Identification of single bacterial cells using digoxigenin-labelled, rRNA-targeted oligonucleotides

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Oligonucleotides were end-labelled with digoxigenin (DIG), chemically at the 5'-end or enzymically at the 3'-end. Following specific *in situ* hybridization of these probes to intracellular rRNA molecules, the hybrids were detected with anti-DIG F_{ab} fragments labelled with fluorescent dyes. The antibody fragments penetrated through the bacterial cell periphery and specifically bound to their antigens. Probe-conferred and non-specific fluorescence per cell were quantified by flow cytometry and compared to values obtained with end-labelled fluorescent probes. The DIG reporter molecules could also be detected in whole fixed cells by antibodies labelled with either alkaline phosphatase or horseradish peroxidase. The penetration of the large antibody–enzyme complexes into the cells required lysozyme/EDTA treatment prior to the hybridization and has so far only been achieved for Gram-negative bacteria. This technique has the potential for significant signal amplification as compared to the fluorescently end-labelled oligonucleotides hitherto used for single cell identification in microbial ecology. Moreover, it can be used instead of fluorescent assays in natural samples showing autofluorescence.

Introduction

The study of microbial ecology requires the accurate and specific detection and enumeration of populations and their changes over time. These data are even more meaningful if they can be determined *in situ* to yield information on the spatial distribution of the individual micro-organisms.

Selective plating has been the classical method used to enumerate specific micro-organisms in an environmental or clinical specimen. However, this approach is hampered by the fact that the number of colony forming units is often only a minor fraction of the cell counts determined by direct microscopic techniques (Jannasch & Jones, 1959). Some of these cells are of course non-viable, others however are just not culturable with today's techniques (Brock, 1987) or have converted into a viable but non-culturable state (Colwell *et al.*, 1985). A second established technique to determine specific cell counts is the immunofluorescence approach. It avoids most of the disadvantages of selective plating and has successfully been used to study the autecology of individual bacterial taxa in microbial communities (Bohlool & Schmidt, 1980; Fliermans *et al.*, 1974). However, antibodies are

commonly raised against surface markers of defined pure cultures and therefore this technique is restricted to bacteria which are sufficiently characterized to grow them in pure cultures.

Over the last decade nucleic acid hybridization has become a powerful tool for the detection of micro-organisms (Saylor & Layton, 1990) and for the monitoring of population dynamics in microbial ecology (Stahl *et al.*, 1988). Specific identification of individual microbial cells was recently achieved by hybridization with radioactively (Giovannoni *et al.*, 1988) or fluorescently (DeLong *et al.*, 1989; Amann *et al.*, 1990*a, b*; Tsien *et al.*, 1990) labelled oligonucleotide probes directed against the naturally amplified, intracellular rRNA. Combined with techniques to retrieve rRNA-sequence information directly from complex ecosystems (Olsen *et al.*, 1986; Weller & Ward, 1989; Giovannoni *et al.*, 1990; Relman *et al.*, 1990), this approach can also be used to monitor non-culturable bacteria (Amann *et al.*, 1991). This is important since there is strong evidence (Torsvik *et al.*, 1990; Giovannoni *et al.*, 1990; Ward *et al.*, 1990) that in many important environments the genetic diversity, as assessed by molecular techniques, far exceeds the microbial diversity determined by culture methods.

However, sensitivity problems have to be solved before the rRNA-based single cell detection will find

Abbreviation: DIG, digoxigenin.

wide applications in microbial ecology. Since the number of bound probe molecules is limited by the number of target molecules, the strength of the hybridization signal is always correlated with the cellular rRNA content. Radioactive labels can be detected *in situ* at very high sensitivity using photographic emulsion but the necessary exposure time of days to weeks is cumbersome and the spatial resolution is limited to one to several microns. Fluorescently labelled oligonucleotide probes yield results not only superior in resolution, but these results may also be evaluated instantaneously using an epifluorescence microscope (DeLong *et al.*, 1989) or flow cytometer (Amann *et al.*, 1990b). The sensitivity is rather limited since the fluorescent probes used so far confer only a single fluorescent dye molecule per ribosome. Their application is therefore restricted to the detection of actively growing micro-organisms with relatively high cellular rRNA content. DeLong *et al.* (1989) have demonstrated a proportional decrease of fluorescence intensity per cell with increasing generation time and have anticipated difficulties in the detection of slowly growing cells. The latter, as well as quiescent cells which are abundant in many important ecosystems (Lewis & Gattie, 1991), could hitherto not be studied due to their low rRNA content.

Any approach that will direct more than a single marker molecule to each target molecule should improve the sensitivity and consequently the applicability of this technique to environmental samples. Oligonucleotide probes covalently bound to multiple fluorescent dye molecules have been synthesized but no increased sensitivity was observed and probe specificity was reduced (D. A. Stahl & R. Amann and R. Amann & G. Wallner; unpublished observations). Simultaneous application of several fluorescently mono-labelled oligonucleotide probes directed to different target sites of one rRNA molecule has increased the signal intensity additively (Amann *et al.*, 1990b), but since the number of sites on the rRNA with a defined specificity is limited, this approach will allow only for a several-fold signal amplification. Probes can also be labelled with non-radioactive reporter groups like biotin (Langer *et al.*, 1981; Leary *et al.*, 1983) or digoxigenin (DIG) (Heiles *et al.*, 1988) and after hybridization the bound reporter groups may be detected with either avidin/streptavidin or a specific antibody. Since these binding proteins may be labelled with several fluorescent molecules or an enzyme detectable by its substrate metabolism, a more significant increase in sensitivity may be achieved. Given the tight network formed by the cell wall it was questionable if the reporter molecules could be detected in whole, fixed bacterial cells by labelled antibodies which are relatively large compared to an oligonucleotide probe. A recent report (Currin *et al.*, 1990) on the

intracellular detection of nitrogenase molecules in bacterial cells stimulated us to perform the experiments discussed in this paper.

Methods

Organisms and growth conditions. *Escherichia coli* (DSM 30083; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Braunschweig, FRG), *Pseudomonas fluorescens* (DSM 50090) and *Bacillus subtilis* (DSM 10) were aerobically grown in YT-broth (g l⁻¹: tryptone, 10; yeast extract, 5; glucose, 5; sodium chloride, 8; pH 7.2) at 37 °C. *Pseudomonas cepacia* (DSM 50181) was cultivated in M1-broth (g l⁻¹: peptone, 5; malt extract, 3; pH 7.0) at 30 °C aerobically. Cells from *Methanococcus vannielii* (DSM 1224) were a generous gift from Dr R. Huber (Department of Microbiology, University of Regensburg, FRG). All cells were harvested at mid-logarithmic phase in order to guarantee a high cellular rRNA content. After centrifugation in an Eppendorf Microfuge (5000 g, 1 min) the growth medium was discarded from the cell pellet. Cells were thoroughly resuspended in PBS (130 mM-sodium chloride, 10 mM-sodium phosphate, pH 7.2).

Cell fixation and preparation of cell smears. The cells were fixed in paraformaldehyde solution as described previously (Amann *et al.*, 1990b) and stored in PBS/ethanol at -20 °C for up to 3 months without any apparent influence on the hybridization results. These fixed-cell suspensions were spotted onto thoroughly cleaned glass slides (Paul Marienfeld KG, Bad Mergentheim, FRG) and allowed to air dry for at least 2 h. After dehydration in 50, 80, and 98% (v/v) ethanol (3 min each) the slides were stored dry at room temperature.

Oligonucleotide sequences. The following oligonucleotides were used in this study: (i) Eub338, a probe complementary to the 16S rRNA of all Bacteria (Amann *et al.*, 1990b); (ii) Pce54a (5'-CCCATCGCATC-TAACAAT-3'), specific for the 23S rRNA of *Pseudomonas cepacia* (Höpfel *et al.*, 1989); (iii) Arch915, a probe complementary to the 16S rRNA of all Archaeobacteria (Stahl & Amann, 1991); and (iv) nonEub338, which is complementary to probe Eub338 and serves as a negative control for non-specific binding.

DIG-labelling of oligonucleotides. 5'-end labelling. The oligonucleotides were synthesized on a DNA synthesizer (Biosearch) and in the last cycle an amino-linker [6-(4-monomethoxytritylamino)hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite; Glen Research] was attached at the 5'-end. Cleavage from the support and deprotection were done according to the manufacturer's guidelines. Approximately 100 µg of crude oligonucleotide in 125 µl 50 mM-sodium borate buffer (pH 8.6) was reacted with 150 µg of digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (DIG-NHS; Boehringer Mannheim) in 125 µl dimethylformamide. This mixture was incubated at room temperature for 14 to 20 h. Unreacted DIG-NHS was separated from the oligonucleotide by size-exclusion-chromatography on Sephadex G25 columns (NAP-5, Pharmacia). Oligonucleotide-containing fractions were loaded on a non-denaturing polyacrylamide gel (15%, w/v; Maniatis *et al.*, 1982) which separated unreacted oligonucleotide from the DIG-oligonucleotide conjugate. The nucleic acids were visualized by UV shadowing and the gel region containing the conjugate was excised with a razor blade. In all cases the conjugate was retarded relative to the unreacted oligonucleotide. The conjugate was passively eluted from the polyacrylamide gel into 1 ml TE buffer (10 mM-Tris/HCl, 1 mM-EDTA, pH 7.2). Finally, salts were removed and the oligonucleotides were concentrated on Nensorb columns (NEN DuPont). After lyophilization the oligonucleotide was dissolved in sterile water (0.2 µm filter) to a final concentration of 50 ng µl⁻¹. The DIG-labelled probe could be stored for at least 6 months without apparent loss of quality.

3'-end-labelling. Single molecules of digoxigenin-dideoxyuridinetriphosphate (Boehringer) were covalently bound to the 3' ends of gel-purified oligonucleotide by the enzyme terminal transferase according to the manufacturer's instructions (Boehringer Mannheim). Using this enzymic approach, no amino-link on the oligonucleotide was required and the labelled probe was available within several hours.

In situ hybridization using digoxigenin-labelled oligonucleotides. An aliquot of 8 µl hybridization solution (0.9 M-sodium chloride, 20 mM-Tris/HCl, 0.01% SDS, pH 7.2) containing 50 ng oligonucleotide probe was applied to each well of the prepared slide and incubated for 2 h at 45 °C in an isotonicity equilibrated humid chamber.

Detection of DIG-labelled oligonucleotides with fluorescently labelled anti-DIG F_{ab} fragments. Carboxyfluorescein or carboxyrhodamine 101-labelled anti-DIG F_{ab} fragments (Boehringer Mannheim) were diluted 1:4 in a solution containing 150 mM-sodium chloride, 100 mM-Tris/HCl (pH 7.5), 0.5% bovine serum albumin and 0.5% blocking reagent (Boehringer Mannheim); 10 µl aliquots of these dilutions were added to each well and incubated with the labelled probe for another hour at 27 °C in the humid chamber. The slide was removed from the humid chamber and immersed in 40 ml washing solution (150 mM-sodium chloride, 100 mM-Tris/HCl, 0.01% SDS, pH 7.4) at 29 °C for 10 min. Slides were briefly rinsed with sterile water (0.2 µm filter), air dried and mounted in Citifluor (Citifluor Ltd, London, UK). The cell smears were viewed with a Plan-Neofluar 100× objective (oil immersion) on a Zeiss Axioplan microscope fitted for epifluorescence microscopy with a 50 W mercury high pressure bulb and Zeiss filter sets 09 and 15. Photomicrographs were done on Kodak T_{max} 400; exposure times were 15 s for epifluorescence and 0.03 s for phase contrast micrographs.

Flow cytometric analysis. Approximately 10^7 to 10^8 fixed cells suspended in 10 µl PBS/ethanol were slowly mixed with 100 µl ice-cold absolute ethanol. The cells were pelleted in an Eppendorf microfuge (5000 g, 5 min) and after removal of the supernatant, thoroughly resuspended in 8 µl of hybridization buffer. DIG- or FLUOS-labelled oligonucleotides (50 ng) were hybridized to the cells for 2 h at 46 °C. Then 10 µl of a 1:4 dilution of FLUOS-labelled anti-DIG F_{ab} fragments were added to the hybridization performed with DIG-labelled probe and incubated for an additional hour at 27 °C. Probe and antibody molecules were removed from the cells by centrifugation, discarding the supernatant. The cells were washed briefly at room temperature in 200 µl hybridization solution, which was discarded after centrifugation, and finally resuspended in 0.5 ml PBS solution. Samples were either examined immediately or stored on ice without apparent changes in signal intensity. Measurements were performed on a FACStar PLUS flow cytometer (Becton Dickinson). The 6W-argon ion laser (Innova; Coherent, Palo Alto, CA, USA) was tuned for 488 nm emission (500 mW), fluorescence was measured as light passing a 530 nm band pass filter (BD530DF, Becton Dickinson). Signals were collected using the 'stream in the air' method at a rate of approximately 200 events s^{-1} to a total of 5000 events.

Detection of DIG-labelled oligonucleotides with enzyme-conjugated anti-DIG F_{ab} fragments. Alkaline-phosphatase- and horseradish-peroxidase-conjugated anti-DIG F_{ab} fragments were purchased from Boehringer Mannheim. The conditions for hybridization and antibody binding are unaltered compared to the use of fluorescent antibodies, however, a pretreatment of the cell smears prior to hybridization was necessary. Fixed cells were incubated with 1 mg ml^{-1} lysozyme in 100 mM-Tris/HCl, 50 mM-EDTA (pH 8.0) for 10 min at 0 °C prior to hybridization. Lysozyme was removed by thoroughly rinsing the slide with H_2O followed by air drying. Alkaline phosphatase was visualized by formation of a colour precipitate. The substrate was a dilution of 45 µl nitroblue tetrazolium (75 mg ml^{-1} in 70% v/v, dimethylformamide) and 35 µl 5-bromo-4-chloro-3-indolylphosphate (50 mg ml^{-1} in

dimethylformamide) (both components from Boehringer Mannheim) in 10 ml 100 mM-Tris/HCl, 100 mM-sodium chloride, 50 mM-magnesium chloride (pH 9.5) as described by Leary *et al.* (1983). An aliquot of 50 µl of the described mixture was applied to each well and incubated at room temperature in a humid chamber for 1 to 12 h. The reaction product was purple and water insoluble, and was visualized by conventional microscopy in bright field or phase contrast configuration.

Horseradish peroxidase was detected accordingly by formation of a purple-blue precipitate from diaminobenzidine using the nickel chloride enhancement technique (Hsu & Soban, 1982). The substrate was a mixture of diaminobenzidine (1.3 mM), H_2O_2 (0.02% v/v), nickel chloride (0.03% w/v), and Tris/HCl (5 mM, pH 7.4). Photomicrographs were taken on Agfapan 25 (Agfa-Gevaert AG, Leverkusen).

Results and Discussion

Specific hybridization in situ of DIG-labelled oligonucleotide to rRNA and detection of the hybrid by fluorescent anti-DIG F_{ab} fragments

Oligonucleotides labelled with DIG at the 5'- or 3'-end hybridized equally well to rRNA molecules in whole fixed cells, and the reporter molecule could be detected *in situ* by fluorescently labelled anti-DIG F_{ab} fragments. In a mixture of pure cultures of the two Gram-negative bacteria *P. cepacia* and *E. coli*, and the Archaeobacterium *Methanococcus vannielii*, the bacterial probe Eub338 hybridized only to cells of *P. cepacia* and *E. coli* (Fig. 1a and b). With probe Pce54a we could specifically detect cells of *P. cepacia* in the same mixture (Fig. 1c and d). Hybridization with the bacterial probe Eub338 assured that the rRNA was accessible and that bound DIG molecules were detectable in both Gram-negative species. Additional experiments using a DIG-labelled oligonucleotide not complementary to rRNA (nonEub) did not show any interfering levels of non-specific binding of DIG-labelled nucleic acid probes and FLUOS-labelled anti-DIG F_{ab} fragments or an elevated level of autofluorescence for any of the cell types used in this study. We could demonstrate accessibility for hybridization probes and anti-DIG antibodies for several more Gram-negative bacteria. However, detection of *M. vannielii* with the Archaeobacteria-specific probe Arch915 was only possible with fluorescently end-labelled probe but not with our indirect approach (data not shown). This may best be explained by free penetration of the low-molecular-mass oligonucleotide probe and exclusion of the higher-molecular-mass antibodies. Previously, detection of Gram-positive bacteria using this indirect labelling approach was only achieved for *Bacillus subtilis* after a partial digestion of the cell wall with lysozyme.

Fig. 1. Specific identification of whole fixed cells with DIG-labelled oligonucleotide probes and fluorescent anti-DIG antibodies. Phase contrast (a, c) and epifluorescence (b, d) photographs are shown for each field. *P. cepacia* (chains of rods), *E. coli* (rods) and *M. vanielii* (cocci) hybridized with DIG-labelled bacterial probe Eub338 (a, b) and with DIG-labelled probe Pce54a specific for *P. cepacia* (c, d). DIG molecules were detected with FLUOS-labelled anti-DIG F_{ab} fragments.

Table 1. Mean fluorescence intensities per cell after *in situ* hybridization as determined by flow cytometry

Probe	Label*	<i>P. cepacia</i>	<i>E. coli</i>	<i>P. fluorescens</i>
Eub338	DIG + A _F	146.6	53.5	15.8
	F	151.6	41.3	20.7
Pce54a	DIG + A _F	106.6	3.9	2.7
	F	57.0	2.2	2.4
nonEub	DIG + A _F	13.0	ND	ND
	F	2.0	ND	ND
-	+ A _F	14.1	3.1	2.9
-	-	2.0	1.8	1.9

ND, Not determined.

* DIG + A_F: probe labelled with DIG at the 5'-end and detected with FLUOS-labelled anti-DIG F_{ab} fragment (A_F). F: probe labelled with FLUOS at the 5'-end.

Flow cytometric analysis

We used a flow cytometer to quantitate the results obtained with direct and indirect labelling. The data are summarized in Table 1. Assuming identical hybridization efficiency of the FLUOS- and DIG-labelled probes, and binding of one multiple labelled antibody to each DIG molecule one could expect a signal increase of three- to fourfold since on average each anti-DIG F_{ab} fragment is labelled with three to four fluorescent dye molecules (according to manufacturer's specifications). However, the mean fluorescence values using the direct and indirect approach were not significantly different. In two comparisons – *P. cepacia* with probe Eub338 and *P. fluorescens* with probe Eub338 – the cells hybridized with directly labelled probe were slightly more fluorescent. In two other cases – *E. coli* with probe Eub338 and *P. cepacia* with probe Pce54a – the indirect approach yielded higher fluorescence. Cells hybridized with probes not complementary to their rRNA in two examples: *E. coli* with probe Pce54a and *P. fluorescens* with probe Pce54a showed slightly higher non-specific fluorescence for the indirect approach (3.9:2.7) as opposed to the directly labelled probes (2.2:2.4) and the mean autofluorescence of unhybridized fixed cells (1.8:1.9). However, in the case of *P. cepacia* cells hybridized with probe nonEub, the non-specific binding was significantly increased to a mean of 13.0 (indirect method) from 2.2 (direct method). This increased fluorescence was not due to binding of the nucleic acid probe but to non-specific binding of the fluorescently labelled anti-DIG F_{ab} fragments, as proved by a control experiment in which the cells were incubated with the antibody without probe addition (mean fluorescence 14.1). This non-specific binding did not appear during the slide assay which included a more rigorous washing

procedure compared to the flow cytometric analysis. This non-specific binding of fluorescent antibody to *P. cepacia* cells could also partially contribute to the higher mean fluorescence of *P. cepacia* detected by Pce54a in the indirect approach (106.6) as compared to the direct approach (57.0).

So far identification of specific cells with DIG-labelled oligonucleotides with subsequent localization of the DIG with fluorescent antibodies does not offer a signal enhancement compared to fluorescently labelled oligonucleotides. We have not determined whether this originates from a lower hybridization efficiency of DIG-labelled oligonucleotides compared to fluorescent oligonucleotides or from incomplete binding of antibodies to the bound DIG reporter molecules. There is a chance that antibodies might be further improved to show a higher binding efficiency or that more fluorescent labels can be attached to one antibody. The non-specific binding of Pce54a to *P. cepacia* cells highlights an intrinsic disadvantage of indirect approaches which are prone to more specificity problems. These can be avoided by adequate washing conditions but controls have to be part of each experiment, especially if complex environmental samples are examined.

It was also obvious from the results that the maximum signal intensity obtained was dependent on the cells examined. The *P. cepacia* cells used in this study yielded a mean fluorescence approximately one order of magnitude higher than the *P. fluorescens* cells. This reflected differences in the cellular rRNA content originating from different cell size and physiological activity. The phenomenon that identical cells do not show identical fluorescence intensities after hybridization to different but perfectly complementary probes was well quantified in this series of experiments. Probe Pce54a conferred less fluorescence to *P. cepacia* cells as compared to the bacterial probe Eub338 in the direct and indirect approach. This might be caused by different accessibilities of the target sites on the rRNA. Higher order structures are probably of more significance for *in situ* hybridizations than for hybridizations to isolated nucleic acid.

Detection of DIG-labelled probe in fixed bacterial cells using enzyme-conjugated anti-DIG-antibodies

Bound DIG-labelled probes could also be detected in whole fixed cells with either horseradish-peroxidase- or alkaline-phosphatase-labelled anti-DIG F_{ab} fragments. The enzyme-labelled antibodies had penetrated into the fixed cells since the substrates were precipitated within the cells. The detection was specific as demonstrated in Fig. 2 where probe Eub338 (*a, b*) has again hybridized to cells of *P. cepacia* and *E. coli*, and probe Pce54a to

Fig. 2. Specific identification of whole fixed cells with DIG-labelled oligonucleotide probes and enzyme-linked anti-DIG antibodies. Phase contrast (*a*, *c*) and bright field (*b*, *d*) micrographs are shown for each field. *P. cepacia* (chains of rods), *E. coli* (rods) and *M. vanielii* (cocci) hybridized with DIG-labelled bacterial probe Eub338 (*a*, *b*) and with DIG-labelled probe Pce54a specific for *P. cepacia* (*c*, *d*). DIG-molecules were detected with alkaline phosphatase-linked anti-DIG F_{ab} fragments. Alkaline phosphatase molecules were localized by substrate precipitation.

P. cepacia (c, d). No epifluorescence is needed to detect the coloured precipitates which will considerably cut down on the cost of equipment needed for single cell detection. Phase contrast microscopy (Fig. 2a and c) visualizes all cells and allows the identification of cells containing precipitate. Bright field illumination (Fig. 2b and d) makes discrimination even easier since only the coloured cells are clearly visible. This approach is not hampered by autofluorescence, known to be a considerable problem in the inorganic and organic fractions of environmental samples as well as in certain microorganisms. Furthermore, enzyme-linked assays have a considerably better sensitivity as compared to fluorescent assays. By prolonging the time of substrate precipitation, the signal may be increased accordingly and it seems likely that cells with low rRNA content may also be detected.

The linkage of enzymes to F_{ab} fragments results in the formation of high-molecular-mass complexes. Not surprisingly the penetration of this detection complex into the cells is even more difficult than for fluorescently labelled F_{ab} fragments. Hitherto we can only show penetration into Gram-negative bacteria after mild lysozyme treatment. The detection of Gram-positive bacteria and Archaeobacteria will only be possible after protocols for partial digestion of the cell walls are developed.

Future directions

This study focussed on the sensitivity problem encountered when rRNA targeted probes are used for the identification of individual cells in natural samples. We have demonstrated that oligonucleotides labelled with DIG can be detected within fixed cells by specific antibodies. Fluorescent antibodies did not increase sensitivity but the enzyme-linked assay has the potential for significant signal amplification. Since penetration of the large antibody-enzyme complex currently limits the application to Gram-negative bacteria, we will try to use probes directly linked to enzyme molecules. A rapid and sensitive method for direct identification and enumeration of micro-organisms seems to be in reach.

Flow cytometric analyses were performed at the GSF-Forschungszentrum für Umwelt und Gesundheit, Institut für biophysikalische Stahlforschung, Arbeitsgruppe Durchflusszytometrie in München-Neuherberg. We thank Dr Beisker for advice and technical assistance. We also thank Dr Hans Paerl (Institute of Marine Science, University of North Carolina, Morehead City, USA) for helpful discussions on cell permeabilization for antibodies and Drs Mühlegger and Walter (Boehringer Mannheim) for supplying us with DIG labelling reagents. This work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

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