Pseudomonas Motility and Antibiotics Resistance

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Abstract

Knowledge about the biofilm formation and antibiotic resistance are resulting in identification of new targets for therapeutics against Pseudomonas infection. These one generally persist despite the use of long term antibiotic therapy. The ability of growing within a biofilm enhances their chances to protect themselves from host defenses, antibiotic therapies, and biocides. A necessary first step towards understanding the susceptibility of biofilms to antibiotics is to understand the mechanisms by which motility behavior is involved. To our knowledge, few studies had been undertaken to compare the implication of swimming-swarming and biofilm in antibiotics resistance. The relationship between these formations is debated in some Pseudomonas species literature. It should be noted that P. aeruginosa has been well-studied as a model organism for the study of these interactions. By contrast, biofilms formation and motility behavior in P. fluorescens has not been extensively analysed. Our data demonstrate that our isolates exhibited an important biofilm mass and were categorized as slime-producers. The morphological and microscopic analysis of biofilm formation in these isolates revealed a very complex, dynamic and biologically exciting view about the architecture. The results indicate that biofilm formation, swimming and swimming motility exhibited a significant effect of resistance toward the β-lactam antibiotics and there is an induced swimming tendril tip bacteria phenotype with the presence of some β-lactam antibiotics.

Keywords: Biofilm; Pseudomonas; Antibiotics resistance; Swimming; Swarming.

Introduction

By targeting various virulence factors novel therapies can be devised for the treatment of severe infections caused by Pseudomonas species. Current therapies focus on the use of antibiotics but the development of antibiotic resistance and expression of multiple virulence factors has led to the ineffectiveness of current therapies [1]. The main virulence determinants of Pseudomonas infections are not only bacterial surface factors, flagella, pili and lipopolysaccharide, but also active processes such as the secretion of toxins, biofilm formation, quorum sensing [2] as well as the involvement of swimming-swarming motility, motility enables P. aeruginosa to colonize different environments, attach to surfaces, and form biofilms [3].

P. aeruginosa and P. fluorescens can become resistant to certain antibiotics which further complicates the treatment of its infections. This resistance arises due to its ability to form biofilm which consist of bacterial communities embedded in an exopolysaccharide matrix (EPS). Because of their high resistance to antimicrobial and cleaning treatments [4,5], their biofilms contribute markedly to the persistence of pathogens on medical devices, leading to critical problems in terms of public health and a potentially major economic impact [6,7].

Biofilm formation is a major virulence factor contributing to the chronicity of infections. It is becoming increasingly clear that biofilm have an enormous impact on medicine [8].

Taken together swimming-swarming motility raises equally interesting implications, previous studies have shown that P. aeruginosa cells in swarming colonies can have distinct phenotypes from planktonic cultures, including increased antibiotic resistance [9]. Biofilm formation, swimming swimming motility and antibiotics resistance are significantly regulated, several studies have examined these phenomena in P. aeruginosa, however, no systematic analysis of swimming motility and its relationship has been carried in P. fluorescens. This study will present a current understanding of how swimming-swarming coordinate biofilm resistance in P. aeruginosa and P. fluorescens. In this regard, if motility participates in antibiotic resistance their flagella orientation may be modified by the presence of certain antibiotics and consequently it should not be random. Thus, a question remains open: have this motility a preferred link with certain antibiotics?

Materials and Methods

Culture preparation

All the reagents, chemicals and media used in the below mentioned experiments were purchased from Sigma Aldrich. The bacterial strains used in this study were: Pseudomonas aeruginosa (PS8) isolated from waste water and three strains of P. fluorescens (PS4, PS9, PS10) from different rhizospheres. Stock cultures were stored at -80°C in Trypticase soy broth and 15% glycerol. Tests conducted for their identification have been based on physiological, nutritional tests [10] and by the use of the Analytical Profile Index (API 20NE; bio Merieux Vitek). Prior to each experiment, a loopful of culture was grown in 10 ml of LB medium with incubation at 28 ± 2°C for 24 h.

Extracellular enzyme activity assay

Activities of extracellular enzymes were evaluated by the inoculation of 50 µl of cell-free, sterile-filtered supernatants from the stationary phase LB cultures (24 h, 37°C, 200 rpm) in holes (0.8 cm in diameter) stamped into substrate agar plates. The plates were incubated for 48 h at 37°C unless stated otherwise. Diameters of clear or turbid halos around the inocula indicated a positive reaction [11].

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Hemolytic activities were determined on LB agar supplemented with 5% sterile-filtered sheep blood. Protease activities were tested on 5% (w/v) skim milk agar plates. Clear zones around the inoculation hole indicated the production of hemolysins and proteases, respectively. A modified method of Habermann and Hardt [12] was employed for the estimation of phospholipases A and C activities. For this purpose, 1% (v/v) egg yolk enrichment was added to 50 mM Na–acetate buffer supplemented with 10 mM CaCl₂ and 1.5% (w/v) agar. A clear halo after 24 h of incubation indicated phospholipase A activity, a white precipitate around the inocula indicated phospholipase C activity. However the pectinolytic activity and starch hydrolysis were undertaken according to [13,14].

Microtitre plate biofilm formation assay

Mucoidy of the strains was assessed on Pseudomonas Isolation Agar. Pseudomonas strains were grown in Luria-Bertani broth at 28°C for 24 h. Cells were harvested by centrifugation (9000 g, 10 min), washed twice with sterile water, and then resuspended in phosphate-buffered saline (PBS) to a DO₀₅₉₀ of 0.4 (~2.10⁸ bacteria/mL). Cultures were transferred to standing culture vessels. Polystyrene 96-well microtiter plates were filled with 100 µL of culture per well. The cultures were allowed to stand at 28°C and 37°C/24 h for the specified times. The micro titer plates wells were gently washed three times with 150 µL of sterile water to remove loosely associated bacteria. After the incubation period, cultures were removed, and then dried at 30°C for 30 min. Samples were stained by the addition of the 1% crystal violet solution (100 µl) to each well above the initial inoculation level and incubated for 30 min. The vessels were then washed three times with 150 µL of sterile water. The intensity of crystal violet staining was measured.

The samples were incubated for 6 h for micro-titer plate for 24 h, after which the OD₅₉₀ values were measured on an ELISA plate reader. All samples were tested in seven independent wells. The amount of surface-attached biofilm was determined by using a modified crystal violet method [3].

Readings of replicates for each isolate were averaged and subtracted from the OD₅₉₀ reading of the negative control (wells containing uninoculated culture medium). OD₅₉₀ was used as indication of biofilm production. Isolates were classified as biofilm producers if OD₅₉₀ was ≥ 0.200 and further classified as strong, moderate, weak, or zero biofilm formers based on their final OD₅₉₀ reading [15].

Antibiotic resistance tests

All the antibiotics used in resistance tests were supplied from oxoid (Table 1), included β-lactams (P5, ATM30, P10, AMP10), Phenicoles (C30), Aminosides (S10), Cyclines (TE, DOX30), sulfamides (SXT), Macrolides (SP10, SP100) and quinolone (NA30). Resistances and susceptibilities to these antibiotics were determined using the disc diffusion method (Oxoid) in accordance with the CDS (Calibrated Dichotomous Sensitivity) method standard [16]. Inocula were prepared by suspending growth from LB agar plates in MHB broth to a starting concentration of 5x10⁸ c.f.u/mL.

Motility assays (swimming, swarming and twitching)

Motility agar plate assays for the determination of bacterial swimming, swarming, and twitching motility were performed as described previously [3,17], with light modifications. Each strain was incubated on LB agar plates free and supplemented with Tween 80 for 24 h at 28°C. Plates of LB medium solidified with 0.3% agar (for the assessment of swimming motility) were inoculated by stabbing colonies

| Class          | Penames                  | Disc  | Reference |
|----------------|--------------------------|-------|-----------|
| β-lactams      | Penicillin G             | P5    | CT0043B   |
|                | Penicillin G             | P10   | CT0043B   |
|                | Amoxicillin–clavulanic   | AMC30 | CT0043B   |
|                | antibiotic               |       |           |
|                | Ampicillin               | AMP10 | CT0030B   |
|                | Monobactames             | Aztreonam | ATM30  |
|                | Carbapenemes             | Imipenem | IPM10 |
| Macrolides     | Spiramycin               | SP10  | CT0232B   |
| Cyclines       | Tetracycline             | TE70  | CT0054B   |
|                | Doxycycline              | DOX30 | CT0018B   |
| Phenicoles     | Chloramphenicol          | CHL30 | CT0013B   |
| Aminosides     | Streptomycin             | STR10 | CT0047B   |
| sulfamides     | Trimethoprim–sulfamethoxazole | SXT25 | CT0052B   |
| Quinolone      | Nalidixic acid           | NAL30 | CT0031B   |

Table 1: List of antibiotics.

with a toothpick and inserting the end of the toothpick just below the surface of the agar. Three colonies were picked from three plates and incubated at 28°C until a migration halo appeared. We then spotted 5 µL of 3 independent suspensions of each strain onto LB medium plus 0.6% agar (swarming motility) and the plates were incubated until a migration halo appeared. For twitching motility, culture was stabbed through agar of LB plates (1% agar) to the bottom of the Petri dish and incubated for 48 h at 37°C as Murray Thomas et al. [18]. After removal of agar, attached cells were stained with crystal violet (1% w/v). Motility was assayed as the radius of the circular expansion of bacterial growth from the point of inoculation.

Statistical analysis

Multi-factor analysis of variance using the software package STATISTICA 7.0 was performed to identify significant differences between strains in biofilm development. Student’s t-test was employed to evaluate the obtained data. Differences were considered statistically significant when P<0.05.

Results and Discussion

Morphological characterization of spreading growth

In general, closely related strains showed comparable phenotypic properties. The isolates were quite similar in their characteristics, with a few noticeable exceptions for hemolytic activity; P. fluorescens strains (S5 and S9) were β-hemolytic, whereas Paeruginosa (S8) and P. fluorescens (S10) were found α-hemolytic positive. They were positive for pectinolytic, starch hydrolysis, proteolysis, lecithinase, esterase and phospholipase C activity. The results, developed in this study, showed that our isolates exhibited an important biofilm mass, the S9 and S10 exhibited increased biofilm formation. The Pseudomonas isolates had OD₅₉₀ readings ranging from 0.21 to 0.35 (Figure 1), according to Perez et al. [19] and Stepanović et al. [15] classification, these isolates were categorized as having moderate biofilm adherence properties; despite they were categorized as slime-producers. Indeed, slime production play an important role in the pathogenesis of infections caused by different microorganisms, and is considered to be a significant virulence factor for some species. Slimes are generally polysaccharide materials, although other polymers may also be present and are probably involved in the protection of microbial cells. However, these molecules are also important in the formation of biofilms on surfaces. Furthermore, exopolymers have been considered to be involved in

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activity of antimicrobial agents that damage the outer membrane is thus
damaged. Toutain-Kidd et al. [26] have suggested its capacity to enhance the
activity of a variety of antibiotics against P. aeruginosa, including chlorhexidine diacetate, benzalkonium chloride, and
antimicrobial activity of PS80. There have been reports on the effects of PS80 on bacteria for more
longer time (6 days) to develop a thick colony. Our results demonstrate
that surfactants such as PS80 do not inhibit bacterial biofilm formation. Moreover, Biofilm growth produced sticky colony
and Nacl. Unsurprisingly, other Pseudomonas isolates exhibited
swimming motility. The Swarming and swimming
biofilm formation in these isolates revealed a very complex, dynamic,
and biologically exciting view about the architecture, and function of
biofilm formation. Moreover, Biofilm growth produced sticky colony
morphology variants. P. fluorescens (S4, S9) and P. aeruginosa (S8) was
strong aggregative phenotype in liquid culture and formed smaller,
rough and wrinkled colonies with dry surface appearance compared to
smooth, dome-shaped, translucent colonies and jelly like consistency with a
regular margins. Besides producing the above mentioned colony phenotypes,
isolates S9 was particularly noted for branched tendril patterns during swimming.

Swarming, swimming motility

Spreading giant colonies were observed with thick bacterial growth
on LB - Polysorbate 80 (PS80) and not observed with thin bacterial
growth on only LB medium plates (Figure 1). The most prominent
spreading growth was observed with LB- Polysorbate 80 (PS80),
containing a higher concentration of the nonionic surfactant in contrast
to nutrient-rich LB medium. PS80 is a nonionic surfactant commonly
added to foods, cosmetics, and pharmaceutical preparations as an
emulsifier and dispersing agent and is considered to be well tolerated when it is delivered to mucosal, intradermal, and intravenous sites
[23], it seemed to have a special enhancing effect on spreading growth,
On the other hand, spreading growth of strains on LB medium took a
longer time (6 days) to develop a thick colony. Our results demonstrate
that surfactants such as PS80 do not inhibit bacterial biofilm formation.
There have been reports on the effects of PS80 on bacteria for more
than four decades. While it possesses little antimicrobial activity
alone, PS80 can increase bacterial cell permeability and enhance the
antimicrobial activity of a variety of antibiotics against P. aeruginosa,
including chlorhexidine diacetate, benzalkonium chloride, and
polymyxin B sulfate [13,24,25]. Consistent with these observations,
Toutain-Kidd et al. [26] have suggested its capacity to enhance the
activity of antimicrobial agents that damage the outer membrane is thus
a reflection of its ability to gain access to the inner membrane, they also
present evidence that P. aeruginosa PA14 can resist the action of PS80
by cleavage of this surfactant by a secreted lipase, showing one possible
resistance pathway for this organism. In this study we have not made
similar observations for the action of PS80 towards biofilms formation.
We hypothesized that the lipases in these strains are not able to cleave
all the PS80 present in the medium. These findings are consistent with
Toutain-Kidd et al. [26] reports for the S. aureus, in which biofilms
formation was enhanced by the presence of PS80, reinforcing the idea
that non-lipase-dependent mechanisms of resistance to PS80 exist.

The first steps of biofilm formation [20]. Previous studies are seldom
encountered, Pringent-Combaret et al. [21] found that the E. coli
exopolysaccharide colanic acid was involved only in the ability of the
cells to produce voluminous biofilm, and not in the adherence of the
cells to plastic surfaces, while Beech and Gaylarde [22] demonstrated
that lipopolysaccharides of the outer membrane of Pseudomonas spp.
and sulphate-reducing bacteria were the important molecules in initial
adhesion to a metal surface.

Figure 1: (a) Biofilm production by pseudomonas isolates, quantitative
measurements of biofilm growth is presented by enumeration of the colony-
forming units (CFU) for OD_{595} readings; (b) swimming and spreading
diameters (cm), the colony types were green moist, smooth, opaque, with a
regular circular or slightly irregular undulated margin which appeared in isolates
S4,S8, S9; an irregular edge formations for S9 while S10 morphotype was more
smooth, dome-shaped, translucent colonies and jelly like consistency with a
regular margins. Besides producing the above mentioned colony phenotypes,
isolates S9 was particularly noted for branched tendril patterns during swimming.

Figure 2: A variable swarming phenotypes displayed by pseudomonas isolates. Swarming motility on TSA medium (1.5% agar) for 72h at 30°C after
a central spot of 5 ml of an overnight bacterial culture in TSB. The formation of
dendritic fractal-like patterns formed by migrating swarms moving away from
an initial location, primary and secondary tendrils are seen to develop at the
swarm edge. Other isolates (data not shown in our ongoing study) exhibited a
coordinated multicellular behavior over the fluid surface of Nutrient agar plates.
Enlarged views of swarming motility are provided in the top.
which the biofilm cells are exposed in *Pseudomonas* isolates.

The scientific literature revealed that many aspects of EPS remain to be addressed. In the context of this complexity, modeling of EPS seems to be an almost impossible task, although it would be extremely helpful in predicting and controlling biofilm processes. It appears that "slime" has been very much underestimated. It may turn out that the EPS matrix is considerably more than simply the glue for biofilms. Rather, it appears to be a highly sophisticated system, which endows the biofilm mode of life with particular, successful features.

Although there is still debate surrounding the steps mechanisms that are involved in the biofilm life, microscopic analysis defined macromolecular “honeycomb” structure, these findings are consistent with reports [27,28]. Taken together, our data suggest that the exact physiological mechanism of biofilm formation has remained elusive and versatile. This premise tries to further understanding the biofilm formation steps, and to explore what the “house of biofilm cells” is built of. Till date, the biofilm life cycle is summarized in three steps: initial attachment events, the growth of complex biofilms, and detachment events. Thus it is unlikely that our data are in discordance with previous observations. If biofilms can be metaphorically called a “city of microbes” [29], the EPS represent the “house of the biofilm cell”. The EPS determine the immediate conditions of life of biofilm cells living in this microenvironment by affecting porosity, density, water content, charge, sorption properties, hydrophobicity, and mechanical stability [30]. Our research has discovered that there is no direct evidence that the initial attachment events are the first step in biofilm cycle, we suggest that these are notable areas for future research.

**The biofilm growth associated to antibiotics resistance**

In the present study, we observed an increased resistance to β-lactam antibiotics, this decreased susceptibility of *Pseudomonas* strains has already been noted by previous researchers [31,32]. Intriguingly, our study showed that the clavulanic acid failed to produce an inhibition zone to imipenem disk with a biofilm formation. In this study, we found an imipenem-resistant strain (S9) which produced a novel beta-lactamase. Our data are consistent with Giwercman et al. [33] reports, in which the exposure of biofilm cells of *P. aeruginosa* [33] reports, in which the exposure of biofilm cells of *P. aeruginosa* and *E. coli* to beta-lactamase inducers triggers the production of the beta-lactamase enzyme that remains associated with the biofilm.

Unsurprisingly, biofilm antibiotic susceptibility has been the subject of intense research and has been the focus of several excellent reviews [34]. The biofilm mode of growth appears to contribute the increased resistance to antibiotics. In addition, Giwercman et al. [33] demonstrated that piperocillin and imipenem were able to induce beta-lactamase production in biofilm and remain associated longer in biofilm than planktonic cells. Coquet *et al* show that there is significant enhancement of beta-lactamase induction [35]. Interestingly, the strains exhibited an extended-spectrum β-lactamases (ESBLs). Furthermore, certain antibiotics, such as macrolides, quinolone, phenicoles and

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**Figure 3:** The EPS matrix of pseudomonas isolates on TSA medium (1.5% agar) with 100X magnification in light microscope on TSA medium (1.5% agar). An increase view of EPS matrix is provided (100X).

**Figure 4:** Macroscopic views of the effect of antibiotics on biofilm formation and motility behavior. The swarming behavior was signaled only for S4 vis a vis IPM and SP. Whereas, a swimming behavior was induced toward AMC and AMP for S8. Switching motility zone was detected for S8 in the presence of IPM. The pigments metabolic pathway is linked to certain antibiotics such as IPM and AMC.

For S9 and S10, the discontinued arrow indicates a twitching motility zones towards AMC and ATM. The swarming behavior was signaled only for P, AMC, SP and AMP. The pigments metabolic pathway is linked to S10 for ATM antibiotic. A Fuzzy zone edges and/or colonies within the zone report as resistant regardless for ATM, SXT and IPM. The carbapenemase is indicated by the small inhibitory zone around imipenem (IPM 10) and resistant colonies within this zone. An optical microscopic picture of the swarm (bull’s eye) is induced for S10 for the case of ATM, in the top the discontinued circles showed (a) an induced twitching morphotype (Expansion profiles of twitching motility. Note the tiny “keyhole” effect between IPM and NA, but the reduced inhibitory zone generated around imipenem (IPM 10) with resistant colonies indicates a carbapenemase. The pigments metabolic pathway is linked to S for P antibiotic; the continued arrow indicates the P and SP induced the same morphotype for S9 and S10.
cycines could induce an important antibiotics susceptibility as shown in Figure 4. Consistent with these observations, have reported that macrolides also appear to alter the formation of the biofilm matrix, which may result in enhanced activity of other antimicrobial agents [36–38]. Therefore, these agents may be effective against biofilm disease caused by P. aeruginosa in different fields. Trends in resistance among Pseudomonas isolates are presented in Figure 5.

The acquisition of β-lactam antibiotics resistance genes by bacterial pathogens is currently a worldwide phenomenon. Beta-lactamases inactivate β-lactam antibiotics by covalently binding the carbonyl moiety of β-lactam ring and hydrolyzing its amino bound [39]. An alternative approach to the β-lactamase problem has been the search for inhibitors of these enzymes which combined with a β-lactamase labile antibiotic and protect it from degradation and allow the β-lactam antibiotics to exert its antibacterial effect [40].

Even drug-susceptible strains of Pseudomonas aeruginosa have considerable defenses. P. aeruginosa has an inducible AmpC β-lactamase and is inherently resistant to those β-lactams that induce this enzyme and are hydrolyzed by it (e.g., cephalothin and ampicillin) [20]. Moreover, many antibiotics are excluded from the pseudomonal cell. This exclusion long was attributed to the cell’s impermeability, although evidence of this was scanty and although the belief proved difficult to reconcile with the discovery that P. aeruginosa copiously manufactures a porin (OprF) that forms large outer membrane pores [41]. In the early 1990s, it began to be realized that much of this “impermeability-mediated resistance” (as it was widely called at the time) actually reflected efflux by MexAB-OprM, a pump system that removes β-lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline, and trimethoprim, as well as various dyes and detergents [42].

Motility behavior associated to antibiotics resistance

Based upon morphotypes observations on Mueller-Hinton surfaces, we show that propagating Pseudomonas (S4 and S8) swarm can collectively change direction towards antibiotic added to an agar plate, and can even reunite and swim as described in Figure 4. We found that S10; P10; AMC30 and ATM30; SP10; NA30; AMP10 can induce a swimming, twitching and swarming phenotype for S9 and S10 respectively (Figure 4). Altogether, our results show that there is an induced swimming tendril tip bacteria phenotype with the presence of some β-lactam antibiotics. Furthermore, our results demonstrate that biofilm formation in S10 and S9 can be a specific, defensive reaction to the presence of antibiotics like Aztreonam and amoxicillin-clavulanic acid. It is important to note that Imipenem, Spiramycine, Aztreonam and amoxicillin-clavulanic acid induced a defensive reaction in S4 and S8. Our data indicate that the branched tendril patterns that are often, but not always, observed in P. aeruginosa swarms can be may be present in the P. fluorescens. Therefore, it is reasonable to propose that an increased basal level of these enzymes is mediated directly by motility behavior and biofilm formation.

The relationship between swarming and biofilm formation is debated in the P. aeruginosa literature [43,44]. Both behaviors are nutrition-ally regulated surface activities that are altered in the absence of motility organelles. Since we observed that the absence of either twitching or swimming potentially altered both biofilm and swarming behaviors, we restricted our subsequent analysis to isolates that were positive for both swimming and twitching. We hypothesized that strains that did not swarm would score higher than swarming isolates in our biofilm assay. We did indeed observe an inverse relationship between swarming motility and biofilm formation among the swim-positive/twitch-positive isolates that grew on Mueller-Hinton plates [45].

Here, we emphasize that swarming motility in P. aeruginosa and P. fluorescens manifests as a rapid, highly organized mechanism of bacterial solid surface translocation. Notably, Pseudomonas aeruginosa often, but not always, forms branched tendril patterns during swarming; this phenomena occurs only when bacteria produce rhamnolipid, which is regulated by population-dependent signaling called quorum sensing. The experimental results of this work show that P. fluorescens (S9) cells propagate as high density waves that move symmetrically as rings within swarms toward the extending tendrils. The study results suggest that both species responds to environmental cues on a very short timescale by actively exploiting local physical phenomena to develop communities and efficiently colonize new surfaces.

Conclusion

In this article, we have assembled some phenotypic processes related to antibiotics susceptibility or resistance in P. aeruginosa and P. fluorescens. Our assessment of the available evidence suggests that the reduced susceptibility of biofilms to antibiotics is linked to the natural process of behavior motility and diversification that is ongoing within the biofilm population. The species displayed severe swarming-swimming and twitching motility in the presence of β-lactam antibiotics. Thus, while behavior motility and biofilm formation seem to be related, the data seem to imply that there is a strong relationship between these processes. Therefore, the behavior motility and biofilm formation will be one of the clinical problems in the therapy of P. aeruginosa and P. fluorescens infections and highlights the need for continuous monitoring this motility in emerging bacterial resistance.

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Conflict Of Interest Statement

Authors declare no conflict of interest.

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