Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung

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*Pseudomonas aeruginosa* colonizing the lung of cystic fibrosis patients is responsible for a decline in health and poor prognosis for these patients. Once established, growth of *P. aeruginosa* in microcolonies makes it very difficult to eradicate the organisms by antimicrobial treatment. An artificial sputum medium was developed to mimic growth of *P. aeruginosa* in the cystic fibrosis lung habitat and it was found that the organisms grew in tight microcolonies attached to sputum components. Several genes, such as *algD*, *oprF* and *lasR* but not *fliC*, were required for tight microcolony formation. Among the sputum components, amino acids, lecithin, DNA, salt and low iron were required for tight microcolony formation. Amino acids were also shown to be responsible for various other cystic-fibrosis-specific phenotypes of *P. aeruginosa*, such as diversification of colony morphology, alterations in LPS structure and hyperexpression of OprF. Since the amino acid content of sputum is elevated in severe lung disease, it is suggested that the tight microcolony biofilm is maintained in these conditions and that they contribute to the vicious cycle of disease severity and failure to eradicate the organism. Thus, growth of *P. aeruginosa* in artificial sputum medium is an appropriate model of chronic lung colonization and may be useful for evaluating therapeutic procedures and studying antibiotic-resistance mechanisms.

INTRODUCTION

Cystic fibrosis (CF), an autosomal inherited disease of Caucasians (Gibson *et al.*, 2003), is caused by dysfunction of the CF transmembrane conductance regulator (CFTR). This disease predisposes patients to a broad spectrum of disease phenotypes including failure of lung function, which is the major cause of mortality among these patients. A limited spectrum of micro-organisms can colonize the CF lung, and *Pseudomonas aeruginosa* in particular is most associated with lung deterioration and a decline in health. Once established, *P. aeruginosa* is often refractory to antibiotic treatment, although high concentrations of some agents such as tobramycin can be achieved in the lung by inhalation (Ramsey *et al.*, 1999). This persistence in the presence of antimicrobial agents is considered to be largely due to a biofilm mode of growth (Lam *et al.*, 1980; Singh *et al.*, 2000), whereby *P. aeruginosa* grows in microcolonies surrounded by an extracellular matrix of the exopolysaccharide alginate. Besides alginate overproduction, *P. aerugi-

Abbreviations: ASM+, basic artificial sputum medium; ASM-, artificial sputum medium without amino acids; CF, cystic fibrosis.
types (Govan, 1975; Ohman & Chakrabarty, 1982; Wang et al., 1996; Yoon et al., 2002).

Current conventional biofilm models observe cells attached to a solid biotic or abiotic surface in steady-state culture or under continuous flow (Christensen et al., 1999; Boddicker et al., 2002). However, P. aeruginosa grows in the CF lung under anaerobic conditions as microcolonies, a biofilm in which bacteria adhere to each other and to sputum components, but not to a surface (Lam et al., 1980; Baltimore et al., 1989; Worlitzsch et al., 2002). Recently, an anaerobic biofilm model was developed in which the bacteria overexpress the outer-membrane protein OprF, a feature that could be correlated with the in situ situation (Yoon et al., 2002). Neither this nor other models could demonstrate the ability of flagella negative strains to form biofilms (O'Toole & Kolter, 1998), but P. aeruginosa isolates from CF patients are frequently non-motile (Mahenthiralingam et al., 1994).

We have developed an artificial sputum medium with a composition that closely resembles CF sputum. In this medium, P. aeruginosa grows in microcolonies, thus presenting a novel model for biofilm formation in the CF lung. Various features of CF isolates, among others, flagella-independent microcolony formation, were displayed by P. aeruginosa grown in the artificial sputum medium.

**METHODS**

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1. Besides FRD1, all isolates were nonmucoid. P. aeruginosa PAO1C (ATCC15692) was purchased from the American Type Culture Collection (ATCC).

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>PAO1C</td>
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<td>This study</td>
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<tr>
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<td>lecA::luxCDABE genomic reporter fusion in PAO1</td>
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<td>PAO1 algD::Gm</td>
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<tr>
<td>PAK</td>
<td>Wild-type</td>
<td>Feldman et al. (1998)</td>
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<td>Woolwine &amp; Wozniak (1999)</td>
</tr>
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<td>FRD875</td>
<td>FRD1 mucA22 algD::xyE aacC1</td>
<td>Woolwine &amp; Wozniak (1999)</td>
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<td>SG50M</td>
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<td>P7-2</td>
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<td>Römling et al. (1994a)</td>
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**Artificial sputum medium and growth conditions.** The basic artificial sputum medium (ASM+) was 5 g mucin from pig stomach mucosa (NBS Biologicals), 4 g DNA (Fluka), 5 mg diethylenetriamine pentaacetic acid (DTPA) (Sigma), 5 g NaCl, 2.2 g KCl, 5 ml egg yolk emulsion (phosphatidylcholine as source of lecithin) (Oxoid) and 5 g amino acids per 1 l water (pH 7.0) modified after Ghani & Soothill (1997). The same medium without amino acids was designated ASM−. For a standard experiment, an inoculum equivalent to OD600 0.05 of an overnight culture of P. aeruginosa PAO1C grown in tryptone soy broth (TSB) was added to 1 ml of sputum medium in 24-well cell-culture plates and incubated for 16 h at 37 °C with gentle shaking. To determine the growth rate, viable counts were performed with cultures grown for 6 h. Cells were treated for 30 min with cellulase (1 mg cellulase ml−1, 400 μg chloramphenicol ml−1 in 0.05 M citrate buffer, pH 4.6) to dissolve the clumps and plated on agar medium. Reconstituted sputum extracts (see below) were also used as growth media and the uninoculated sputum extract was used as a control. Viable and dead bacteria were discriminated using the LIVE/DEAD BacLight Bacteria Viability Kit (Molecular Probes) according to the instructions of the manufacturer. This kit is based on the membrane-permeant SYTO 9 dye, which labels live bacteria with green fluorescence, and propidium iodide, which labels membrane-compromised (non-viable) bacteria with red fluorescence.

**Adherence to surface.** Adherence of P. aeruginosa to polystyrene wells was investigated by crystal violet staining in 24-well plates as described previously (Römling et al., 1998).

**Assessment of phenotypic diversity.** For long-term incubation up to 45 days, standing cultures were set up in a conical flask 60 % filled with relevant medium. These were subcultured daily up to day 15, and at day 30 and day 45. Approximately 1000 colonies were examined for changes in colony morphology after overnight growth on tryptone soy agar (TSA) plates.

**Transposon mutagenesis.** Transposon mutants were created by introduction of the Tn5-transposon-based transposome using EZ::TN Tnp transposome (Epiconcentre) into P. aeruginosa PAO1C by...
Electroporation (2-0 kV, 25 μF, 200 Ω). *P. aeruginosa* transformants were selected for kanamycin resistance, restreaked, transferred to 96-well plates containing ASM+ and incubated overnight at 37 °C with shaking. Genomic DNA was isolated from appropriate mutants using the Nucleospin Tissue kit (Macherey-Nagel), digested with *PstI* and ligated under conditions favouring intramolecular ligation. Insertion points of the kanamycin-resistance cassette were identified by sequencing of the fragment obtained by inverse PCR using primers provided by the manufacturer.

**Preparation of sputum extracts.** Sputum extracts were prepared by adding an equal volume of sterile double-distilled water to the sputum. After centrifugation for 10 min at 14,000 g, the supernatant was passed through a 0.45 μm membrane filter (Millipore) and the pellet was saved. The filtrate was subsequently used for amino acid analysis or as bacterial growth medium when reconstituted with 20 μl of the resuspended pellet.

**Quantification of free amino acids in sputum.** Free amino acids in the sputum extracts were quantitatively derivatized at the amino group, either primary or secondary, using phenylisothiocyanate (PITC) in the presence of a base, disopropylethylamine (DIEA) (Applied Biosystems 420A Derivatizer). The derivatives, phenylthiocarbamyl-amino acids (PTC-aa) were analysed by HPLC (Applied Biosystems 130A Separation system) and quantified by comparison to standard concentrations of corresponding amino acids.

**Analysis of LPS.** LPS from *P. aeruginosa* was isolated as described elsewhere (Hitchcock & Brown, 1983) and preparations were analysed by SDS-PAGE using 15 % discontinuous gels (Tsai & Frasch, 1982). Low-molecular-mass core-lipid A bands were resolved in 16-5 % Tricine SDS-PAGE gels (Lesse et al., 1990). For Western blot analysis, LPS bands from conventional and Tricine SDS-PAGE were transferred to polyvinylidenefluoride membranes (Millipore) and blocked with 5 % skimmed milk. The blotted membrane was incubated with mAbs 177, 7-4, 101 and N1F10 specific for lipid A, inner core, outer core and A-band (common conserved antigen), respectively (de Kievit & Lam, 1994), and a polyclonal antibody against the B-band (the variable O-antigen with O-chain repeating units). The blots were developed at room temperature with goat anti-mouse F(ab')2 conjugated antibody and detected using Lumilight (Roche).

**Proteome analysis.** Whole-cell proteins were extracted, isoelectrically focused in a linear pH range 4–7 in the first dimension and mass-separated in a 12–15 % gradient polyacrylamide gel in the second dimension (Gorg et al., 2000). The most prominent spot was identified as OprF. Tryptic digest of the eluted protein was performed and analysed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), where eight of 19 peptides matched. Sequence analysis of peptides was done by MS/MS using Micromass Q-Tof-2 (Micromass) and the peptide sequences DLVNYEYGVEGGR and QYPSTSTTVEGHTD(SV)GYTGD supported identification of OprF.

**Electron microscopy.** Bacteria in microcolonies were fixed, embedded and ultrathin sectioned (Yakimov et al., 1998). Samples for scanning electron microscopy were prepared as described previously (Lünsdorf et al., 2001).

**RESULTS**

**Microcolony formation of *P. aeruginosa* PAO1C in artificial sputum medium**

To study biofilm formation of *P. aeruginosa* under conditions that closely resemble the CF lung habitat, we developed an artificial sputum medium (ASM+) that contained components such as mucin, DNA, surfactant, salt ions, iron and amino acids in concentrations found in an average sputum of a patient with CF (Ghani & Soothill, 1997). When *P. aeruginosa* PAO1C was grown in this medium, the cells formed macroscopically visible clumps (microcolonies), which could not be disrupted even by vigorous pipetting (Fig. 1). Light microscopy verified the formation of tight microcolonies with almost no planktonic cells. Staining of the well by crystal violet showed that the bacterial cells did not attach to a polystyrene and glass surface (Fig. 1, data not shown). The microcolonies formed by growth in ASM+ were termed ‘tight’ and those in ASM− were ‘loose’.

A similar pattern of growth in ASM+ was observed for one CF clinical isolate and three environmental isolates of *P. aeruginosa*. Each of the isolates grew in the loose microcolony mode in ASM−.

To investigate the components in the ASM+ that influenced the formation of tight microcolonies, *P. aeruginosa* PAO1C was grown in artificial sputum medium with individual components omitted stepwise (Fig. 2a). When amino acids were not available, the visible microcolony appeared smaller due to the increased number of planktonic cells (Fig. 1). The cells were easily disrupted by pipetting, confirming that they were only loosely connected, and light microscopy showed the bacterial cells to be associated loosely with each other in a background of planktonic cells (Fig. 1).

In the absence of mucin there was little growth, suggesting that mucin is a major energy source for *P. aeruginosa* in this medium. The lack of mucin also caused significant adherence of *P. aeruginosa* PAO1C to the walls of the polystyrene well, with the formation of a conventional biofilm. Lecithin, DNA, the salt components and a low iron content were required to a variable extent for the formation of tight microcolonies (Fig. 2a). In addition, we observed that a change in the surface area to volume ratio from 2 to 3:3 influenced tight microcolony formation (data not shown), suggesting a role for oxygen in the aggregative behaviour of the bacteria (Worlitzsch et al., 2002). In conclusion, all the components in the artificial sputum medium contributed to tight microcolony formation of *P. aeruginosa* PAO1C (Figs 1 and 2).

A dose-dependent correlation between amino acid content and tight microcolony formation was observed (Fig. 2b). Tight microcolonies began to be formed in approximately 0.5 mg amino acids ml−1. This observation likely reflects the situation of bacteria–host interactions *in vivo*, since tight microcolony formation of *P. aeruginosa* PAO1C was found to correlate with the amount of amino acids in sputum extracts from CF patients (Fig. 2c).

Individual amino acids had complex effects on growth and the degree of tight microcolony formation in the artificial sputum medium (Fig. 2d). Tryptophan was found to be required for tight microcolony formation; however, bacterial growth was restricted when tryptophan was used alone as the amino acid supplement.
Genes involved in *P. aeruginosa* microcolony formation in ASM+

To determine which gene products contribute to tight microcolony formation, selected genes previously reported to be required for conventional biofilm formation were investigated (Fig. 3). First, strains deficient in the production of structural components, such as extracellular polysaccharide alginate (*algD*) mutant, type IV pili (*pilB*) mutant and the flagellum (*fliC*) mutant, were compared with their respective isogenic wild-type strains (Table 1, Fig. 3 and data not shown). While alginate and pili affected tight microcolony formation, the lack of the flagellum had no effect. Since there is currently a debate about whether alginate production is required for conventional biofilm formation (Wozniak et al., 2003), we tested another *algD* mutant in a different strain background [FRD1 and FRD875 (*algD::xylE*)], with essentially the same results (data not shown).

Quorum sensing signals play a role in the development of biofilm architecture and their absence leads to less-severe chronic infections in mice (Wu et al., 2001). Therefore, *lasR* (senses the quorum sensing signal molecule 3O-C12-HSL) and *rhlR* (senses C4-HSL) mutants and their respective wild-type strains were tested. While *lasR* was required for tight microcolony formation, the *rhlR* mutant behaved similarly to the wild-type (Fig. 3).

To find novel genes, a panel of 270 transposon mutants were screened for the loss of tight microcolony formation. Transposon insertions were identified in *oprF*, *rmlA* and *sucC* (Table 1).

**CF-specific phenotypes of *P. aeruginosa* grown in artificial sputum medium**

We tested whether cultivation of *P. aeruginosa* in artificial sputum medium with (ASM+) and without (ASM−) amino acids resembles growth in the CF lung. *P. aeruginosa* PAO1C grew slowly in both media, with generation times of 2.3 h (ASM+) and 3.4 h (ASM−). After 16 h of growth, few dead cells were detected in ASM−, but a significant number of cells...
were not viable in ASM+. Such a slow growth rate and nonviable cells might reflect the situation in the lung. Otherwise the high number of bacterial cells that are reached in the lung would probably be fatal for the patient. Scanning and transmission electron microscopy showed an abundance of extracellular matrices around the cells in ASM+ medium and in a sputum extract, but these structures were rare around cells grown in ASM− (Fig. 4).
In vitro diversification of *P. aeruginosa* colony morphology

A unique characteristic of *P. aeruginosa* growing in the CF lung is phenotypic diversification. To test the effect of the growth medium on colonial variation, *P. aeruginosa* PAO1C was incubated in TSB, ASM+ and ASM− up to 45 days. After 45 days no change in colony morphology was observed on subculture from TSB or ASM− (Fig. 5). However, cells grown in ASM+ yielded mucoid colonies after only 1 day. After 7 days of growth in ASM+, colony variation occurred in approximately 5–10% of colonies. After 45 days, the majority of the colonies were dwarf or mucoid, but mucoidy was unstable and lost on subculture. Rough colonies and small-colony variants were other morphologies that closely resembled forms frequently isolated from CF patients.

**Modification of LPS in artificial sputum medium**

The LPS is also subject to adaptive mutations in the CF lung habitat (Ernst *et al.*, 1999). Following growth of *P. aeruginosa* PAO1C in ASM+ and ASM−, lipid A modification became apparent as lipid A was not detectable with the lipid A-specific mAb 177 (Fig. 6). A similar observation was made when *P. aeruginosa* PAO1C was grown in one of the sputum extracts (Fig. 6a). A low abundance of A-band antigen, the common antigen, was observed in ASM+ as well as when *P. aeruginosa* PAO1C was grown in the sputum extracts. Core-plus-one O-antigen repeat (core +1) was overexpressed when PAO1C was grown in ASM with and without amino acids as well as in one of the sputum extracts.

**Proteomics**

The protein expression patterns of *P. aeruginosa* PAO1C in TSB, ASM+ and ASM− were compared by two-dimensional gel electrophoresis. Abundant expression of OprF was found only in ASM+ (Fig. 7).

**DISCUSSION**

In this study a novel biofilm model, tight microcolony formation in artificial sputum medium, that mimics *P. aeruginosa* growth in the CF lung is presented. *P. aeruginosa* exhibited similar characteristics when grown in artificial sputum medium and in sputum extracts. In addition, PAO1C, a burn wound isolate, grown in artificial sputum medium displayed characteristics of isolates from the CF lung.

For some time *P. aeruginosa* has been the model organism for biofilm formation and its growth in microcolonies in the CF lung has been the paradigm of a chronic disease caused by biofilm-forming bacteria (Lam *et al.*, 1980). Several biofilm models have been developed for *P. aeruginosa* over time, and most of them measured biofilm formation on a solid surface (Yoon *et al.*, 2002). However, several studies have shown that *P. aeruginosa* grows in the CF lung in microcolonies attached predominantly to mucin (Baltimore *et al.*, 1989; Lam *et al.*, 1980; Wörlich et al., 2002). We were able to demonstrate that *P. aeruginosa* grows readily as microcolonies when grown in a medium that reflects the chemical environment in the CF lung. Such a special biofilm, in which cells adhere to each other but not to the surface, is unconventional but in agreement with the definition of Costerton *et al.* (1995) that biofilms are ‘matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces’.

Although the flagellum has been shown to have a role in biofilm formation, the fact that the majority of CF isolates are usually non-motile and do not express the flagellin protein (Mahenthiralingam *et al.*, 1994) raises the question about the direct involvement of the flagellum in CF-associated *P. aeruginosa* biofilms. Since in the artificial sputum medium
tight microcolonies were formed without the requirement of the flagellum, this evidence supports the non-motile attributes of isolates from CF sputum samples and further contradicts the necessity of the flagellum for biofilm formation (Yoon et al., 2002). However, this does not rule out the role of the flagellar cap protein FliD for mucin adhesion by P. aeruginosa (Arora et al., 1998).

Nevertheless, our model system showed similarities to previous models (O’Toole & Kolter, 1998; Yoon et al., 2002). Structural components such as the exopolysaccharide alginate, the type IV pili, LPS or rhamnolipids and the outer-membrane protein OprF are required for tight microcolony formation. In contrast to Mahenthiralingam et al. (1994), we rarely observed complete loss of twitching motility in isolates of a prevalent CF clone (unpublished data), which supports an in vivo role of type IV pili in microcolony formation. LPS and rhamnolipids have been shown to contribute to conventional biofilm formation (Beveridge et al., 1997; Davey et al., 2003). RmlA encodes the first enzyme in the biosynthesis of deoxythymidine-diphosphate (dTDP)-rhamnose, the precursor of L-rhamnose, a component of the core oligosaccharide of LPS and secreted rhamnolipids.

Changes in cell metabolism also appear to contribute to tight microcolony formation. SucC encodes the beta chain of succinyl-CoA synthetase, an enzyme of the Krebs cycle, which, in other systems, has been found to be required for

Fig. 4. Electron microscopy of P. aeruginosa PAO1C microcolonies. (a) Scanning electron micrographs of P. aeruginosa PAO1C microcolonies established in (1) ASM+, (2) ASM− and (3) sputum extract 5. (b) Transmission electron micrographs of P. aeruginosa PAO1C microcolonies established in (1) ASM+ and (2) ASM−. Bars: (a) 2 µm, (b) 1 µm.

Fig. 5. Morphological changes after long-term cultivation of P. aeruginosa PAO1C in (1) ASM+, (2) ASM− and (3) TSB for 45 days. Arrows: black, dwarf morphotype; dark grey, brown-pigmented morphotype; light grey, mucoid colony.
biofilm formation (Solano et al., 2002) or be upregulated in biofilms (Beloin et al., 2004). We also demonstrated the contribution of the 3O-C12-HSL quorum sensing signalling pathway to tight microcolony formation and a similar production of signalling molecules in ASM+ and a sputum extract. It has been demonstrated that N-acyl homoserine-lactones are produced in vivo (Middleton et al., 2002).

The most intriguing finding was that almost all of the sputum components were required for tight microcolony formation, for example eukaryotic DNA. Recently, it was shown that bacterial DNA was required for biofilm formation (Whitchurch et al., 2002), but in sputum, eukaryotic DNA released by neutrophils is much more abundant. Inhalation of DNase I is an established therapy that has been shown to improve lung function and reduce pulmonary exacerbations in CF patients. Generally, the mechanism of DNase I therapeutics is the reduction of sputum viscosity, thereby facilitating expectoration (Gibson et al., 2003). However, reduced microcolony formation, which facilitates eradication of bacteria, might also contribute to improved health development after DNase I treatment.

Besides being required for attachment, mucin was found to be a major nutrient source, but lecithin was also required. Based on previous studies, the sputum of CF patients is a nutrient-rich environment (Barth & Pitt, 1996; Ohman & Chakrabarty, 1982); however, an abundance of nutrients does not necessarily cause rapid growth of the organisms. Indeed, a slow growth rate of P. aeruginosa was observed with and without amino acids.

The non-specific porin OprF was upregulated only in artificial sputum medium with amino acids. Thus, the presence of amino acids might be a signal for enhanced OprF expression. Alternatively, the effect of amino acids might only be indirect. Recently, it has been shown that OprF is highly expressed in anaerobic biofilms, where it is required for anaerobic respiration (Yoon et al., 2002). In our model, the formation of tight microcolonies might already provide an environment with a sufficiently low oxygen tension.

We are aware of the fact that the artificial sputum medium described here is not entirely representative of sputum in the lung because other biologically active components, which could also serve as nutrients, are present in the sputum, including lactoferrin, oligopeptides and other lipids besides lecithin (Ohman & Chakrabarty, 1982; Sahu & Lynn, 1978). Nonetheless, P. aeruginosa grown in artificial sputum medium and sputum extracts exhibited similar characteristics to patients’ isolates.

The sputum from each individual patient has a unique composition, which varies with the underlying CF phenotype and disease progression. Since the amino acid content correlates with disease severity (Thomas et al., 2000) and tight microcolony formation of P. aeruginosa (this study), we envisage the initiation of a vicious cycle in the patient.
whereby a more severe disease phenotype reduces the probability of its eradication from the lung. The isolation of mucoid colonies from CF patients indicates that P. aeruginosa has established itself as a persistent resident and this correlates with poor prognosis. We speculate that the emergence and establishment of the mucoid form might also be connected with changes in amino acid content of the sputum.

Clearly, the artificial sputum medium does not resemble the acute phase of the infection but the established colonization of P. aeruginosa. In this phase of the infection process, antibiotic resistance of P. aeruginosa is the problem of utmost clinical importance. However, clinical observations did not find a correlation between resistance of P. aeruginosa to tobramycin and effective treatment of patients (Smith et al., 2003). Using the artificial sputum medium antibiotic-resistance mechanisms of P. aeruginosa forming microcolonies can be systematically investigated.

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