Chitin Nanowhiskers Mediate Transformation of *Escherichia coli* by Exogenous Plasmid DNA

**Alisa Mera**¹, Jun Araki², Takashi Ohtsuki², Makoto Shimosaka³ and Naoto Yoshida⁴*

¹Department of Biochemistry and Applied Biosciences, University of Miyazaki, 1-1 Gakuen Kibanadai-Nishi, Miyazaki 889-2192, Japan
²International Young Researchers Empowerment Center, Shinshu University, Tokida 3-15-1 Ueda, Nagano 386-8567, Japan
³Graduate School of Medicine and Engineering, University of Yamanashi, Kofu 400-8511, Japan
⁴Division of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, Tokida 3-15-1, Ueda, Nagano 386-8567, Japan

**Abstract**

A colloidal solution of chitin nanowhiskers containing pUC18 plasmid DNA and cells of *Escherichia coli* was placed on an agar hydrogel and stimulated by sliding friction applied between the agar hydrogel and polystyrene stir stick, leading to transformation of the bacteria to antibiotic resistance. The combination of chitin nanowhiskers and sliding friction was necessary for genetic transformation, indicating that the chitin nanowhiskers induced the Yoshida effect, resulting in the formation of *E. coli* cells penetration-intermediates. The pUC18 adsorbed onto the chitin nanowhiskers is presumably taken up through the penetration-intermediates, leading to transformation. The transformation efficiency changed when the number of recipient cells and amount of chitin nanowhiskers were varied. The maximum number of penetration-intermediates acquiring pUC18 DNA was obtained with concentrations of agar hydrogel, chitin nanowhiskers, and recipient *E. coli* cells of 2.5%, 5.0 μg/ml, and 4.8 x 10⁹ colony forming units/μg of pUC18.

**Key words:** Chitin nanowhisker; *Escherichia coli*; Plasmid; Transformation; Yoshida effect

**Introduction**

Chitin is the most abundant natural amino polysaccharide, with more than 10¹¹ tons of chitin produced annually [1]. There is currently great interest in chitin, not only as an underutilized resource, but also for its potential to serve as a valuable new multifunctional material. The current literature contains ample information regarding the purification [2,3], modification [4,5], degradation [6,7], and physical properties of chitin [8,9]. Because of their antibacterial, moisture retaining, and healing characteristics, chitin and chitosan (partially deacetylated chitin) are often utilized in water purification [10,11] or as additives in cosmetics [12,13], antibacterial agents [5,14], and pharmaceutical adjuvants [4,15,16]. However, many applications do not effectively utilize chitin, and a considerable amount goes to waste.

Heterogeneous acid hydrolysis of purified crab shell chitin produces fragmented microfibrils known as crystallites [17]. These partially acetylated crystallites are rod-like colloids produced by chain cleavage occurring at random locations along the microfibrils. Protonation of amino groups on the crystallites provides a positive surface charge and stabilizes the colloids due to repulsive forces between crystallites [18]. Li et al. [18] also reported that the degree of deacetylation, zeta potential, weight loss, and crystallinity of chitin nanowhiskers produced from crab shell chitin that has been deacetylated using a NaOH treatment can be controlled by manipulating the reaction time and conditions of acid hydrolysis.

When a colloidal solution consisting of nano-sized acicular material and bacterial cells is placed in a sliding friction field applied between a hydrogel and an interface forming material, the nano-sized acicular material and bacterial cells form a complex called a penetration-intermediate. This phenomenon is referred to as the Yoshida effect [19], and has been referenced by several microbiotechnology researchers [20,21]. Bacterial cells readily take up exogenous DNAs through penetration-intermediates by the Yoshida effect. Uptake of plasmid DNA adsorbed on chrysotile by the penetration-intermediate of *Escherichia coli* has been shown to alter its antibiotic resistance [22]. As a result of the discovery of the Yoshida effect, the penetration-intermediate has become a useful tool for introducing exogenous genes into bacteria [23], and for applications such as quantitative detection of asbestos species in the environment [24].

The occurrence of the Yoshida effect can be ascertained by assessing the transformation efficiency of the penetration-intermediate using plasmid DNA. The Yoshida effect has been confirmed using nano-sized acicular materials including multi-walled carbon nanotubes, maghemite (γ-Fe₂O₃), α-sepiolite, and chrysotile with a diameter of 10–50 nm. Whichever nano-sized material is employed, a hydrogel, an interface-forming material, and an energy source for generating sliding friction are essential to produce the Yoshida effect.

The shape of chitin nanowhiskers prepared by Li et al. [18] and Revol and Marchessault [25] closely resembles that of the nano-sized acicular materials that induce the Yoshida effect. In this study, we evaluated whether chitin nanowhiskers are sufficient to produce the Yoshida effect by investigating the frequency of plasmid uptake in penetration-intermediate *E. coli* cells. We also determined the optimum conditions needed to form penetration-intermediates capable of acquiring plasmid DNA.

*Corresponding author: Naoto Yoshida, PhD, Department of Biochemistry and Applied Biosciences, University of Miyazaki, 1-1 Gakuen Kibanadai-Nishi, Miyazaki 889-2192, Japan, Tel:+81-985-58-7218; E-mail: a04109u@cc.miyazaki-u.ac.jp

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mid DNA in order to establish a genetic transformation method in *E. coli* dependent on chitin nanowhiskers and the Yoshida effect.

**Materials and Methods**

**Preparation of a chitin nanowhisker suspension**

An aqueous suspension of chitin nanowhiskers was prepared by acid hydrolysis of commercial α-chitin powder (Tokyo Chemical Industry Co., Ltd.) obtained from crab shells, according to a previously reported method [23]. Typically, 10 g of air-dried α-chitin powder was treated for 90 min with 100 ml of boiling 3 M HCl under reflux and with vigorous stirring. The sample was then washed with distilled water by repeated centrifugation (1600 x g, 5 min)-dilution cycles until the supernatant reached a pH of about 2. Above pH 2, the coarse aggregations from the hydrolysis residue began to disperse to yield a turbid supernatant containing colloidal particles of chitin nanowhiskers. Combined aliquots of the turbid supernatants from several centrifugation cycles were thoroughly dialyzed against distilled water until reaching a neutral pH, yielding a suspension with a solid content of 0.5–1%. The yield of nanowhiskers relative to the amount of starting α-chitin powder was around 70%.

**Transmission electron microscopy of chitin nanowhiskers**

The morphology of the chitin nanowhiskers was observed using transmission electron microscopy (TEM). A drop of a dilute (< 0.1%) suspension of chitin nanowhiskers was deposited on a carbon-coated Formvar sheet spread on a copper grid (Okenshoji Co., Ltd., Tokyo, Japan). The dried sample was observed at 80 kV without staining and using a defocus contrast technique using JEOL JEM-2100 transmission electron microscope.

**Fluorescent microscopic observation of chitin nanowhiskers**

A chitin nanowhisker colloidal solution (2.8 mg/ml) was mixed with 0.5 µM synthetic DNA oligonucleotide (5’-CTA CCG CTT CGT GGA GCA GCC CGC CC-3’) tagged at the 5’ end with fluorescein isothiocyanate (FITC) (Sawady Co., Japan), and then observed directly using fluorescence microscopy (Axioskop2plus, Zeiss). For fluorescence studies, B-2A (BP 460–500 nm; DM 505 nm; BA 510–560 nm) filter blocks (Nikon) were used to visualize FITC. The experiments were documented using a Nikon digital camera.

**Determining the amount of pUC18 adsorbed onto chitin nanowhiskers**

Colloidal solutions with varying concentrations of chitin nanowhiskers (0.35-1.40 mg/ml) were mixed with pUC18 DNA (at a final concentration of 25 µg/ml) and centrifuged at 15,000 x g for 10 min. The concentration of pUC18 DNA remaining in supernatant was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, DE, USA) equipped with DNA measurement software. The wavelength spectrum of the supernatant was determined by wavelength scanning using the NanoDrop ND-1000 to confirm the presence of an absorbance peak at 260 nm. Wavelength scans were performed from 230 to 300 nm. The amount of pUC18 adsorbed onto the chitin nanowhiskers was calculated from the difference between the initial and the remaining amount of pUC18 in the supernatant.

**Cultivation of recipient cells**

*Escherichia coli* JM109 served as the recipient. Cells grown on Luria-Bertani (LB) plates [26] were inoculated into LB broth and cultured for 14–18 h at 30°C with aeration (provided by shaking at 150 rpm). The culture broth was directly subjected to the Yoshida effect when the cell density reached an OD600 of 3.0 (6.0 x 10^6 cells/ml), as determined by spectrophotometry.

**Factors needed to induce the Yoshida effect**

To investigate the occurrence of the Yoshida effect by using chitin nanowhiskers, *Escherichia coli* JM109, agar (Nacalai Tesque, Japan), polystyrene stir sticks (Sarstedt, Nünbrecht, Germany), and an automatic turning table equipped with a Gene Injector (Preci Co. Ltd., Japan) served as the plasmid recipient cells, the hydrogel, the interface forming material, and the energy source to produce a sliding friction field, respectively. pUC18 DNA was used as the donor plasmid. *E. coli* cells were transformed with pUC18 to express a phenotype of ampicillin resistance.

**Standard protocol for inducing the Yoshida effect**

Agar hydrogel plates were prepared by autoclaving a 1.5-3.5% agar (Nacalai Tesque, Japan) solution containing LB nutrient at 121°C for 15 min. Ampicillin was then added to a final concentration of 100 µg/ml and the agar was allowed to solidify in Petri dishes (8.5 cm diameter). The surfaces of the agar hydrogel plates were rapidly dried in a clean room to remove all visible water condensation before applying sliding friction. A 50 µl volume of chitin nanowhisker colloidal solution (0.1–50 µg/ml) containing 1.0 ng of pUC18 DNA was placed on the agar hydrogel, followed by 50 µl of *E. coli* culture broth. Sliding friction was applied to the surface of the agar hydrogel without disrupting the agar for 60 seconds using a polystyrene stir stick by rotating the plate on an automatic tuning table set at 90 rpm. The vertical reaction force of the polystyrene stir stick was maintained at 40 g/cm² using the Gene Injector [23].

**Evaluation of the occurrence of the Yoshida effect**

The frequency of penetration-intermediate formation is reflected by the number of colony forming units transformed to ampicillin resistance. After sliding friction was applied, the agar hydrogel plates were incubated at 37°C for 15–18 hr, after which the number of colonies demonstrating ampicillin resistance was determined. The transformation efficiency was expressed as the number of transformant colonies per microgram of pUC18 DNA.

**Results**

**Transmission electron microscopy of chitin nanowhiskers**

The colloidal chitin nanowhiskers obtained from crab chitin hydrolyzed with 3 M HCl was observed under a scanning electron microscope. As shown in Figure 1A, the presence of acicular and high crystallinity particles with an axial ratio of about 21 was confirmed. As the average size of a typical chitin nanowhisker was 7 x 7 x 150 nm, the number of chitin nanowhiskers was estimated to be approximately 9.55 x 10^11 per mg.

**Observation of chitin nanowhiskers using fluorescence microscopy**

Chitin nanowhiskers mixed with DNA oligonucleotide tagged at the 5’ end with FITC were observed under a fluorescence microscope. Chitin nanowhiskers fluoresced green (Figure 1B). It should be noted that significant amount of chitin nanowhiskers aggregated themselves, but acicular crystalline material was also observed. These results indi-
cated that nucleic acid was effectively adsorbed onto the surface of the chitin nanowhiskers.

Amount of pUC18 DNA adsorbed onto chitin nanowhiskers

Mixing pUC18 DNA with chitin nanowhiskers resulted in adsorption of pUC18 onto the nanowhisker surfaces and a decrease in the amount of free pUC18. The pUC18 solution (23.5 µg/ml) solution without chitin nanowhiskers showed a large adsorption peak at 260 nm (Figure 2, inset). This peak decreased as the chitin nanowhisker concentration increased, and the DNA concentration in the supernatant was 3.55 and 2.78 µg/ml when the concentration of chitin nanowhisker was 0.46 and 0.56 mg/ml, respectively. No free pUC18 DNA was detected in the supernatant containing 0.70 mg/ml or more of chitin nanowhiskers (Figure 2). From these results, the maximum amount of pUC18 adsorbed onto the chitin nanowhiskers was calculated to be 35.7 µg/mg of chitin nanowhiskers.

Occurrence of the Yoshida effect induced by chitin nanowhiskers

When 50 µl of a mixture containing 30 µg/ml of chitin nanowhiskers, 200 ng/ml of donor pUC18, and 50 µl of E. coli culture broth was placed in a sliding friction field applied between an agar hydrogel (2.5%) and polystyrene stir stick, 1.5 x 10^4 colonies transformed to ampicillin resistance were obtained per microgram of pUC18 (Table 1). Under similar conditions but lacking either chitin nanowhiskers or pUC18 DNA, no ampicillin resistant colonies appeared. These results indicated that the transformation of the E. coli cells to antibiotic resistance was not due to chemical mutation induced by the chitin nanowhiskers. Instead, intracellular uptake of exogenous pUC18 facilitated the transformation of the E. coli cells to antibiotic resistance. Similarly, an elimination of E. coli cells did not bring any transformed colonies, indicating that the E. coli transformants observed in the previous experiments were not environmental contaminants. As an additional control, a removal of sliding friction was found to produce no ampicillin resistant colonies. It is clearly shown that the combination of chitin nanowhiskers and sliding friction are essential for E. coli transformation with pUC18 DNA. The occurrence of genetic transformation of E.coli through the intracellular acquisition of pUC18 indicated that a penetration-intermediate was formed and that chitin nanowhiskers had induced the Yoshida effect in the E. coli cells. Next, we optimized the conditions to maximize the transformation efficiency with pUC18 in E. coli.

Effect of hydrogel hardness on transformation efficiency

A 50 µl chitin nanowhisker colloidal solution containing pUC18 and 50 µl of E. coli culture broth was placed on variable concentration (1.5–3.5%) agar hydrogels, after which sliding friction was applied to the hydrogel surface. The concentrations of chitin nanowhiskers and E. coli cells were 30 µg/ml and 6.0 x 10^8 cells/ml, respectively. As shown in Figure 3, all concentrations of the agar hydrogel enabled the penetration-intermediate to acquire pUC18 with a transformation efficiency of more than 10^5 cells/µg of pUC18. The 2.5% agar provided the optimal hardness for transformation of the penetration-intermediate, producing a transformation efficiency of 2.6 x 10^5 cells/µg of pUC18.

Effect of chitin nanowhisker concentration on transformation efficiency

Colloidal solutions (50 µl) containing pUC18 and different concentrations of chitin nanowhiskers (0–150 µg/ml) were placed on agar hydrogels with 50 µl of E. coli culture broth, after which sliding

![Figure 1: Transmission electron microscopic observation of chitin nanowhiskers (A). Fluorescence microscopic observation of chitin nanowhiskers (B). Image shows crystals excited at 480 ± 20 nm.](image)

![Figure 2: Detection of free pUC18 DNA in the presence of varied concentrations of chitin nanowhiskers. Inset: UV spectrum of the supernatant containing free pUC18 DNA.](image)

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<th>Chitin nanowhisker (µg/ml)</th>
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Table 1: Combination of factors needed to introduce donor pUC18 DNA into recipient E. coli cells.
friction was applied. The concentrations of agar and *E. coli* cells were 2.5% and 6.0 x 10^8 cells/ml, respectively. As shown in Figure 4, at chitin nanowhisker concentrations of 0.1 and 1.0 µg/ml, the transformation efficiency of the penetration-intermediate was relatively low, with 3 x 10^3 and 1.7 x 10^4 ampicillin-resistant colonies formed/µg of pUC18, respectively. On the other hand, at chitin nanowhisker concentrations between 5.0 and 50 µg/ml, the transformation efficiency rose to more than 10^5 ampicillin-resistant cells formed/µg of pUC18. The colloidal solution containing 5.0 µg/ml of chitin nanowhiskers was optimal for transformation of *E. coli* cells, resulting in a transformation efficiency of 5.0 x 10^5 ampicillin-resistant colonies formed/µg of pUC18.

**Effect of recipient *E. coli* cell concentration on transformation efficiency**

Colloidal solutions (50 µl) of chitin nanowhiskers (5.0 µg/ ml) containing pUC18 were placed on agar hydrogel with 50 µl of culture broth containing varying the concentration of *E. coli* cells from 1.2 x 10^8 to 6.0 x 10^8 cells/ml, after which sliding friction was applied. The optimal culture broth concentration of *E. coli* for transformation of the penetration-intermediate is 4.8 x 10^8 cells/ml, which represents 2.4 x 10^8 cells in 50 µl of culture broth. This concentration of *E. coli* cells produced a transformation efficiency of 1.2 x 10^8 ampicillin-resistant colonies formed/µg of pUC18. (Figure 5).

**Discussion**

When a colloidal solution consisting of nano-sized acicular material and bacterial cells is stimulated by sliding friction at the interface between the hydrogel and the interface-forming material, where the frictional coefficient rapidly increases, the nano-sized acicular material forms a chestnut–bur shaped complex. This complex grows larger and penetrates the bacterial cells due to the driving force derived from the sliding friction, a process known as the Yoshida effect [27]. Chrysotile and α-sepiolite are nano-sized acicular materials that have been used for transformation with plasmid DNA through the Yoshida effect. The ability of chrysotile and α-sepiolite to adsorb DNA was demonstrated by Yoshida and Ide [22]. We mixed chitin nanowhiskers with oligonucleotide tagged at the 5’ end with FITC and observed them under a fluorescence microscope. This experiment indicated that the chitin nanowhiskers have a high affinity for adsorbing nucleic acid that makes them suitable for transformation using the Yoshida effect (Figure 1B).

Xu et al. [28] reported that the positive charge of chitosan acts to condense the relatively large negative charge of the plasmid DNA, such that it can be incorporated into the chitosan nanoparticles. Incorporation of the plasmid into the chitosan nanoparticles does not affect the structural integrity of the plasmid, as demonstrated by gel electrophoresis. The chitin nanowhisker surface leaves amino groups due to acid hydrolysis-induced deacetylation. Conductometric titration [29,30] indicated that acid hydrolysis-induced deacetylation resulted in 299 µmol of amino groups/g chitin nanowhiskers. The surface charge density of chitin nanowhiskers was demonstrated to be 0.45/nm², which is close to the value of 0.5–0.6/nm² previously reported by Li et al. [29]. The protonation of chitin nanowhisker surface amino groups imparts a positive surface charge that interacts with anionic polymers such as nucleic acids to form ionic complexes. Plasmid DNA adsors onto the surface of chitin nanowhiskers due to the negative charge of the DNA. The maximum amount of pUC18 DNA that will adsorb onto chrysotile whiskers at pH 7.0 is 1.1 µg/mg of chrysotile [22]. In contrast, the maximum amount of pUC18 DNA that adsors onto chitin nanowhiskers at pH 7.0 is estimated to be 35.7µg/mg of chitin nanowhiskers. The amount of
nucleic acid that would adsorb onto chitin nanowhiskers is significantly greater than that adsorbs onto chrysolite whiskers. Our experiments reveal that chitin nanowhiskers form a chestnut–bar shaped complex that grows larger as a result of sliding friction and penetrates E. coli cells due to the driving force derived from the sliding friction. Plasmid DNA adsorbed onto the chitin nanowhiskers is then introduced into the bacterial cells through the perforations.

Several groups have reported genetic transduction using chitin, including Richardson et al. [31], Mao et al. [32], and Bozik and Saka [33]. Bozik and Saka [33] demonstrated that deacetylated chitin nanoparticles substituting for virus particles are safe for use as gene delivery vehicles. Plasmid DNA was encapsulated in 450–820 nm chitin nanoparticles prepared according to the solvent evaporation and complex coacervation methods. The authors confirmed that the chitin nanoparticles protect the encapsulated plasmid DNA from nuclease attack, and also reported that the efficiency of chitin nanoparticle-mediated transformation of E. coli is significantly higher than transformation with naked DNA. However, they did not report the efficiency of transforming E. coli with plasmid DNA.

The efficient introduction of DNA into bacteria is a phenomenon of great practical importance in genetic engineering and molecular biology. The introduction of exogenous DNA into E. coli was first demonstrated by Mandel and Higa [34], who observed that incubating a suspension of E. coli cells and bacteriophage λDNA in a solution of CaCl2 at 0°C resulted in the subsequent appearance of infectious centers. They further showed that a heat pulse, in which the mixture of cells and DNA was briefly incubated at 42°C, chilled on ice, and then diluted into growth medium, improved the frequency of transfection. Since the first demonstration of Ca2+-dependent DNA transfer into E. coli by Cohen [35], Hanahan's protocol has been rigorously developed to achieve maximum transformation efficiency, and is now one of the best transformation methods available [36,37]. This method has been successfully applied to E. coli strain DH5α, yielding transformation efficiencies of 1010–1013 per microgram of plasmid DNA. We determined that E. coli JM109 can be transformed to ampicillin resistance via plasmid DNA and chitin nanowhiskers through the Yoshida effect, with a maximum transformation efficiency of 2.1 x 1010 per microgram of pUC18. The transformation efficiency we achieved using chitin nanowhiskers was comparable to that obtained using conventional chemical methods.

A great deal of effort has gone into the development of transformation technology, resulting in a large body of knowledge and a diverse array of methodologies. Some methods are very simple but are not widely applicable. Other methods may be more widely applicable but are more complex or difficult. Our newly developed method is simple, reliable, and highly reproducible, with the additional benefit that plating and transformation occur simultaneously. Interest in using chitin nanowhiskers and sliding friction force for gene introduction rather than chemical transformation is certain to increase as more researchers become aware of the method's low toxicity and technical simplicity.

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References


