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Research Paper

Conjugative antibiotic-resistant plasmids promote bacterial colonization of microplastics in water environments

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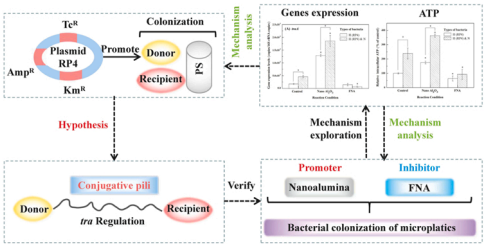
HIGHLIGHTS

- RP4 promoted bacterial colonization of microplastics.
- Nano-alumina promoted and FNA inhibited bacterial colonization.
- Bacterial colonization of microplastic was related to conjugative pili.

GRAPHICAL ABSTRACT



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ABSTRACT

Both microplastic and bacterial antibiotic resistance have attracted attention worldwide. When microplastics coexist with antibiotic-resistant bacteria (ARB), which carry antibiotic resistance genes (ARGs), ARB colonize the surface of microplastics, and a unique biofilm is formed. The ARB and ARGs in biofilms are denser and more difficult to remove. However, studies on the factors influencing the formation of microplastic biofilms are limited. In this study, plasmid RP4, which appeared in wastewater treatment plants, was found to be able to promote irreversible bacterial colonization of microplastics, and the hypothetical reason was conjugative pili expression. Then, the potential conjugative pili synthesis promoter "nanoalumina" and inhibitor "free nitrous acid" (FNA) were selected to test this hypothesis. Simultaneously, nanoalumina promoted and FNA inhibited bacterial colonization when RP4 existed. Combined with the gene expression and ATP analysis results, this hypothesis was confirmed, and the mechanism of RP4 on bacterial colonization was related mainly to conjugative pili protein synthesis and intracellular ATP. In this study, the effects of plasmid RP4, nanoalumina, and FNA on the formation of microplastic biofilms were reported, which has a certain reference value for other researchers exploring microplastic biofilms.

1. Introduction

The plastic industry has been developing rapidly since 1950. The annual output of plastics in the world has exceeded 320 million tons (Wright and Kelly, 2017) and may reach 33 billion tons by 2050 (Rochman et al., 2013a). However, mass production, wide use, rapid disposal of plastics, and strong resistance to degradation have led to the large-scale release of plastics into the water environment, where the plastics can persist for many centuries. The most common plastics detected in the environment are polyethylene (PE), polyvinyl chloride

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(PVC), polypropylene (PP), polyethylene terephthalate (PET), and polystyrene (PS) (de Ruijter et al., 2020; Rochman et al., 2013b). These pieces of plastic debris can be decomposed into large plastic (>25 mm), medium plastic (5–25 mm), microplastic (0.1 μm-5 mm), and nanoplastic (<100 nm) (Jahnke et al., 2017). Plastic debris, one of the rapidly growing pollution sources, has become an issue of high concern causing environmental problems, among which microplastics are of high interest to researchers (Cunningham et al., 2020; Huang et al., 2021; Lapointe et al., 2020). At present, microplastics are detected in marine environments, fresh water, sewage treatment plants, and other water environments (Blair et al., 2019; Klein et al., 2015; Song et al., 2018).

Microplastics are difficult to degrade in water environments and can interact with inorganic particles, organic matter, and microorganisms (Jin et al., 2020b; Parrish and Fahrenfeld, 2019; Sun et al., 2020). Different sizes and types of microorganisms (including protozoa, algae, bacteria, fungi, and even viruses) can adhere to the surface of microplastics (McGivney et al., 2020; Mughini-Gras et al., 2021; Wu et al., 2019; Zhang et al., 2021) and colonize to form biofilms. Generally, the development of a biofilm consists of four stages: the colonization stage of reversible adhesion, the colonization stage of irreversible adhesion, the maturation stage of biofilm, and the shedding and recolonization stage of microorganisms (Carniello et al., 2018; Donlan, 2001; Furuichi et al., 2020; Hall-Stoodley and Stoodley, 2002; Hoffman et al., 2005; Hughes and Berg, 2017), and each stage shows different characteristics. Previous studies showed that the α diversity (richness and evenness) of communities in microplastic (PE and PP) biofilms was lower than the diversity in natural substrates (pebbles and wood), and the biofilm on the surface of microplastics had its own uniqueness (Miao et al., 2019). Microplastic biofilms can cover the surface of microplastics, affecting their deposition, migration, and many other environmental behaviours (Wieczorek et al., 2019). Tu et al. (2020) found that the microplastic biofilm formed altered the functional groups of PE and subsequently reduced the hydrophobicity. The results of Johansen et al. (2019) showed that the biofilms formed on microplastics could enhance the adsorption of radioactive pollutants (strongly cationic Cs and weakly cationic Sr). Wang et al. (2020b) observed that microplastic biofilms significantly enhanced the adsorption and stability of copper(II) and tetracycline on microplastics. The influence of microplastic biofilm formation in the water environment has been researched, but research on the factors and mechanisms affecting the formation of microplastic biofilms in water environments is still rare (Bank et al., 2020).

Antibiotics are one of the greatest human-made inventions of the 20th century. With the extensive use of antibiotics, bacterial antibiotic resistance also appears (Calero-Caceres and Muniesa, 2016). Antibiotic resistance genes (ARGs) were also proposed as new environmental pollutants in 2006 and have attracted immense attention worldwide (Pruden et al., 2006). Several studies have confirmed surface water, groundwater, aquaculture wastewater, sewage treatment plants, and other water environments as important reservoirs of ARGs (Czekalski et al., 2014; Knapp et al., 2010; Li et al., 2015; Zhang et al., 2013). ARGs can be transmitted in the water environment through horizontal transfer (conjugation, transformation, and transduction) and vertical transfer (ARGs are replicated via natural proliferation of bacteria) (Dodd, 2012; Jin et al., 2020a; Wang et al., 2021). The coexistence of microplastics, antibiotic-resistant bacteria (ARB), and ARGs in the water environment may enrich ARB and ARGs on microplastic biofilms, and the ARB and ARGs in biofilms are denser and more difficult to remove, which poses a greater threat to human health (Di Pippo et al., 2020). The results of Lu et al. (2019) revealed that the absolute abundance of ARGs on microplastics in recirculating water of a mariculture system was 10^9 copies/g, and the content of ARGs on the surface of microplastics was 10^3 times higher than the content of ARGs in water. The relationship between microplastic biofilms, ARB, and ARGs has attracted much attention (Sun et al., 2021; Wang et al., 2020a). However, studies on the factors that may affect the formation and development of microplastic biofilms are limited.

Accidental experiments have shown that the conjugative antibiotic resistant plasmid RP4 might promote irreversible bacterial colonization of microplastics in this study. Previous studies have shown that most ARGs in wastewater treatment plants are carried by plasmids (Che et al., 2019); RP4 is a plasmid that appears in wastewater treatment plants (Guo et al., 2015). Therefore, the role of RP4 in the transmission of antibiotic resistance should not be ignored. RP4, with the genes for producing conjugative pili, is a conjugative pili synthesis-repressed plasmid. The analysis of the possible reason for RP4 promoting bacterial colonization might be that the RP4 plasmid carried the genes that could express conjugative pili because Ghigo (2001) found that biofilm formation declined after they artificially disabled genes (*traA*), which controlled conjugative pili synthesis, and the conjugative pili protein could be used as an adhesion factor by bacteria, which promoted bacterial colonization (Ghigo, 2001; May and Okabe, 2008). The composition of the conjugative pili is the same as the composition of common pili, but its structure is longer and thicker than the structure of the common pili, and it is the pathway of gene conjugative transfer. Gram-negative bacteria all have common pili, while only bacteria carrying conjugative plasmids synthesize conjugative pili. Compared with common pili, conjugative pili can connect bacteria with bacteria, and they have the potential to promote biofilm formation (Clarke et al., 2008; Costa et al., 2016; Lederberg and Tatum, 1946). In this study, only conjugative pili were explored, and the next mentioned pili all referred to conjugative pili. Previous studies have shown that nanometal oxides (nanoalumina and nanotitanium dioxide) promoted and free nitrous acid (FNA) inhibited the expression of some genes related to conjugative pili synthesis (Han et al., 2020; Huang et al., 2019; Qiu et al., 2012). Nanoalumina and FNA are pollutants that may exist in water or may be used as water treatment agents, so their possible effects in water have attracted attention from researchers. These materials might affect bacterial colonization of microplastics by affecting conjugative pili synthesis in water environments and might change the conjugative transfer of ARGs in the biofilm. However, there are limited studies on this topic. Therefore, potential conjugative pili synthesis promoters (nanoalumina) and inhibitors (FNAs) were selected to verify whether RP4 promoted bacterial colonization on the microplastic surface by conjugative pili protein expression and to study their effects on bacterial colonization.

In this study, the irreversible colonization ability of the same number but different types of bacteria (which carried or did not carry RP4) on microplastics was explored. Second, the effects of nanoalumina and FNA on bacterial colonization on the microplastic surface and ARG conjugative transfer in the biofilm were studied. Third, the gene expression levels that control conjugative pili protein synthesis and intracellular ATP content were analysed. Finally, the mechanism by which RP4 promotes bacterial colonization of microplastics is summarized. After adding nanoalumina and FNA, the conjugative pili synthesis promoter (nanoalumina) promoted and inhibitor (FNA) inhibited bacterial colonization exactly, which verified that RP4 promoted bacterial colonization on microplatics by conjugative pili protein expression.

2. Materials and methods

2.1. Bacterial strains and microplastics

The “H (RP4)” donor strain was *E. coli* HB101 carrying plasmid RP4 with ampicillin, kanamycin, and tetracycline resistance (Amp^R, Km^R, and Tc^R). The plasmid RP4 was obtained from the Tianjin Institute of Health and Environmental Medicine. The “H” strain was *E. coli* HB101, without the RP4. The “N” strain was *E. coli* NK5449 (CGMCC NO. 1.1437), obtained from the China General Microbiological Culture Collection Center (CGMCC), which encodes high-level resistance to rifampicin and nalidixic acid. The “H (RP4)” donor strain and the “N” recipient strain were used in conjugation experiments, and the system had been proven to be suitable for conjugation tests in the previous literature (Guo et al., 2015; Zhu et al., 2020). The “N (RP4)” strain was

E. coli NK5449, which carried RP4.

The PS microplastics, purchased from Tsinghua Tongfang Teaching Instrument., Ltd., Beijing, China, were cylindrical particles with a bottom diameter of 3 mm and a height of 4 mm.

2.2. Bacterial culture

All glycerol tubes of bacterial strains stored at −80 °C were thawed. Subsequently, 50 μL "H (RP4)" bacterial suspension was added to Luria-Bertani (LB) broth containing 32 mg/L tetracycline, 50 mg/L kanamycin, and 100 mg/L ampicillin; "H" bacterial suspension was added to LB broth without antibiotics; 50 μL "N" bacterial suspension was added to LB broth that contained 160 mg/L rifampicin; and "N (RP4)" bacterial suspension was added to LB broth containing all of the above antibiotics. After shaking for 16 h in a shaker incubator (180 rpm) at 37 °C, the mixture was centrifuged at 6000 rpm for 10 min. The cells were washed three times to remove residual growth medium constituents and then suspended in phosphate buffer solution (PBS, pH = 7.4) with different concentrations of bacterial suspension.

2.3. Detection of bacterial number on microplastics

Four single factors (reaction time, bacterial concentration, temperature, and microplastic concentration) were optimized to obtain the bacterial colonization conditions on microplastics for the following experiments. The details of this process are described in the Supporting Information (Fig. S1).

The six types of bacterial experiments were "H (RP4)", "H", "N (RP4)", "N", "H" & "N" and "H (RP4)" & "N". According to the test methods previously studied (Guo et al., 2015; Lin et al., 2016; Wang et al., 2015), an equal total amount of bacterial suspension was mixed in phosphate-buffered saline (PBS) solution containing 1% LB medium (bacterial concentration was approximately 10^8 colony forming units (CFU)/mL, H/N ratio = 1:1, temperature = 37 °C, and pH = 7.4). Then, microplastics were added to the mixture such that the final solution contained 1.0 particles/mL microplastics. After shaking for 24 h in a shaker incubator (180 rpm), three microplastics were randomly selected from the reaction system, and each microplastic was added to 5 mL PBS, washed slowly for 3–5 minutes and repeated 3–5 times to ensure that no bacteria were detected in the final PBS washing, which proved that the bacterial adhesion on the microplastics was irreversible. Previous research methods were used to elute the bacteria from the microplastics, which were resuspended in PBS solution (Arias-Andres et al., 2018; Zhang et al., 2020), the details were described in the Supporting Information Text 1. After gradient dilution, the bacteria were cultured for 24 h on plates containing the corresponding antibiotics, and their colonies were counted. Each experiment was repeated in triplicate.

2.4. Effect of nanoalumina and FNA on bacterial colonization and conjugative transfer

Two types of representative materials, nanoalumina (nanomaterial, conjugative pili promoter) and FNA (conjugative pili inhibitor), were selected for testing (Huang et al., 2019; Qiu et al., 2012). The specific concentration range was selected according to the test results in previous studies, and the unit used was also consistent with previous studies. Nanoalumina (particle size, 10 nm) was purchased from Aladdin, Ltd. (Shang, China). The FNA concentration was calculated according to Eq. (1).

$$FNA \quad (as \quad HNO_2 - N) = \frac{NO_2 - N}{K_a \times 10^{pH}}, \quad K_a = e^{-\frac{2300}{270+T(^\circ C)}} \tag{1}$$

The three types of bacterial experiments were "H (RP4)", "H" & "N", and "H (RP4)" & "N". Equal total amounts of each bacterial suspension were mixed in PBS solution containing 1% LB medium (bacterial concentration was approximately 10^8 CFU/mL, H/N ratio = 1:1, temperature =37 °C, and pH = 7.4). Next, microplastics were added to the mixture so that the final solution contained 1.0 particles/mL microplastics. Then, nanoalumina (0, 0.05, 0.5, 2.5, 5.0 mmol/L) and FNA (0, 0.001, 0.005, 0.01, 0.02 mg/L) were added to the mixture. Then, according to the aforementioned methods, the bacteria were eluted from the microplastics and resuspended in PBS solution. After gradient dilution, the bacteria were cultured on plates containing the corresponding antibiotics for 24 h, and their colonies were counted. Each experiment was repeated in triplicate. The details of conjugative transfer analysis are described in the Supporting Information.

2.5. mRNA analysis

The conjugative pili synthesized by the RP4 plasmid and F plasmid were essentially the same and were controlled mainly by *tra* genes, including *traA*, *traB*, *traC*, and *traX*. In this study, mRNAs (*traA*, *traB*, and *traC)* were selected, and the corresponding gene sequences were collected from the National Center for Biotechnology Information (NCBI) database. The 16 S rRNA was selected as an internal reference (Qiu et al., 2012). Polymerase chain reaction (PCR) primers and PCR conditions are shown in Table S1, and the details of the quantitative (qPCR) process are described in Supporting Information Text 3, Fig. S4 and Fig. S5.

Since the "H" & "N" reaction system did not contain RP4, this system, in theory, cannot synthesize conjugative pili. Through the control test, *traA*, *traB*, and *traC* were not detected in the reaction system. Therefore, in the final test results, only two types of bacterial experiments, "H (RP4)" and "H (RP4)" & "N", were presented. The reaction solution was prepared according to the abovementioned bacterial mixing methods. Based on the previous test results, nanoalumina (0 and 0.5 mmol/L) and FNA (0 and 0.01 mg/L) were selected. After the reaction, the RNA in the mixture was extracted using the RNAprep Pure Cell/Bacteria Kit (Tiangen Biotech, China), the mRNA was reverse-transcribed into DNA using the FastKing RT Kit (Tiangen Biotech, China), and the four standard plasmids were prepared. The concentration of standard plasmids was measured using a microspectrophotometer (NanoDrop 2000C, NanoDrop Technologies, Wilmington, DE, USA), and the standard plasmid copies were calculated. The standard plasmids were subsequently diluted 10 times, and the standard curves were elucidated using a real-time fluorescence quantitative PCR instrument (ABI7500, Applied Biosystems, USA). Finally, based on the standard curves, the copy number of genes was calculated using the Ct (cycle threshold) value of the test samples. Each experiment was repeated in triplicate. Gene expression levels were calculated using Eq. (2).

$$Gene \quad expression \quad levels = \frac{Gene \quad expression \quad copies \quad (copies/\mu L)}{16S \quad rRNA \quad copies \quad (copies/\mu L)} \tag{2}$$

2.6. Other analytical methods and data analyses

Adenosine triphosphate (ATP) was detected, and bacterial colonization in microplastics was observed using scanning electron microscopy (SEM), as shown in the Supporting Information. After the reaction, the intracellular ATP concentration was assayed according to the manufacturer's instructions. All kits were purchased from the Nanjing Jiancheng Bioengineering Institute. Each experiment was repeated in triplicate. The relative analysis of the conjugative pili protein is shown in Fig. S3. The details of this process are described in the Supporting Information.

Each experiment was repeated at least in triplicate. Student's *t*-test was used to evaluate statistically significant differences ($P < 0.05$) between the experimental and control groups. Student-Newman-Keuls (S-N-K) tests were used for multiple comparisons between experimental groups. All statistical corrections were conducted in SPSS 19.0. The null hypothesis that the frequency was not different between different

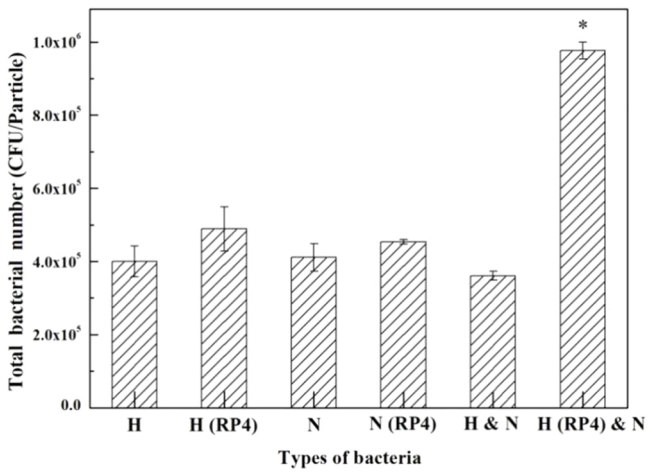

Fig. 1. Total bacterial colonization number on microplastics under different types of bacteria. The error line indicates the standard deviation of the three repeated measurements. "* " indicates that there are significant differences between the experimental groups and all the other groups.

samples was rejected at a value less than or equal to 0.05.

3. Results

3.1. Effect of RP4 plasmid on bacterial colonization

First, the effects of a single factor on bacterial colonization were investigated (Fig. S1, Supporting Information). The details are described in the Supporting Information. To facilitate the experiment, the reaction time was eventually selected as 24 h, the initial concentration of bacteria was 10^8 CFU/mL, the temperature was 37 °C, and the microplastic concentration was 1.0 particles/mL. Next, the total bacterial colonization number of microplastics with different bacterial types was studied under the above selected conditions (Fig. 1). Under the same initial bacterial count, the bacterial number colonized on microplastics in the "H (RP4)" & "N" reaction system was 2.1–2.5 times higher than the bacterial colonization numbers of the other five types. Bacterial colonization of microplastics was observed using SEM (Fig. 2). In the "H" & "N" reaction system, the bacteria were loosely adhered on the microplastic surface and were arranged in a single layer. In the "H (RP4)" & "N" reaction system, the density of bacteria on the surface of the microplastics was evidently higher than the density of bacteria on the surface of the microplastics in the former system, and the bacteria were densely distributed, displaying a multilayered arrangement. The biomass of biofilms determined by the crystal violet assay is shown in Supporting Information Text 8 and Fig. S6. All the total bacterial colonization data on microplastics counted by plates with and without antibiotics are listed in Supporting Information Tables S2, S3 and S4. The results illustrated that there was no significant difference in the colony count data of ARB on plates with or without corresponding antibiotics, and inaccurate colony counts caused by selective pressure of antibiotic plates were excluded.

3.2. Effect of nanoalumina and FNA on bacterial colonization and conjugative transfer

As shown in Fig. 3 (A), in the "H", "N" and "H" & "N" reaction systems, no significant difference was observed between the experimental and control groups in the total bacterial number on the microplastics. When the concentration of nanoalumina exceeded 0.5 mmol/L, the bacterial colonization of microplastics in the experimental group increased significantly by 1.6–2.6 times compared with the control group of the "H (RP4)", "N (RP4)" and "H (RP4)" & "N" reaction systems. Compared with the "H (RP4)" reaction system control group (Not adding nanoalumina), the addition of nanoalumina and recipient bacteria increased bacterial colonization of microplastics by a factor of 6.6. As shown in Fig. S2 (A) (Supporting Information), compared with the control group, the addition of nanoalumina (>0.5 mmol/L) significantly increased the conjugation frequency of the RP4 plasmid with suspended bacteria, up to approximately 50 times. When the concentration of nanoalumina was lower than 0.5 mmol/L, there was no significant difference between the experimental and control groups; only when the concentration of nanoalumina exceeded 2.5 mmol/L was the conjugation frequency of the experimental group in the biofilm state significantly higher than the conjugation frequency of the experimental group in the biofilm state of the control group, but the highest promotion was only approximately 2 times, which was far less than 50 times the value in the suspended state.

As shown in Fig. 3 (B), in the "H (RP4)" & "N" reaction system, when the concentration of FNA was more than 0.01 mg/L, the bacterial colonization of microplastics in the experimental group was significantly lower than the bacterial colonization of microplastics in the control group, and the bacterial colonization with 0.02 mg/L FNA was only 32% of the control. In other reaction systems, there was no significant difference in the total bacterial number on microplastics between the experimental and control groups. As shown in Fig. S2 (B), compared with the control group, the addition of FNA (>0.005 mg/L) significantly reduced the RP4 conjugative transfer frequency in the suspended state by up to 8 times. When the concentration of FNA was lower than 0.1 mg/L, there was no significant difference in the

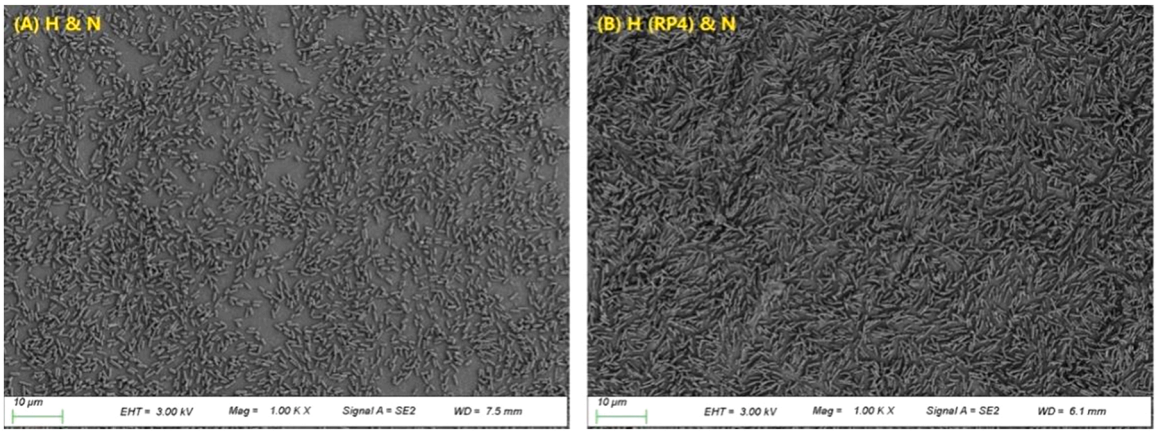

Fig. 2. Bacterial colonization on microplastics was observed using scanning electron microscopy (SEM). (A) Surface of microplastics in the "H" & "N" reaction system. (B) Surface of microplastics in the "H (RP4)" & "N" reaction system.

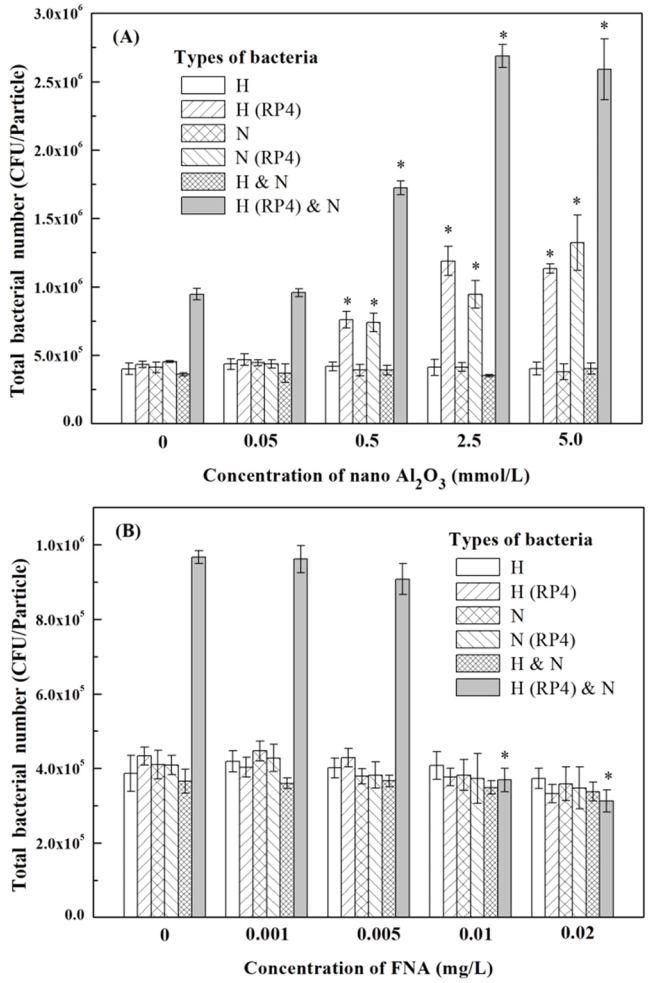

Fig. 3. Effect of nanoalumina and FNA on bacterial colonization on the microplastic surface. The error lines indicate the standard deviation of the three repeated measurements. "* " indicates that there are significant differences between the experimental groups and the control groups.

conjugation frequency between the experimental and control groups in the biofilm state. When the concentration of FNA was 0.02 mg/L, the conjugation frequency of the experimental group in the biofilm state was significantly lower (only approximately 1.6 times) than the conjugation frequency of the control group. The survival rate of bacteria after FNA treatment is shown in Supporting Information Table S5. The final FNA concentrations after the reaction are listed in Supporting Information Table S6.

3.3. mRNA analysis and ATP analysis

As shown in Fig. 4, the detected expression levels of *traA, traB*, and *traC* were mainly consistent with the previous test results (Fig. 3), and the subtle differences are examined in the discussion section. In the control group, compared with the "H (RP4)" system, the *traA* expression levels in the "H (RP4)" & "N" system increased significantly. Compared with the control group, the addition of nanoalumina significantly increased the *traA* expression levels up to 4.1 times in the "H (RP4)" & "N" and "H (RP4)" systems. The addition of FNA decreased the *traA* expression levels in the "H (RP4)" & "N" systems by a factor of 7.4. Compared with the control group, there was no significant difference in *traB* expression levels in the "H (RP4)" & "N" and "H (RP4)" systems (Fig. 4 (B)). As shown in Fig. 4 (C), after the addition of FNA, the *traC* expression levels in the "H (RP4)" system decreased by 1.8×10^{-4} copies/16 S rRNA copies compared with the control group. In addition

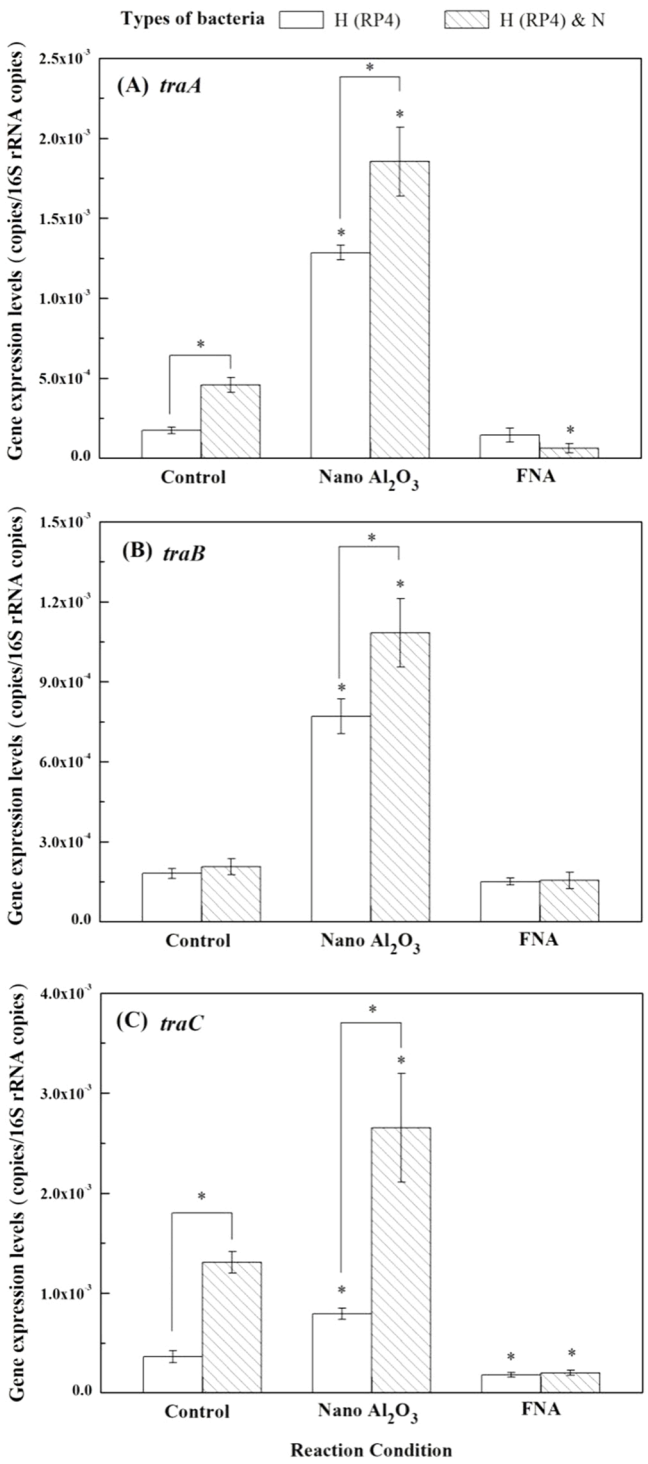

Fig. 4. Expression of genes related to pili under different reaction conditions. (A) *traA* expression levels. (B) *traB* expression levels. (C) *traC* expression levels. Nanoalumina (0.5 mmol/L) and FNA (0.01 mg/L) were used in the experiment. The error lines indicate the standard deviation of the three repeated measurements. "* " indicates that there are significant differences between the experimental groups and the control groups.

to the above differences, the results of *traB* and *traC* were essentially similar to that of *traA*. As shown in Fig. 5, in the control group, the relative concentration of intracellular ATP in the "H (RP4)" & "N" system was 2.4 times higher than the relative concentration of intracellular ATP in the "H (RP4)." When nanoalumina was added, the intracellular ATP concentration in the experimental group was significantly higher

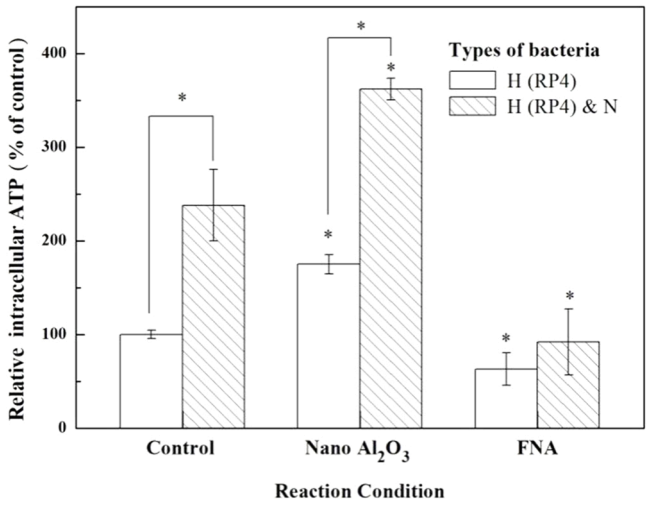

Fig. 5. Relative intracellular ATP under different reaction conditions. Relative intracellular ATP (% of control) = [the detected intracellular ATP concentration/the control ("H (RP4)") intracellular ATP concentration] × 100%. Nanoalumina (0.5 mmol/L) and FNA (0.02 mg/L) were used in the experiment. The error lines indicate the standard deviation of the three repeated measurements. "* " indicates that there were significant differences between the experimental and control groups.

than the intracellular ATP concentration in the control group. However, the intracellular ATP concentration decreased significantly after the addition of FNA.

4. Discussion

4.1. RP4 promoted bacterial colonization of microplastics

As shown in Fig. 1, the significant difference between "H (RP4)" and "H (RP4)" & "N" shows that the existence of recipient bacteria could improve bacterial colonization in the presence of RP4 plasmid, while the significant difference between experiments "H" & "N" and "H (RP4)" & "N" shows that RP4 has the ability to promote bacterial colonization of microplastics. The same can be inferred from Fig. 2. In the "H (RP4)" & "N" reaction systems, the surface of the microplastics exhibited denser bacterial aggregation, with blurred bacterial boundaries. All aforementioned results demonstrated that RP4 could promote bacterial colonization of microplastics, thus promoting microplastic biofilm development. Both F and RP4 plasmids could evidently improve the ability for bacterial colonization (Ghigo, 2001). Conjugative pili, as an adhesion factor, increased the effective surface area and adhesion ability of biofilms, which adhered more to bacteria suspended in water. The pili of the two plasmids were regulated and synthesized mainly by the *tra* gene region on the plasmid. However, in contrast to the F plasmid, the RP4 plasmid belongs to the repressed plasmid, which can inhibit the synthesis of pili. In other words, in the absence of external stimulation, the single bacteria carrying the RP4 plasmid can express and synthesize a limited number of pili (Bradley et al., 1980; Ghigo, 2001). Therefore, with only "H (RP4)", there was no significant difference in the bacterial colonization ability on microplastics among experiments. The addition of recipient bacteria can facilitate the transfer of the conjugative plasmid RP4; for conjugative transfer to occur, the gene transfer channel should be established by the conjugative pili protein, during which there might be a mechanism that controls the expression levels of pili synthesis genes, and the increase in protein synthesis of pili ultimately promotes bacterial colonization of microplastics. Therefore, potential conjugative pili synthesis promoters (nanoalumina) and inhibitors (FNAs) were selected to verify whether RP4 promoted bacterial colonization on the microplastic surface by conjugative pili protein expression and to study their effects on bacterial colonization. Meanwhile, the gene expression levels controlling pili synthesis in different reaction systems were analysed to verify the above hypothesis.

4.2. Effect of nanoalumina and FNA on bacterial colonization and conjugative transfer

From Fig. 3, nanoalumina (0–5 mmol/L) was observed to have a negligible effect on bacterial colonization in the absence of the RP4 plasmid. When the RP4 plasmid was present, nanoalumina (>0.5 mmol/L) promoted bacterial colonization in the "H (RP4)", "N (RP4)" and "H (RP4)" & "N" reaction systems. These results indicated that nanoalumina promoted bacterial colonization by affecting RP4-related factors. When there are no recipient bacteria, nanoalumina may also reduce the inhibition of RP4 on pili synthesis and ultimately promote bacterial colonization. It was hypothesized that nanoalumina could promote the expression of pili regulatory genes in the RP4 plasmid. When the concentration of nanoalumina reached 0.5 mmol/L, the synthesis of pili protein increased, which promoted bacterial colonization of microplastics. Similarly, a previous study showed that nanoTiO_2 had the ability to increase pili regulatory gene expression of the F plasmid, which was consistent with the conjecture of this study(Han et al., 2020). Therefore, mRNA analysis experiments were subsequently conducted to verify this conjecture. In the absence of the RP4 plasmid or only in the presence of donor bacteria, FNA (0–0.02 mg/L) had a negligible effect on bacterial colonization (Fig. 3). In the "H (RP4)" and "N (RP4)" reaction systems, the accelerant nanoalumina promoted bacterial colonization, while the inhibitor FNA did not inhibit bacterial colonization, possibly because when the donor bacteria existed alone, there was no stimulation from the recipient bacteria. RP4 itself belongs to the pili synthesis-repressed plasmid. Because few pili were synthesized and had no significant effect on bacterial colonization, although FNA may inhibit pili synthesis, FNA does not inhibit bacterial colonization. In the "H (RP4)" & "N" reaction systems, FNA (>0.01 mg/L) inhibited bacterial colonization. A possible reason for this is that the RP4 plasmid could express and synthesize more pili in the presence of the recipient. At this time, FNA exhibited an inhibitory effect to reduce bacterial colonization to a level comparable to that of the control group, and subsequent mRNA was analysed to verify the hypothesis.

The effect of nanoalumina and FNA on conjugative transfer is described in detail in the Supporting Information. Similar to a previous study (Qiu et al., 2012), nanoalumina promoted conjugative transfer in suspended bacteria (Fig. S2). In this study, biofilm formation promoted conjugative transfer, which was similar to previous research (Arias-Andres et al., 2018). Conjugation in biofilms was less affected by nanoalumina than in suspension. Similar to previous studies (Huang et al., 2019), FNA inhibited conjugative transfer in suspended bacteria (Fig. S2). Similar to nanoalumina, the influence of FNA on biofilm bacteria was considerably less than the influence of FNA on biofilm bacteria in the suspended state, possibly due to the accumulation in the biofilm of microorganisms, which were encapsulated in extracellular polymer substances (EPS) and had their own resistance to external pressure (e.g., nanoalumina) (Zhang et al., 2020). Meanwhile, the formation of biofilms did not necessarily promote conjugative transfer under certain conditions; for example, in the presence of high concentrations of nanoalumina (>2.5 mmol/L), compared with the suspended state, where the frequency of conjugative transfer on biofilms decreased significantly.

4.3. Mechanism by which RP4 promotes bacterial colonization of microplastics

In previous studies on the effects of nanoalumina and FNA on conjugative transfer, there have been few reports on gene expression related to pili. Few studies have focused on *traG* and *traF* (Huang et al., 2019). The main function of the *traG* protein is to bind to the relaxor and

transport it across the bridge (this function is crucial for conjugative transfer rather than pili synthesis). *TraF* is involved mainly in the assembly of pili. However, pili are composed mainly of one fimbriae protein subunit, which is the expression product of *traA*. The complete *traA* gene encodes 121 amino acids with a molecular weight of 13 Ku, of which the first 51 amino acids are the leading peptide sequence, and the mature pili protein contains 70 amino acid residues with a molecular weight of 7 Ku (Frost et al., 1994). Approximately 14 proteins are involved in the formation and function of pili, such as *traA, traQ, traL, traE, traB, traV, traC, traW, traU, traH, traX, traF,* and *traG* (Lin and Liu, 2008). *TraA* is evidently the main component of pili, but the expression of *traA* is sometimes excluded in previous reports. Therefore, in this study, *traA*, *traB*, and *traC* were selected because *traA* protein is the main component of pili, *traB* participates in the assembly of pili, and *traC* is involved in ATPase activity, which is used for energy supply in the synthesis of pili and can reflect the energy metabolism in the process of pili synthesis to a certain extent (Frost et al., 1994).

Under similar conditions, the total bacterial number on the microplastics was consistent with the trend in *traA* expression levels (Fig. 4). The addition of the recipient could significantly increase the synthesis of pili and promote bacterial colonization of microplastics. In addition, nanoalumina significantly increased the synthesis of pili. Furthermore, FNA could significantly inhibit the synthesis of pili in the presence of both donor and recipient bacteria. These results are consistent with previous experimental phenomena.

Compared with the control group, there was no significant difference in *traB* expression levels in the “H (RP4)” & “N” and “H (RP4)” systems (Fig. 4 (B)), and *traB* did not regulate the synthesis of the main proteins of pili. Therefore, the expression of *traB* can be used only as a reference for pili protein synthesis and not as a decisive factor. It is also possible to synthesize different quantities of pili protein under the same *traB* expression levels. In addition to the above differences, the results for *traB* and *traA* were similar. As shown in Fig. 4 (C), after the addition of FNA, the *traC* expression levels in the “H (RP4)” system decreased by 1.8×10^{-4} copies/16 S rRNA copies compared with the control group, possibly FNA itself could significantly reduce intracellular ATP concentrations by varying intracellular Fe^{2+} and Mg^{2+} concentrations (Huang et al., 2019). The main function of the *traC* protein is ATPase activity, and the expression of *traC* decreased when the concentration of intracellular ATP decreased. From the previous analysis, the presence of donor bacteria itself was observed to inhibit the synthesis of pili, and the amount synthesized was limited, during which pili did not demonstrate the ability to promote bacterial colonization of microplastics. Therefore, the decrease in *traC* expression level insignificantly affected bacterial colonization. In addition to the above differences, the results for *traC* and *traA* were essentially similar.

The M13 phage can infect only bacteria that express the conjugative pili, which can delay the growth of infected bacteria. Increased synthesis of conjugative pili proteins by bacteria significantly delays bacterial growth in the medium (Lin et al., 2011). Therefore, M13 can relatively quantify the amount of pili protein expressed by bacteria. Referring to a previous study (Wang et al., 2015), detailed test methods are described in the Supporting Information. As shown in Fig. S3 (A) (Supporting Information), in the absence of M13, the growth curves of “H (RP4)” were similar under different reaction conditions. As shown in Fig. S3 (B), in the presence of M13, the growth rate of bacteria in the “H (RP4)” system treated with nanoalumina decreased significantly compared with the growth rate of bacteria in the control group, while FNA did not significantly change bacterial growth, which was consistent with the results in Fig. 4. The results showed once again that nanoalumina could promote the overexpression and synthesis of conjugative pili proteins in donor bacteria.

From the results of Fig. 3, nanoalumina and FNA did not significantly affect bacterial colonization of microplastics in the “H” & “N” reaction system, while nanoalumina significantly promoted and FNA significantly inhibited bacterial colonization in the “H (RP4)” & “N” reaction system. The above analysis showed that nanoalumina and FNA affected bacterial colonization by affecting factors related to RP4, which excluded the effects of these two compounds themselves on colonization. Simultaneously, the gene expression analysis results were consistent with previous experimental results, which indicated that nanoalumina could promote and FNA could inhibit the synthesis of pili. In summary, the addition of promoter nanoalumina improved the bacterial colonization ability on microplastics, and the addition of inhibitor FNA reduced the bacterial colonization ability on microplastics to the level of the control group, which confirmed that the conjugative antibiotic-resistant plasmid RP4 could promote bacterial colonization of microplastics by increasing the synthesis of pili.

ATP analysis indicated that the addition of recipient bacteria significantly increased intracellular ATP levels. Nanoalumina could increase the intracellular ATP concentration and provide more sufficient energy for pili synthesis, simultaneously promoting the expression of *traA*, *traB*, and *traC* and finally promoting the synthesis of pili. However, FNA inhibits ATP synthesis, reduces the energy required for pili synthesis, and then inhibits the expression of *traA* and *traC*, thereby inhibiting the synthesis of pili.

4.4. Relationship between bacterial colonization and conjugative transfer

The donor-carried conjugative pili synthesis-repressed RP4 and produced few conjugative pili. However, when the donor encountered the recipient, the rare accidental encounter between transfer-proficient bacteria carrying inhibited plasmids and potential recipients initiated the cascade of conjugative transfer because pili synthesis was transiently derepressed, which promoted pili synthesis (Ghigo, 2001). This phenomenon, known as "epidemic transmission", continued until all potential recipients obtained plasmids. Therefore, when donor and recipient bacteria coexisted, RP4 promoted bacterial colonization of microplastics by increasing the synthesis of conjugative pili protein. Simultaneously, bacterial colonization greatly increased the bacterial density on the surface of microplastics, increased the contact frequency between donors and recipients, and then promoted the conjugative transfer of ARGs. In summary, the increase in conjugative pili promoted bacterial colonization of microplastics and conjugative transfer, and a high bacterial colonization frequency could also promote conjugative transfer. Conjugative transfer promoted the synthesis of conjugative pili by transiently activating pili synthesis-repressed plasmids and then promoted bacterial colonization. However, for nonpili synthesis-repressed plasmids, such as the F plasmid, conjugative transfer did not significantly affect biofilm formation because the donor carrying the F plasmid can synthesize many pili by itself (Ghigo, 2001).

4.5. Implications of this study

In a real water environment containing several bacteria, recipient bacteria must be present. The RP4 plasmid could promote irreversible bacterial colonization of microplastics and promote the formation of mature microplastic biofilms. In particular, the conjugative resistant plasmid containing ARGs not only promotes bacterial colonization of microplastics but also promotes ARG conjugative transfer in the microplastic biofilm. Moreover, some studies have demonstrated that most ARGs in wastewater treatment plants are carried by plasmids (Che et al., 2019). Therefore, conjugative plasmids carrying ARGs increase the risk of ARG transmission in the water environment, and a strategy to control this type of plasmid should be seriously considered. In the water environment, nanoalumina can promote ARG conjugative transfer and can also promote bacterial colonization of microplastics; hence, entry of ARG into the water environment should be controlled. In contrast, FNA can inhibit ARG conjugative transfer and bacterial colonization; hence, it can be considered to control the transmission of ARGs.

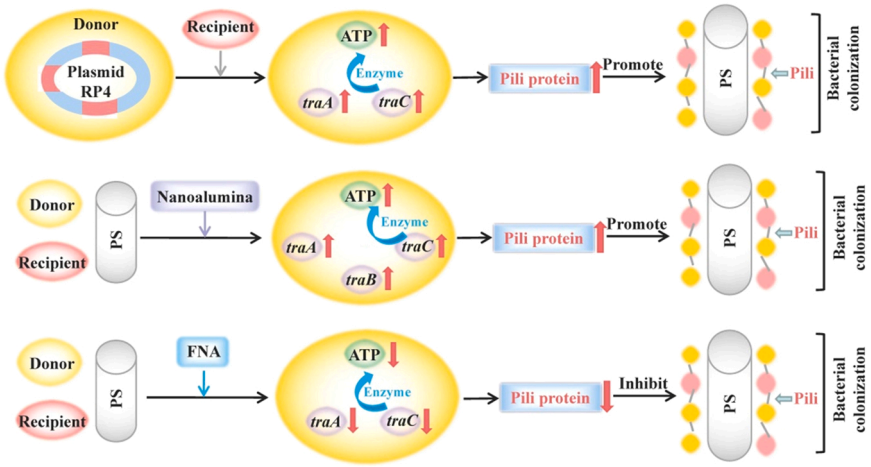

Fig. 6. Mechanism by which RP4 promotes bacterial colonization on the microplastic surface.

5. Conclusions

This study demonstrates the effects of RP4, nanoalumina and FNA on bacterial colonization on microplastic surfaces. Fig. 6 explains the mechanism by which the conjugative antibiotic resistant plasmid RP4 promotes irreversible bacterial colonization of microplastics in the early stage of biofilm formation in the water environment. When donor bacteria carrying RP4 exist alone, they have a negligible effect on bacterial colonization of microplastics because they themselves inhibit the synthesis of conjugative pili. When donors and acceptors coexist, the presence of recipients increases the intracellular ATP concentration and intracellular energy supply, simultaneously increasing the expression level of conjugative pili synthesis genes and promoting the synthesis of conjugative pili. At this time, conjugative pili can be used as an adhesion factor to promote bacterial colonization. Similarly, nanoalumina and FNA can promote and inhibit bacterial colonization of microplastics, respectively, by affecting intracellular ATP concentration and conjugative pili synthesis.

CRediT authorship contribution statement

Zhang Guosheng: Conceptualization, Methodology, Data curation, Writing – review & editing, Writing – original draft. **Chen Jiping:** Conceptualization, Methodology, Visualization, Investigation, Supervision, Validation. **Li weiying:** Visualization, Investigation, Supervision, Validation,

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.128443.

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