td>Centrifugation</td>
<td>12.9</td>
<td>1.5</td>
<td>Sheng et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>70.3</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaOH</td>
<td>159.2</td>
<td>16.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heating</td>
<td>71.6</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Culture pure of <em>Klebsiella aerogenes</em></td>
<td>Centrifugation</td>
<td>179.0</td>
<td>1.9</td>
<td>Brown &amp; Lester (1980)</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaOH</td>
<td>332.0</td>
<td>13.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heating</td>
<td>233.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Activated sludge†</td>
<td>Centrifugation</td>
<td>21.0</td>
<td>1.9</td>
<td>Comte et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>100.0</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaOH + formaldehyde</td>
<td>318.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heating</td>
<td>64.0</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Activated sludge‡</td>
<td>Centrifugation</td>
<td>25.7</td>
<td>1.0</td>
<td>Liu &amp; Fang (2002)</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>146.8</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaOH + formaldehyde</td>
<td>164.9</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cation exchange resin</td>
<td>57.8</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 | Comparison of total EPS (carbohydrate + protein) extracted from various cultures

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*mg g⁻¹ dry cells.
†mg g⁻¹ DW EPS.
‡mg g⁻¹ volatile suspended solids.
N.D.: Not Determined.
of Acidiphilium 3.2Sup(5) cultures before and after EPS extraction by EDTA, centrifugation, NaOH and heating. Table 2 shows bacterial concentration of cultures after EPS extraction.

The degree of turbidity in bacterial cultures submitted to extraction with centrifugation and NaOH was different to the spectrum corresponding to the pure culture without treatment (Figure 4). In the former case, the lower absorbance would be related to lysis of cells during centrifugation. The cellular lysis process causes a decrease of the number of cells as shown in Table 2 (Sesay et al. 2006). Brown & Lester (1980) have reported similar results after EPS extraction by centrifugation from pure cultures of Klebsiella aerogenes showing that the action of shearing forces provokes a high extraction of EPS and cell lysis (Table 1). The lower absorbance observed after NaOH extraction would be a consequence of the acidity change of the medium that would have affected the structural stability of cell membrane provoking a strong cellular disruption (see Table 2). Sheng et al. have reported similar results for the EPS extraction with NaOH from bacterial cultures of Rhodopseudomonas acidophila (see Table 1) (Sheng et al. 2005).

Unlike in previous cases, the spectra of bacterial cultures extracted with EDTA and by heating were practically identical to the untreated culture. In spite of that, Table 2 shows that the extraction by heating provoked a significant decline in the bacterial cell population related to cell lysis phenomena. Hence, in this case, the optical density would be associated to both cells remaining in the bulk solution and polymeric substances extracted. By contrast, after extraction with EDTA the number of cells in the culture remained almost constant (Table 2). That suggests that this treatment had an insignificant effect on cell concentration and cellular lysis can be discarded.

### Table 2 | Concentration of culture after extraction

<table>
<thead>
<tr>
<th>Method</th>
<th>Number after extraction (cells/mL)</th>
<th>Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>$2.12 \times 10^9$</td>
<td>89.8</td>
</tr>
<tr>
<td>Heating</td>
<td>$1.32 \times 10^9$</td>
<td>55.9</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>$1.62 \times 10^9$</td>
<td>68.6</td>
</tr>
<tr>
<td>NaOH</td>
<td>$2.52 \times 10^8$</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Density culture without extraction (cells/mL) = $2.36E + 09$.

Attachment of bacteria Acidiphilium 3.2Sup(5) on carbon supports

Another interesting aspect of the process would be to obtain knowledge about the evolution of the biofilm formation and the impact on them of extracellular substances excreted by bacteria. For that, FE-SEM scanning electron microscopy was performed on carbon solid supports (carbon fibre cloth and graphite rods) previously in contact with cells of Acidiphilium 3.2Sup(5) at two different growth times: 144 and 216 hours.

Figure 5 shows the bacterial attachment to carbon fibre cloth. Besides certain degree of cellular attachment over the fibre, the presence of EPS aggregates is also observed. Micrograph 5(left) shows bacterial cells attached
to carbon fibres, with a preferential colonization in interfibre spaces. Morphologically, cells are homogeneous as corresponds to a pure culture. The presence of organic tissues would be attributable to the generation of EPS. Therefore, at that cultivation time, attached bacteria were in an initial stage of generation of such substances. The bacterial attachment increased over time, Figure 5(right). Again, the higher cellular density took place preferentially into the space between fibres. It is obvious the formation of EPS networks, with a gel-type aspect as referred by (Wingender et al. 1999). Those substances are shown in detail in Figure 5(upper) and would confirm the preferential attachment of cells to interstices between microfibres through the formation of EPS, which serve as anchorage sites for cells.

Figure 6 shows the attachment of cells on graphite rods. The graphite surface is largely colonized with cells placed on surface irregularities, Figure 6(left). The increasing bacterial population due to bacterial overgrowth is clear in Figure 6(right) with a high density of attached cells in pores and surface rugosities. Furthermore, there is a complex network of EPS on the support surface, as shown in more detail in Figure 6(upper).

TEM microscopy of Acidiphilium 3.2Sup(5)

TEM microscopy of bacteria allowed a better comprehension of the relationship between cells and extracellular polymeric substances excreted by them. Figure 7 is a detail of a cell of A. 3.2Sup(5) showing the presence of a kind of “cloud” surrounding one side of the cell. The “cloud” that contains inner filaments and has a spherical shape, possibly is formed by different extracellular polymeric substances excreted by the own bacteria.
FT-IR spectroscopy of Acidiphilium 3.2Sup(5) EPS

The Fourier transform infrared spectra of EPS extracted with EDTA (the best method of extraction tested) and by the control method (centrifugation) are compared in Figure 8. This analysis could be useful for elucidating an eventual contamination of the EPS obtained by the extractant reagent.

In spite that both spectra are different they have matching bands. For example, both spectra present pronounced bands between 3,200 and 3,320 cm$^{-1}$. Those bands are characteristic of the O-H symmetric stretch vibration in polymeric compounds, which could be due to the presence of residual water in the sample (Schmitt & Flemming 1998). Another coincident band appears at values between 1,069 and 1,099 cm$^{-1}$, which is characteristic of the O-H symmetric stretch vibration in polysaccharides and their derivatives (Sheng et al. 2005). In addition, in both spectra there are bands at values between 1630 and 1657 cm$^{-1}$, characteristic of the C=O and C-N symmetric stretch vibration (peptidic bond of proteins corresponding to the group of amides I) (Jiang et al. 2004). Finally, the bands at 1,403 y 1,418 cm$^{-1}$ for both spectra would be related to the symmetric stretch vibration of C=O (Comte et al. 2006). Therefore, in both spectra the main functional groups are hydroxyl, carboxyl and amino, which correspond mostly to the presence of carbohydrates and proteins in the EPS obtained, as shown in Figures 2 and 3 and Table 3.

CONCLUSIONS

The extraction of extracellular polymeric substances, EPS, both amount and chemical composition, was clearly affected by the method used. The methodology able to obtain the highest extraction and a lower intracellular contamination was the treatment with EDTA (2%) for 3 h and at 4°C, using a dose of de 3.2 g/g-DW. Unlikely, the presence of solid supports substantially modified the extraction of EPS in terms of both, amount, being lower for the supports and growth times evaluated, and composition, since in all cases the amount of proteins increased. That is an indication that, in such conditions, the degree of cellular lysis increased.

Table 3 | Main functional groups observed from IR spectra of EPS extracted

<table>
<thead>
<tr>
<th>Wave number (cm$^{-1}$)</th>
<th>Band assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,200 – 3,320</td>
<td>$\nu_{s}$ of O–H into polymeric compounds</td>
</tr>
<tr>
<td>1,630 – 1,660</td>
<td>$\nu_{s}$ of C=O and C–N of amides associated with proteins (amide I)</td>
</tr>
<tr>
<td>1,400 – 1,420</td>
<td>$\nu_{s}$ of stretching vibration of C=O in carboxylates</td>
</tr>
<tr>
<td>1,060 – 1,100</td>
<td>$\nu_{s}$ of OH (fingerprint) of polysaccharides</td>
</tr>
<tr>
<td>&lt;1,000</td>
<td>Several bands visible: phosphate or sulphur functional groups</td>
</tr>
</tbody>
</table>

$\nu_{s}$ symmetric stretch; $\delta_{s}$ symmetric deformation (bend).
Microscopy observations showed that bacteria attachment increased with bacterial growth time. The bacterial attachment would be related to the generation and development of an EPS network interweaving the surfaces of carbon supports with bacterial cells. Furthermore, those substances tend to grow preferentially on sites between microfibres when using carbon fibre cloth as support, or on pores and surface roughness when using graphite rods.

EPS are mainly constituted by hydroxyl, carboxyl and amino functional groups according to the high content of proteins and especially carbohydrates detected by chemical analysis. The presence of those anionic groups allows its interaction with heavy metals such as iron, and that opens new interesting studies in order to elucidate its use as an eventual agent for preferential nucelation.

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