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# *Moraxella catarrhalis* Coaggregates with *Streptococcus pyogenes* and Modulates Interactions of *S. pyogenes* with Human Epithelial Cells

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**The pathogens *Streptococcus pyogenes* and *Moraxella catarrhalis* colonize overlapping regions of the human nasopharynx. We have found that *M. catarrhalis* can dramatically increase *S. pyogenes* adherence to human epithelial cells and that species-specific coaggregation of these bacteria correlates with this enhanced adherence.**

In most environments, the interaction of individual microbes with other microbial species can have a profound effect on the colonization, persistence, and survival of the microorganisms. The importance of polymicrobial interactions has also been documented in human and animal diseases (5). In the case of periodontal disease, the binding or coaggregation of *Porphyromonas gingivalis* to *Streptococcus gordonii* enhances the colonization of the tooth surface by *P. gingivalis* (19). This coaggregation is complex and is mediated by at least two adhesin-receptor pairs (9, 19). Although much is known about polymicrobial interactions in the human colon and oral cavity, little work has been done investigating polymicrobial interactions among bacteria that colonize or infect the human nasopharynx.

As a model for possible polymicrobial interactions in the human nasopharynx, we investigated interactions between *Streptococcus pyogenes* and *Moraxella catarrhalis* for several reasons. First, *S. pyogenes* and *M. catarrhalis* are human-specific pathogens that colonize the nasopharynx, causing a variety of diseases (4, 7, 8, 13, 17, 21, 23, 28, 33). Their carriage rates among asymptomatic individuals can be very high, and the adherence of both organisms to epithelial cells is critical for their pathogenesis and they adhere to the same human epithelial cell lines (11, 15, 16, 22, 31).

***M. catarrhalis* increases *S. pyogenes* adherence to human epithelial cells.** To assess whether *S. pyogenes* (strain 1881, serotype M1) (Table 1) and *M. catarrhalis* (strain 035E) interact, we measured their adherence to A549 (lung) and Chang (conjunctival) human epithelial cells alone or in combination with a quantitative adherence assay that we previously described (18). The presence of *S. pyogenes* had a small (less-than-threefold) negative influence on the binding of *M. catarrhalis* to both cell lines. However, *M. catarrhalis* substantially increased *S. pyogenes* adherence to Chang cells (22-fold) and to A549 cells (15-fold) (Fig. 1A and B). In contrast, a nonadherent *Escherichia coli* strain had no effect on *S. pyogenes* adherence. *M. catarrhalis* had a modest effect (threefold) on the adherence of the closely related species *S. agalactiae*. In addition, we observed that the adherence of two other gram-positive cocci (*S. aureus* and *S. pneumoniae*) was not significantly affected by *M. catarrhalis* (Fig. 1C). Thus, the dramatic effect of *M. catarrhalis* on *S. pyogenes* adherence is not shared by other gram-positive human pathogens.

Microscopic examination of *M. catarrhalis* and *S. pyogenes* bound to human cells suggested that the bacteria were colocalized on the eukaryotic cell surface (data not shown). We therefore hypothesized that *M. catarrhalis* was acting as an adapter, binding to both human cells and *S. pyogenes*. To test this, we measured the ability of an *M. catarrhalis* adherence-negative *uspA1* mutant to modulate the binding of *S. pyogenes* to Chang cells. As previously reported (18), the *uspA1* mutation decreased the binding of *M. catarrhalis* to Chang cells 10-fold (Fig. 2A). When *S. pyogenes* was coinfected with the *M. catarrhalis* wild-type strain, *S. pyogenes* adherence was increased by 18-fold (Fig. 2B) while coinfection of monolayers with the *M. catarrhalis uspA1* mutant increased *S. pyogenes* adherence only 2-fold (Fig. 2B). Similar results were seen following coinfection of A549 cells with an adherence-negative *M. catarrhalis hag* mutant that was recently demonstrated to be a

TABLE 1. Bacterial strains used in this study

| Strain | Description | Source or reference(s) |
|---|---|---|
| *M. catarrhalis* O35E | Wild type | 2 |
| *M. catarrhalis* O35E *hag* (O35E.TN2) | Adhesin mutant; decreased adherence to A549 cells | 16 |
| *M. catarrhalis* O35E *usp*A1 (O35E.1) | Adhesin mutant; decreased adherence to Chang cells | 1 |
| *M. catarrhalis* O35E *usp*A2 | Decreased serum resistance | 1 |
| *S. pyogenes* 1881 | Opacity factor (−); *emm1* | 27 |
| *S. pyogenes* 90–226 | Opacity factor (−); *emm1* | 10, 12 |
| *S. pyogenes* SF370 | Opacity factor (−); *emm1* | 30 |
| *S. pyogenes* CS101 | Opacity factor (+); *emm49* | 25 |
| *S. aureus* RN6390 | Wild type | 24 |
| *S. agalactiae* | Wild type | Laboratory stock |
| *S. pneumoniae* | Wild type | 20 |

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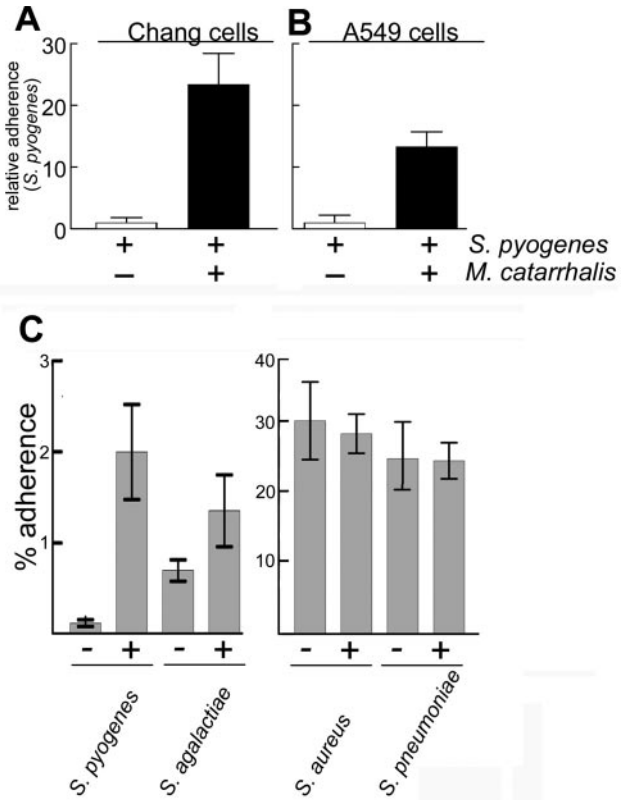


FIG. 1. *M. catarrhalis* specifically enhances the binding of *S. pyogenes* to human cells. *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, or *S. aureus* ($\sim 2 \times 10^7$ CFU) was incubated with human cells alone or mixed with *M. catarrhalis* ($\sim 2 \times 10^7$ CFU). Results represent the mean of two to four independent experiments done in duplicate. Error bars represent the standard deviations. (A and B) *M. catarrhalis* enhanced *S. pyogenes* binding to A549 and Chang cells (closed bars) >15-fold compared to *S. pyogenes* alone (open bars). (C). *M. catarrhalis* had no effect on *S. agalactiae*, *S. pneumoniae*, or *S. aureus* adherence to A549 cells.

major *M. catarrhalis* adhesin for A549 cells (data not shown) (16).

***M. catarrhalis* and *S. pyogenes* form coaggregates.** To test whether these bacteria directly interact, we developed a quantitative assay to measure their coaggregation. First, bacterial cell surfaces were labeled with biotin with the membrane-impermeable biotin derivative sulfo-NHS-LC-biotin (EZ-Link; Pierce, Rockford, Ill.). Freshly prepared 1.7 mM sulfo-NHS-LC-biotin solution in water (0.5 ml) was added to plate-grown bacteria ($10^8$ CFU in 1 ml of PBSG [11.9 mM phosphate, 137 mM NaCl, 2.7 mM KCl, 0.15% gelatin type B from bovine skin, pH 7.4]), and the cells were incubated at room temperature for 30 min. Excess biotinylation reagent was removed by four washes with PBSG.

To measure coaggregation, biotinylated ($10^7$ CFU) or non-biotinylated *M. catarrhalis* cells were mixed with streptavidin-magnetic beads (10 μl of a 50% slurry; Cortex Biochem, San Leandro, Calif.) and incubated with gentle agitation for 30 min at 37°C in microcentrifuge tubes. The tubes were placed in a magnetic separator (Cortex Biochem), and after 30 s, the supernatant was aspirated and the beads were resuspended in 1 ml of PBSG. The beads were washed four more times and resuspended in 1 ml of PBSG. Unlabeled *S. pyogenes* was mixed with *M. catarrhalis*-magnetic beads and incubated for 30 min. Unbound bacteria were removed by five rounds of washing (changing tubes each time), and bound bacteria were enumerated by serial dilution and outgrowth on selective medium. We recovered $(3.8 \pm 0.72) \times 10^6$ *S. pyogenes* CFU when using biotinylated *M. catarrhalis* cells (Fig. 3A, +bMc). In contrast, about 40-fold less *S. pyogenes* [$(1.0 \pm 0.17) \times 10^5$ CFU] was recovered when the cells were mixed with unlabeled *M. catarrhalis* (Fig. 3A, +Mc). The reverse experiment, with biotinylated *S. pyogenes* and measurement of *M. catarrhalis* recovery, gave a comparable result (Fig. 3A). These results are not due to growth effects during the outgrowth step since similar results were obtained when bacterial numbers were directly quantitated by quantitative real-time PCR and an *S. pyogenes*-specific probe (29) (data not shown). Coaggregation was not due to nonspecific clumping of the bacteria since *Staphylococcus aureus* was not copurified with biotinylated *M. catarrhalis* (Fig. 3A). It is worth noting that neither *S. pyogenes* nor *M. catarrhalis* coaggregated with biotinylated *E. coli* (data not shown), ruling out the possibility of a surface avidin-like protein mediating the coaggregation.

*M. catarrhalis* also coaggregated with three other *S. pyogenes* strains (serotype M1 strains SF370 and 90-226 [10, 12, 30] and serotype M49 strain CS101 [25]), suggesting that coaggregation with *M. catarrhalis* is widespread among *S. pyogenes* strains (Fig. 3B).

To test whether the *M. catarrhalis* adhesins necessary for increasing *S. pyogenes* adherence (Fig. 2) are also necessary for coaggregation, we used the coaggregation assay to measure the binding of *M. catarrhalis hag*, *uspA1*, and *uspA2* mutants to biotinylated *S. pyogenes*. These mutants bound as well as wild-type *M. catarrhalis* (100- to 1,000-fold increased recovery) to

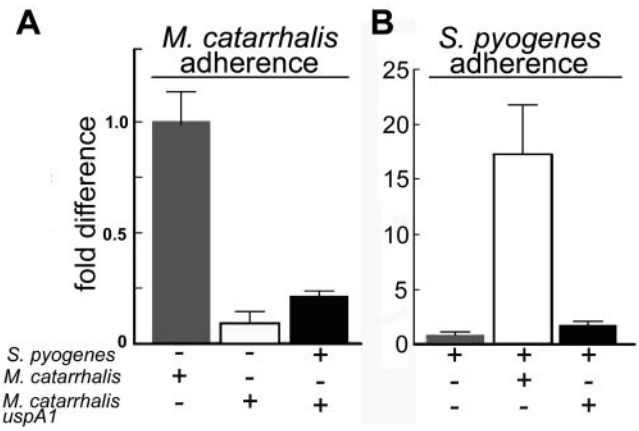


FIG. 2. The *M. catarrhalis* adhesin UspA1 was necessary for enhanced adherence to Chang cells. *S. pyogenes* ($\sim 2 \times 10^7$ CFU) and *M. catarrhalis* ($\sim 2 \times 10^7$ CFU) were incubated with Chang cells either alone or mixed as described in Materials and Methods. Results represent the mean of two to four independent experiments done in duplicate. Error bars represent the standard deviations. (A) The adherence of a *uspA1* mutant of *M. catarrhalis* to Chang cells (open bar) was only 10% of that of the isogenic wild-type strain (gray bar), as previously reported (1). Coinfection with *S. pyogenes* had a small effect on the adherence of the *M. catarrhalis uspA1* mutant (closed bar). (B) Wild-type *M. catarrhalis* enhanced the adherence of *S. pyogenes* 18-fold (gray bar versus open bar). The *M. catarrhalis uspA1* mutant enhanced the adherence of *S. pyogenes* only twofold (closed bar).

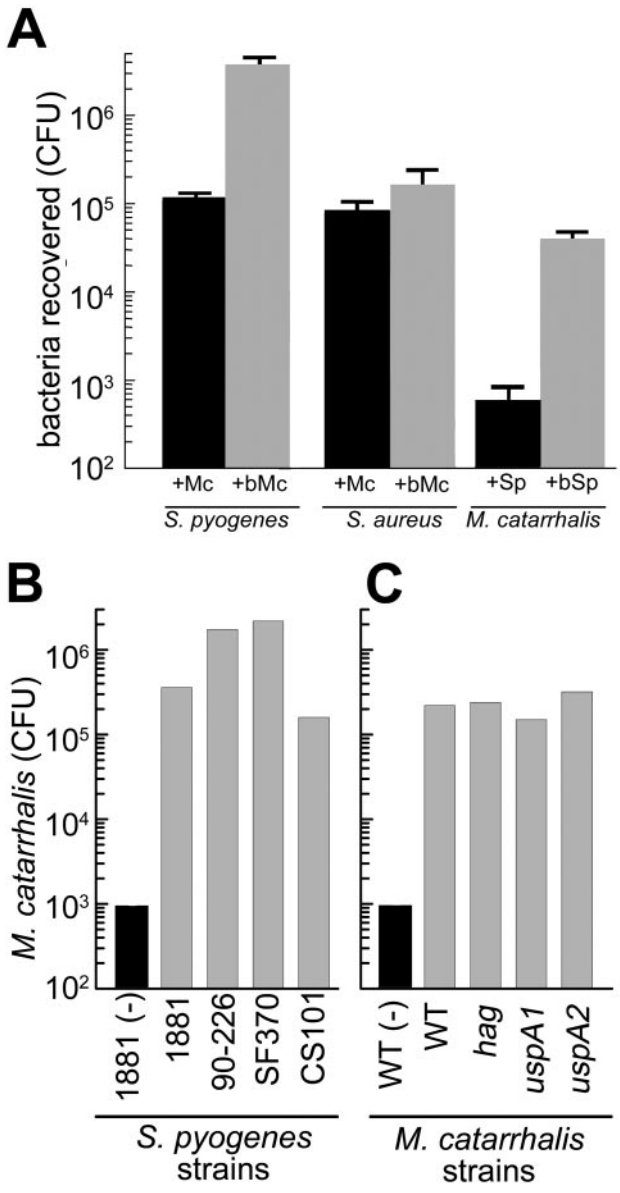


FIG. 3. Quantitation of *M. catarrhalis* and *S. pyogenes* coaggregation. (A) biotinylated (+bMc, gray bar) or nonbiotinylated (+M, dark bar) *M. catarrhalis* was incubated with *S. pyogenes* or *S. aureus*. Nonbiotinylated *S. pyogenes* (+Sp, dark bar) or biotinylated *S. pyogenes* (+bSp, gray bar) was incubated with *M. catarrhalis*. Avidin-conjugated magnetic beads were added, and biotinylated bacteria were purified by extensive washing on a magnetic separator. Purified aggregates were serially diluted and plated on appropriate media. The bars represent CFU of the nonbiotinylated bacteria recovered after purification. Error bars represent the standard deviation of three independent experiments. In each case, the difference between biotinylated and nonbiotinylated bacteria was significant ($P < 0.001$) as determined by Student's *t* test. More than 30-fold more *S. pyogenes* was recovered with the streptavidin-magnetic beads in the presence of biotinylated *M. catarrhalis* (gray bars) than in the presence of nonbiotinylated *M. catarrhalis* (dark bars). No enhancement of binding was observed with *M. catarrhalis* and *S. aureus* (data not shown). Biotinylated *S. pyogenes* (gray bar) was also able to enhance the recovery of nonbiotinylated *M. catarrhalis* (dark bar) by 100-fold. (B) *S. pyogenes* strains (gray bars) were biotinylated and incubated with *M. catarrhalis* O35E, and coaggregation was measured as described above. *M. catarrhalis* was also incubated with beads in the absence of *S. pyogenes* (dark bar). *M. catarrhalis* coaggregated with each of the *S. pyogenes* strains. (C) Wild-type (WT) and adhesin mutant *M. catarrhalis* O35E bacteria were incubated with biotinylated *S. pyogenes* 1881, and coaggregation was measured as described above. Wild-type *M. catarrhalis* O35E was also incubated with beads in the absence of *S. pyogenes* (dark bar). All of the *M. catarrhalis* adhesin mutants coaggregated with *S. pyogenes*, suggesting that these adhesin proteins do not mediate coaggregation of these bacteria.

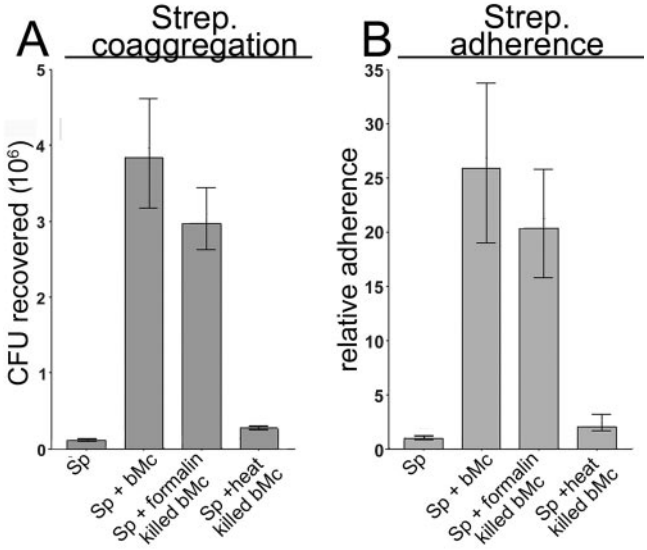


FIG. 4. Effects of heat and formalin on the coaggregation and coadherence of *M. catarrhalis* and *S. pyogenes*. *S. pyogenes* (Sp) was incubated alone or with biotinylated *M. catarrhalis* (bMc), heat-killed biotinylated *M. catarrhalis*, or formalin-killed, biotinylated *M. catarrhalis*. Cells were washed, and half of the cells were purified with streptavidin- magnetic beads and the other half were used in an adherence assay with A549 cells as described in the text. (A) *S. pyogenes* coaggregated with the live and formalin-killed, biotinylated *M. catarrhalis* cells but not with the heat-killed, biotinylated *M. catarrhalis* cells. (B) *S. pyogenes* adherence to A549 cells was also enhanced by the live and formalin-killed biotinylated *M. catarrhalis* cells but not by the heat killed, biotinylated *M. catarrhalis* cells. Strep., streptococcal.

biotinylated *S. pyogenes* (Fig. 3C). This supports our hypothesis that *M. catarrhalis* acts as an adapter that can mediate *S. pyogenes* adherence to human cells. In addition, these results indicate that the *M. catarrhalis* surface molecules UspA1, UspA2, and Hag do not mediate coaggregation with *S. pyogenes*.

**Killed *M. catarrhalis* coaggregates with *S. pyogenes*.** To further investigate the *M. catarrhalis* modulation of *S. pyogenes* adherence, we tested whether heat-killed (60°C for 10 min) or formalin-killed (10% formalin in PBSG for 10 min, followed by extensive washing) *M. catarrhalis* eliminates this effect. Heat-killed *M. catarrhalis* no longer coaggregated with *S. pyogenes* or enhanced its adherence to A549 cells (Fig. 4). Microscopic examination showed few heat-killed *M. catarrhalis* bacteria bound to lung cells (data not shown), suggesting that the *M. catarrhalis* molecule(s) involved in this process was heat labile. Formalin-killed *M. catarrhalis* cells coaggregated with *S. pyogenes* and enhanced adherence to levels similar to those observed with viable *M. catarrhalis* (Fig. 4). Thus, neither de novo protein synthesis by *M. catarrhalis* nor a soluble factor produced by *M. catarrhalis* was necessary for enhancing *S. pyogenes* adherence or coaggregation.

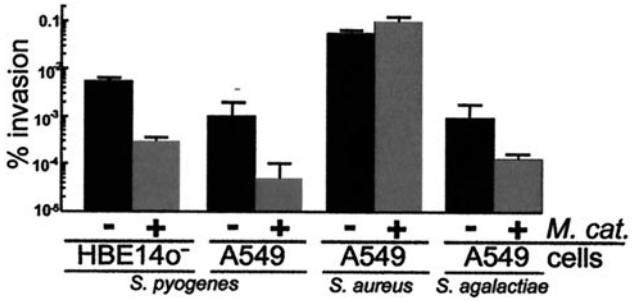


FIG. 5. *M. catarrhalis* inhibited *S. pyogenes* and *S. agalactiae* invasion of human epithelial cells. Human cell lines 16HBE14o$^-$ (polarized bronchial epithelial cells) and A549 (lung epithelial cells) were incubated with *S. pyogenes*, *S. aureus*, or *S. agalactiae* alone (light gray bar) or with *M. catarrhalis* ($\sim 2 \times 10^7$ CFU/ml, dark bar). *M. catarrhalis* inhibited *S. pyogenes* (25- to 28-fold) and *S. agalactiae* (10-fold) invasion but not *S. aureus* invasion. None of the bacteria tested had a significant effect on the adherence or invasion of *M. catarrhalis*. Bar height is the mean of two or three independent experiments, each done in duplicate. Error bars represent the standard deviation.

***M. catarrhalis* inhibits streptococcal invasion of human epithelial cells.** Since *S. pyogenes* is also an invasive pathogen (10, 12) and adherence is a necessary first step for invasion, we tested whether *M. catarrhalis* affects *S. pyogenes* invasion. With a gentamicin resistance invasion assay (26), we found that invasion of A549 cells by *S. pyogenes* was reduced 25-fold in the presence of *M. catarrhalis* (Fig. 5). Similar results were obtained with a human bronchial epithelial cell line that forms polarized monolayers (16HBE14o$^-$), which are more similar to in vivo epithelial layers (31, 32) (Fig. 5). *M. catarrhalis* was not acting as a general inhibitor of invasion, since it had no effect on *S. aureus* invasion (Fig. 5). Interestingly, *M. catarrhalis* also decreased *S. agalactiae* invasion of A549 cells by 10-fold. Since *M. catarrhalis* has only a small effect on *S. agalactiae* adherence (Fig. 1), our results imply that the effects of *M. catarrhalis* on the invasion of *S. agalactiae* and *S. pyogenes* was at least partly independent of its ability to coaggregate.

**Summary.** In this paper, we have shown that *M. catarrhalis* has striking effects on *S. pyogenes* adherence to and invasion of human epithelial cell lines. This remarkable enhancement was specific and widespread among *S. pyogenes* strains. Our results are consistent with the hypothesis that *M. catarrhalis* and *S. pyogenes* form coaggregates and that these coaggregates bind to human cells via *M. catarrhalis* adhesins. Together, our data suggest that prior colonization by *M. catarrhalis* could have a profound effect on the binding to, as well as the invasion of, mucosal surfaces by *S. pyogenes*. Since there are no reports of *M. catarrhalis-S. pyogenes* coinfections, we instead hypothesize that colonization by *M. catarrhalis* could increase the probability of colonization by *S. pyogenes* or the progression of disease. Because more than 90% of clinical isolates of *M. catarrhalis* produce β-lactamases (14), another significant issue is the possibility that in coaggregates *S. pyogenes* is more resistant to β-lactam antibiotics, the recommended antimicrobial treatment for *S. pyogenes* infections (3). This possibility is supported by the observation that *M. catarrhalis* increased the resistance of *S. pneumoniae* to β-lactam antibiotics in continuous-biofilm studies (6). Furthermore, because *M. catarrhalis* appears to inhibit streptococcal invasion in vitro, prior colonization by *M. catarrhalis* might promote an *S. pyogenes* surface infection rather than a deep-tissue, invasive disease. Thus, coaggregation with *M. catarrhalis* is one of many factors that could contribute to the pathogenicity of *S. pyogenes*.

In addition to the potential clinical consequences of this coaggregation and coadhesion, these results highlight the potential importance of microbial interactions among bacteria in the nasopharynx. We are currently identifying the bacterial molecules that mediate this polymicrobial interaction to better understand the mechanisms involved.

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