

Acid stimulus-triggered acquisition of competency in *Escherichia coli*

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ABSTRACT

This work describes a novel phenomenon in which exposure of *Escherichia coli* to low concentrations of citric acid induces the uptake of exogenous plasmid DNA and transformation to antibiotic resistance. Transformation to ampicillin resistance using pUC18 was observed at an efficiency of 10^4 cells per microgram DNA. This acid stimulus-triggered acquisition of competency in *E. coli* was caused not only by citric acid but also by lactic acid, succinic acid, and malic acid. Transformation efficiency was not adversely affected by the washing of *E. coli* cells with 200 mM NaCl immediately after exposure to citric acid. Thus, DNA uptake can occur concurrently with stimulus by citric acid. The acid stimulus-triggered transformation was investigated using transposon insertion-disrupted *E. coli* strains. The transformation efficiency of an *ompA* disruptant decreased to 5% of that of the wild-type parent, suggesting that OmpA is involved in acid stimulus-triggered acquisition of competency in *E. coli*.

KEYWORDS: *Escherichia coli*, citric acid, plasmid, transformation, OmpA.

1. INTRODUCTION

Much attention has been paid to the effects of organic acids on bacterial cell growth, especially in the context of techniques for the prevention of food spoilage [1]. Organic acids such as acetic acid,

lactic acid, and citric acid are well known to have inhibitory effects on bacterial growth [2]. The antibacterial activity of an organic acid is affected by its adhesion to the microbe, membrane permeability, and hydrophilicity/hydrophobicity. When in a dissociated state (alkaline side of pKa), the organic acid is ionized and adsorbs on the cell surface. When in a non-dissociated state (acid side of pKa), the organic acid permeates the cell membrane and enters into the cell easily. Influxed organic acid typically dissociates at cellular pH, releasing a proton. The resulting decrease of intracellular pH can cause cell death due to inactivation of enzymes, or can inhibit cell growth due to excessive consumption of ATP to efflux intracellular protons. Notably, citric acid has antibacterial activity against Gram-positive bacteria and has been used for microbial control in refrigerated processed meats [3].

Previously, we reported a novel phenomenon in which the interaction (*via* sliding friction) of bacterial cells and nano-sized acicular material (such as sepiolite) results in the formation of a fusion body called the penetron [4]. The penetron was applied as part of a newly developed method to detect chrysotile asbestos-containing industrial waste materials with high sensitivity [5]. We observed that if an unknown sample includes chrysotile asbestos, sliding friction results in the formation of a penetron. The penetron is known to facilitate transformation to antibiotic resistance by enhancing the bacterial uptake of exogenous plasmid DNA. Thus, we are able to assess the amount of chrysotile contained in an unknown sample by counting the

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number of transformants, following the exposure of the bacterial sample. However, while the newly developed method permitted high-sensitivity detection of chrysotile, sensitivity to crocidolite and amosite asbestos was much lower. To detect crocidolite and amosite asbestos at high sensitivity, we investigated the pH range of our technique's reaction conditions and found that low pH, adjusted by citric acid buffer, yielded what appeared to be highly sensitive detection. However, we subsequently discovered that this improvement did not reflect an actual change in sensitivity, because transformation was observed with citric acid alone, even in the absence of asbestos. We realized that the transformation mediated by citric acid was occurring *via* an unknown bacterial process that enables the uptake of exogenous plasmid DNA. That is, separate from its antibiotic effects, citric acid renders *E. coli* competent.

In this report, we identify and characterize this novel phenomenon by which citric acid stimulus induces the transformation of *E. coli* by exogenous plasmid DNA. We propose a transformation mechanism whereby acid stimulus facilitates bacterial uptake of plasmid DNA *via* outer membrane transporter protein channels.

2. MATERIALS AND METHODS

2.1. Culture of *Escherichia coli*

Escherichia coli JM109 (*el4*⁺, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, Δ (*lac-proAB*), [F', *traD36 proAB*, *lacI*^f Δ M15]) [6] was used as plasmid recipient strain. The strain was inoculated onto an LB agar plate and cultured for 24 hr at 37 °C.

2.2. Acid stimulus-triggered transformation

E. coli cells from plate-grown colonies were suspended in distilled water to final absorbance at 600 nm of 2, and then used as the *E. coli* suspension. Acid solution, *E. coli* suspension, and plasmid DNA (pUC18) were mixed in a tube to yield final concentrations of 2-10 mM, 2×10^7 /mL, and 0.5 μ g/ml, respectively, and then the mixture was spread onto an LB agar plate containing 100 μ g/mL of ampicillin. The number of colonies transformed to ampicillin resistance was counted after incubation at 37 °C for 14-18 hr. The acid solutions were prepared using citric acid, sodium citrate, L-malic acid, succinic acid, lactic acid, oxalic acid, or DL-tartaric acid.

2.3. The effect of ATPase inhibitor on acid stimulus-triggered transformation

Citric acid solution (10 mM), *E. coli* (2×10^7 /mL), plasmid DNA (pUC18) (0.5 μ g/mL), and ATPase inhibitor were mixed in a tube, and then the mixture was spread onto an LB agar plate containing 100 μ g/mL of ampicillin. The LB agar plate was incubated at 37 °C for 14-18 hr. The number of colonies transformed to ampicillin resistance was compared with that obtained from a parallel transformation performed without ATPase inhibitor. Potassium cyanate (Wako, Japan) and sodium azide (Wako, Japan) were used (separately) as ATPase inhibitors, with KCN tested at concentrations of 4.0 or 20 mM and NaN₃ concentrations of 1.0 or 5.0 mM. *N,N'*-Dicyclohexylcarbodiimide (DCCD) (Wako, Japan), an uncoupling reagent, was tested at concentrations of 100 or 500 μ M.

2.4. Acid stimulus-triggered transformation of transposon insertion disruptants

The transposon insertion disruptants derived from *E. coli* KP700 (shown in Table 1) were purchased from National BioResource Project (NIG, Japan): *E. coli*. Each transposon insertion disruptant was inoculated onto LB agar medium containing 50 μ g/mL of kanamycin and incubated at 37 °C for 24 hr. A loopful of a resulting *E. coli* colony was suspended in sterilized distilled water and then used as the *E. coli* suspension. An aliquot (25 μ L) of this *E. coli* suspension was combined with 475 μ L of 10 mM citric acid solution and 0.5 μ L (0.25 μ g) of pUC18 DNA, and 50 μ L of the mixture was spread onto LB agar medium containing ampicillin (100 μ g/mL). After incubation at 37 °C for 14-18 hr, the colonies transformed to ampicillin resistance were counted.

3. RESULTS

3.1. Acid stimulus-triggered transformation

A 10 mM citric acid solution containing 0.5 μ g/mL of pUC18 DNA was rapidly mixed with an *E. coli* suspension. The reaction mixture was immediately spread onto LB agar medium containing ampicillin. After incubation at 37 °C for 18 hr, *E. coli* colonies transformed to ampicillin resistance emerged on the LB agar medium. The size of pUC18 extracted from transformants was confirmed by agarose gel electrophoresis (data not shown), indicating that

Table 1. *E. coli* KP700 transposon insertion disruptants screened for acid stimulus-triggered transformation.

Strain no. ¹⁾	Genotype	Insertion site (inserted after)	Disrupted gene
KP7600	Wild type: F, <i>lacF</i> ^o , <i>lacZAM15</i> , <i>galK2</i> , <i>galT22</i> , lambda-in (<i>rrnD-rrnE</i>)1		
JD20807	Transposon insertion disruptant	584779	<i>ompT</i>
JD20835	Transposon insertion disruptant	645521	<i>citT-3</i>
JD20836	Transposon insertion disruptant	645696	<i>citT-4</i>
JD21009	Transposon insertion disruptant	985724	<i>ompF</i>
JD21084	Transposon insertion disruptant	1380025	<i>ompG</i>
JD21989	Transposon insertion disruptant	1019184	<i>ompA</i>
JD22456	Transposon insertion disruptant	1434867	<i>ompN</i>
JD24803	Transposon insertion disruptant	4062200	<i>ompL</i>
JD25109	Transposon insertion disruptant	3534176	<i>ompR</i>
JD26455	Transposon insertion disruptant	1312403	<i>ompW</i>
JD27510	Transposon insertion disruptant	849779	<i>ompX</i>
JD20145	Transposon insertion disruptant	259303	<i>phoE</i>
JD21298	Transposon insertion disruptant	1912485	<i>prc</i>
JD22156	Transposon insertion disruptant	1162114	<i>lpoB</i>
JD24024	Transposon insertion disruptant	3176309	<i>tolC</i>
JD25333	Transposon insertion disruptant	4241286	<i>malG</i>
JD25334	Transposon insertion disruptant	4244248	<i>malE</i>
JD25336	Transposon insertion disruptant	4245041	<i>malK</i>
JD25338	Transposon insertion disruptant	4246351	<i>lamB</i>
JD27395	Transposon insertion disruptant	4242696	<i>malF</i>

¹⁾Designated by “NBRP-*E. coli* at NIG”.

E. coli was transforming to ampicillin resistance by incorporation of pUC18 DNA. Table 2 shows that citric acid mediates the transformation of *E. coli* by exogenous plasmid DNA. Transformation efficiency was 10⁴ colony-forming units (cfu) per µg of pUC18. Washing the *E. coli* cells with 200 mM NaCl following exposure to citric acid containing pUC18 did not affect the transformation efficiency (Table 2). These observations indicated that *E. coli* exposed to citric acid rapidly reacted to uptake pUC18. These phenomena did not occur in reaction mixtures lacking citric acid (Table 2).

As shown in Table 3, citric acid was the most potent organic acid for the induction of acid stimulus-triggered transformation in *E. coli*. L-Malic acid, succinic acid, and lactic acid yielded lower transformation efficiencies than the same concentration of citric acid. Sodium citrate did not

enable *E. coli* transformation by uptake of pUC18. Solutions of 10 mM citric acid, L-malic acid, succinic acid, or lactic acid all exhibited pHs of approximately 2.1-3.1. Sodium citrate is a salt composed of a weak acid and a strong base, and thus typically exhibits slight alkalinity (pH 7.7) in water. Acidity of the reaction mixture appears to be critical for citric acid stimulus-triggered acquisition of competency.

The optimum concentration for citric acid, for acid stimulus-triggered transformation was determined using *E. coli* JM109 and pUC18 DNA (Fig. 1). Exposure to citric acid at concentrations up to 0.5 mM did not provide stimulation of transformation to ampicillin resistance. Citric acid at 1.0 mM yielded 17000 ± 400 ampicillin-resistant colonies per µg pUC18. The highest number (23000 ± 400 cfu/µg pUC18) of *E. coli* transformants was

Table 2. Transformation of *Escherichia coli* JM109 to ampicillin resistance following exposure to citric acid in the presence of plasmid DNA.

Treatment with citric acid ¹⁾	Washing ²⁾	Transformation efficiency (cfu/ μ g pUC18)
+	-	10 ⁴
+	+	10 ⁴
-	-	ND ³⁾
-	+	ND

¹⁾10 mM citric acid.

²⁾*E. coli* JM109 cells were washed using 200 mM NaCl and centrifugation.

³⁾ND, not detected.

Table 3. Efficiency of acid stimulus-triggered transformation in the presence of various chemicals.

Chemicals ¹⁾	No. of transformants ²⁾ / μ g pUC18
Citric acid	16000 \pm 300
Sodium citrate	ND ³⁾
L-malic acid	1500 \pm 100
Succinic acid	1700 \pm 150
Lactic acid	1600 \pm 100
Oxalic acid	ND
DL-tartaric acid	ND

¹⁾10 mM each.

²⁾Colonies transformed to ampicillin resistance. Values indicate the mean \pm SD of three independent experiments.

³⁾ND, not detected.

obtained at 2.0 mM citric acid, and transformation efficiencies progressively decreased as the concentration of citric acid was further increased from 3.0 to 10 mM citric acid. Thus, under our experimental conditions, the optimal concentration of citric acid for acid stimulus-triggered transformation was 2.0 mM.

3.2. Effect of exposure time on transformation efficiency

As shown in Fig. 2, three reagents (10 mM citric acid, *E. coli* JM109, and pUC18 DNA) were mixed and held at room temperature for 0 to 100 min prior to being spread onto LB agar. When the mixture was spread onto LB agar immediately after mixing (0 min), 16000 \pm 300 cfu/ μ g pUC18 were obtained. Notably, the number of transformants decreased to 6800, 4800, 2600, and 1400 when the mixture was held for 20, 40, 60, and 100 min

before spreading, respectively. In such acidic conditions, the viability of *E. coli* cells was not affected by citric acid exposure. These results suggested that *E. coli* was releasing pUC18 once taken up into the cell.

3.3. Attenuation of acid stimulus-triggered acquisition competency

To determine the continuity of the competent state, the 10 mM citric acid and *E. coli* JM109 cells were combined, and the mixture was divided and stored in separate tubes and were incubated for 20 to 80 min at room temperature. Following this incubation, pUC18 (0.5 μ g/ml) was added to each incubated tube, and each mixture sample was immediately plated onto LB agar. As shown in Fig. 3, the number of transformants decreased with increasing incubation time prior to DNA exposure. These data showed that the state of acid

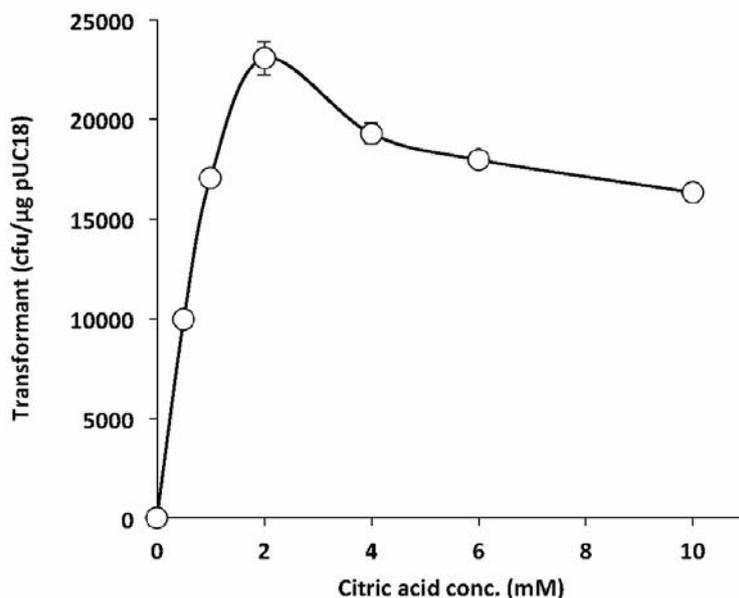


Fig. 1. The effect of citric acid concentration on acid stimulus-triggered transformation. *E. coli* and pUC18 were mixed in citric acid solution (final concentration 0-10 mM), then immediately spread onto LB agar. The transformant number (cfu/plate) indicates the number of colonies transformed to ampicillin resistance that emerged on one LB agar plate. Data are presented as the average value of three independent experiments.

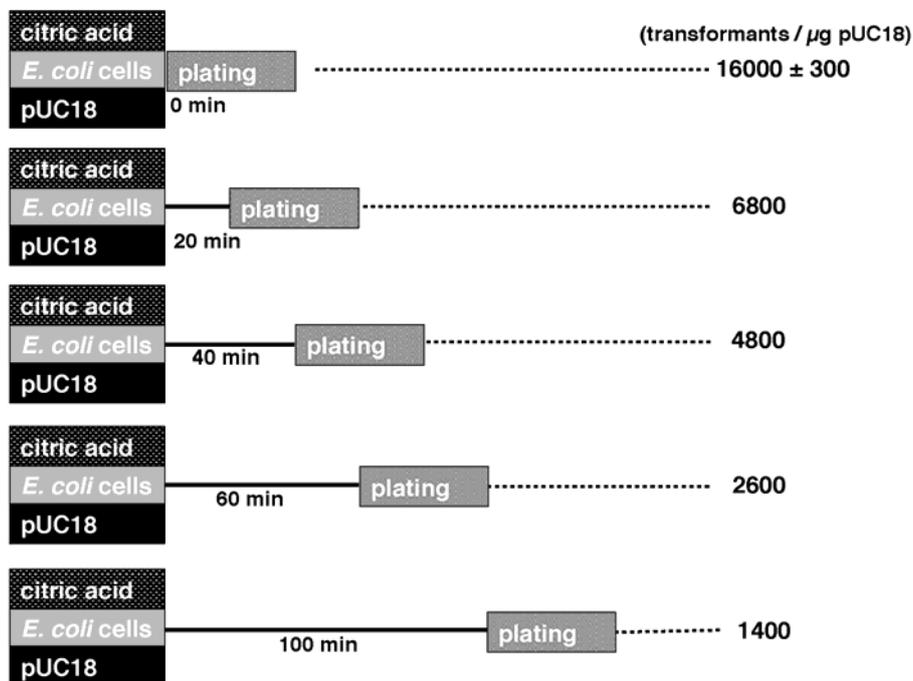


Fig. 2. Schematic representation of the excretion of plasmid that had been imported following acid stimulus. The numerical values show the colony forming units per μg pUC18; data are presented as the mean value ± SD of three independent experiments.

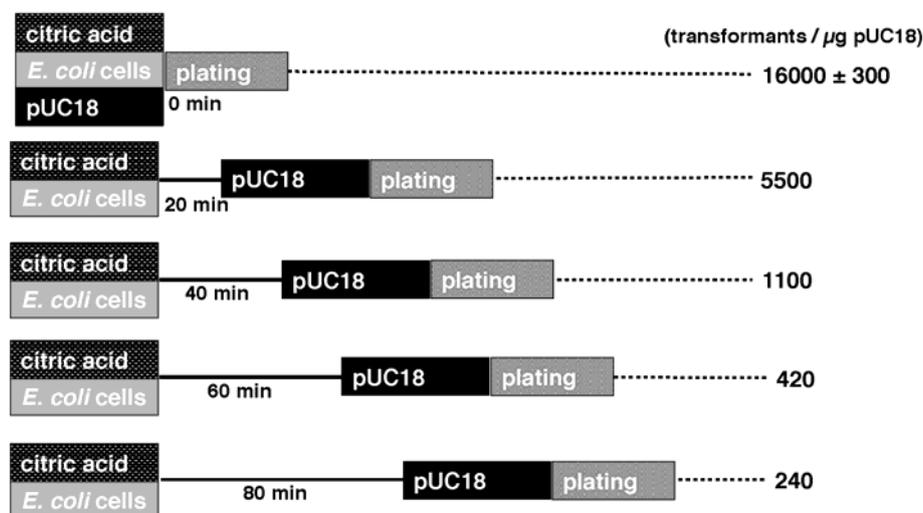


Fig. 3. Schematic representation of the attenuation of acid stimulus-triggered competency. The numerical values show the colony forming units per μg pUC18; data are presented as the mean value \pm SD of three independent experiments.

stimulus-triggered competency was induced in *E. coli* cells immediately upon exposure to citric acid, and then was gradually attenuated with longer time courses.

3.4. Recovery of competency

The above results demonstrated that *E. coli* cells acquired competency for transformation to antibiotic resistance upon acid stimulus followed by neutralization (spreading onto LB agar). The competency of *E. coli* was investigated by repeating a series of stimuli. Fig. 4 shows the time course and plasmid acquisition profile of *E. coli* treated with three cycles of citric acid stimulation at 120 min (2-hr) intervals. As above, 50 μl of stimulated *E. coli* was spread onto an LB agar plate containing 100 $\mu\text{g}/\text{mL}$ of ampicillin after each round of stimulation. The transformation efficiency of *E. coli* cells in the first cycle (mix 1) reached the maximum value (10^4 cfu/ μg pUC18) but progressively fell during subsequent cycles. However, *E. coli* cells re-exposed to citric acid after washing with 200 mM NaCl (mix 1-2, mix 1-3) recovered competency to levels similar to that seen in the first cycle.

3.5. The effect of ATPase inhibition on the acid stimulus-triggered transformation

In many instances, organisms uptake exogenous substances *via* ABC transporters, whereby permeation

is coupled to ATP degradation [7, 8]. To understand whether acid stimulus-triggered transformation was dependent on ATP consumption, we investigated the phenomenon using 3 different mixtures containing potassium cyanide [9], sodium azide [10], and *N,N'*-dicyclohexylcarbodiimide (DCCD), respectively [11]. The results showed that there was no statistically significant difference between the number of transformants generated by acid stimulus-triggered transformation in the presence or absence of ATP inhibitors (data not shown). Therefore, plasmid uptake by acid stimulus-triggered transformation appears not to depend on ATP consumption.

3.6. Acid stimulus-triggered transformation of transposon insertion disruptants

To identify pathways possibly involved in acid stimulus-triggered transformation, competency in *E. coli* KP7600 (wild strain) was compared with that in various transposon insertion disruptants (mutant strains) [12]. To facilitate comparisons, the number of transformants obtained with each strain was normalized to the number obtained from the wild-type parent strain, which was defined as 100 (i.e., 100%). Using normalization, the numbers from *ompF* and *ompG* mutant strains were increased to 151 and 163, respectively. Notably, the numbers of transformants obtained

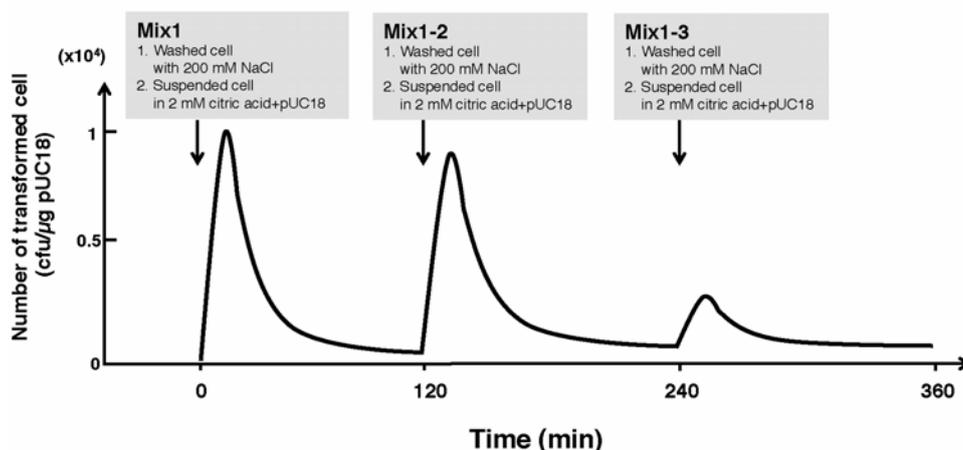


Fig. 4. The competency of *E. coli* when subjected to repeated cycles of acid stimulation and neutralization. *E. coli* grown on LB agar medium was washed with 200 mM NaCl solution. The washed *E. coli* was suspended in 500 μ L of a 2 mM citric acid solution containing 0.5 μ g/mL of pUC18 to prepare a mixed solution (Mix1). At 120 minutes after preparation of Mix1, the *E. coli* in Mix1 was again washed by centrifugation, resuspension in 200 mM NaCl, and re-centrifugation followed by resuspension in 500 μ L of 2 mM citric acid solution containing 0.5 μ g/mL of pUC18 to generate a mixed solution (Mix1-2). At 240 minutes after preparation of Mix1, *E. coli* in Mix1-2 was again washed (as above) with 200 mM NaCl solution and resuspended in 500 μ L of 2 mM citric acid solution containing 0.5 μ g/mL pUC18 to generate a mixed solution (Mix1-3). The mixture was allowed to stand at room temperature (25 $^{\circ}$ C) throughout all experiments.

from *ompW* and *ompX* mutant strains were similar to that from the parent strain. Thus, the *ompW* and *ompX* appear not to be involved in acid stimulus-triggered transformation. In contrast, transformation of *cit-3*, *cit-4*, or *ompR*-deficient mutant strains was decreased to 39-16% of that of the wild-type strain. Most strikingly, the transformation efficiency of the *ompA* mutant strain was decreased to 5% (Fig. 5).

4. DISCUSSION

We demonstrated that *E. coli* uptakes plasmid DNA immediately upon exposure of the bacterium to 1-10 mM citric acid. The efficiency of acid stimulus-triggered transformation was 10^4 cfu per μ g of pUC18 DNA. Some unknown interaction between the acid stimulus-triggered reaction and the DNA polymer must exist. *E. coli* is assumed to uptake exogenous DNA by a mechanism that couples the process with the uptake of the polyvalent carboxylic acid. It is suggested transporter proteins that react with the acid stimulus are present in the outer membrane of cells.

The phenomena shown in Fig. 2 indicated that the number of transformants was decreased as the interval before plating was increased, thus suggesting that

E. coli excretes incorporated pUC18 extracellularly as the time of exposure increases. Incorporated pUC18 should be temporarily retained in the periplasmic space. We hypothesize that *E. coli* determine whether to import or export plasmid DNA by sensing the extracellular citric acid concentration.

As shown in Fig. 3, when the time of *E. coli* exposure to citric acid solution was increased, the number of transformants decreased. The acid stimulus-triggered acquisition of competency in *E. coli* peaked immediately after exposure to citric acid, gradually falling thereafter. Longer exposure of *E. coli* to citric acid yielded decreased transformation efficiencies. Presumably, when the citric acid flow into periplasm reaches saturation while high-level citric acid is still present outside of the cell, the equilibrium is inclined in the efflux direction. We hypothesize that when citric acid is removed from outside of the *E. coli* cells, gates (transporters) that had been opened by the acid stimulus became closed, blocking the continued influx of citric acid. The “plating” depicted in the Figs. 2 and 3 presumably released *E. coli* from citric acid exposure and shifted the reaction solution to a neutral state. This change in stimulus

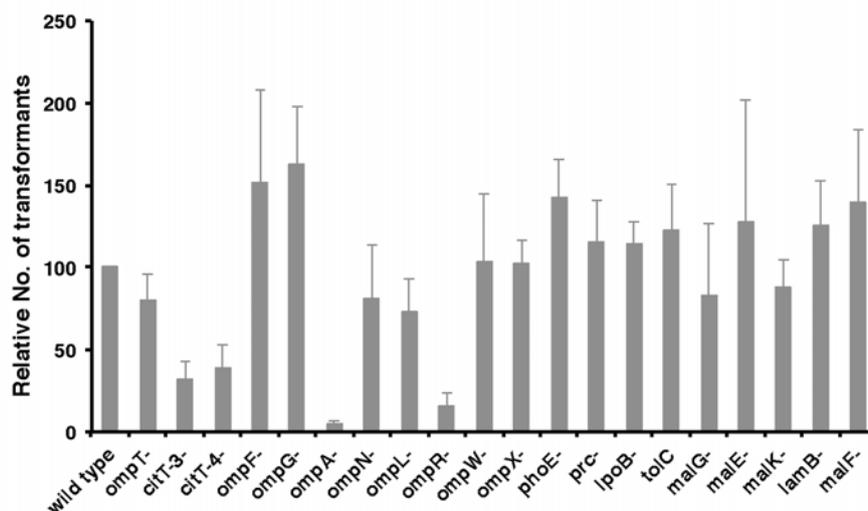


Fig. 5. The number of transformants obtained from each transposon insertion disruptant was normalized to the number obtained with the wild-type strain (defined as 100). Values are plotted as mean \pm SD obtained from three independent experiments.

was transmitted to the channel in the inner membrane, promoting the importation of plasmid DNA from the periplasm into the cytoplasm.

The highest transformation efficiency ($10^4/\mu\text{g}$ pUC18) was obtained when *E. coli* cells were spread onto LB agar medium immediately after exposure to citric acid solution containing pUC18; subsequently the number of transformants decreased due to excretion of pUC18 into the extracellular space. However, we found that if *E. coli* cells were re-stimulated with citric acid, competency was restored (Fig. 4). This phenomenon supports the hypothesis that *E. coli* incorporates or excretes plasmid DNA depending on the extracellular environment.

The National BioResource Project: *E. coli* (National Institute of Genetics, Mishima, Japan) has made available a collection of 6294 transposon insertion disruptants in the *E. coli* KP7600 background; each of these strains harbors a single insertion into the chromosome of a transposon (mini-Tn10(KmR)) encoding kanamycin resistance. A search of the database for strains carrying lesions in genes encoding outer membrane proteins (e.g., *ompA*) yielded a panel of 18 disruptants. We evaluated acid stimulus-triggered transformation using these *omp*-deficient mutants of *E. coli* KP7600. Two citrate transporter genes (*citT*) are known to be present on the *E. coli* chromosome [13]. TolC has been

well characterized as a protein involved in the efflux of antibiotics and bile acid, and in the secretion of lethal factors like enterotoxins and cytolytic toxins [14]. LamB is well known as the receptor for adhesion of phages and polysaccharides [15]. We evaluated acid stimulus-triggered transformation among transposon insertion disruptants as shown in Table 3. If the number of transformants from a transposon insertion disruptant was lower than that from wild type, it could be concluded that the disrupted gene product is a membrane protein that is deeply involved in the uptake of plasmid, by acid stimulus. The results indicated that acid stimulus-triggered transformation was most severely decreased in the *E. coli ompA* mutant. OmpA is one of the most abundant outer membrane proteins in *E. coli*, and is known to be highly expressed during exponential growth phase but only moderately expressed during stationary phase. OmpA is involved in importing conjugative plasmid DNA and bacteriophage DNA [16], as well as in the transport of bacteriocins, a group of toxic peptides [17]. However, it is still not understood whether OmpA is related to genetic transformation by exogenous DNA.

DNA uptake has been characterized to various extents in both Gram-positive and Gram-negative bacteria. The first step in this process is the binding of double-stranded DNA, which requires

the presence of a species-specific uptake sequence. Evidence for import of single-stranded fragments [18] and for linear uptake has been obtained in *Streptococcus pneumoniae* and *Bacillus subtilis* [19]. In the case of acid stimulus-triggered transformation, *E. coli* imports circular-state pUC18 DNA, because no transformants were found when using pUC18 linearized by digestion with the restriction endonuclease *HindIII* (data not shown). We presume that pUC18 is drawn into the periplasm through a channel protein with a shutter function.

Fig. 6 shows the predicted mechanism for DNA uptake by acid stimulus-triggered transformation, a process in which OmpA is implicated. Fig. 6A postulates a flow model of citric acid through OmpA. Exposure to citric acid stimulates opening of the OmpA gate, and citric acid flows into the periplasm through this transporter. Once citric acid concentration in the periplasm exceeds some threshold, *E. coli* will begin to excrete citric acid by flow in the opposite direction, again mediated by OmpA. Sensing of a subsequent fall in citric

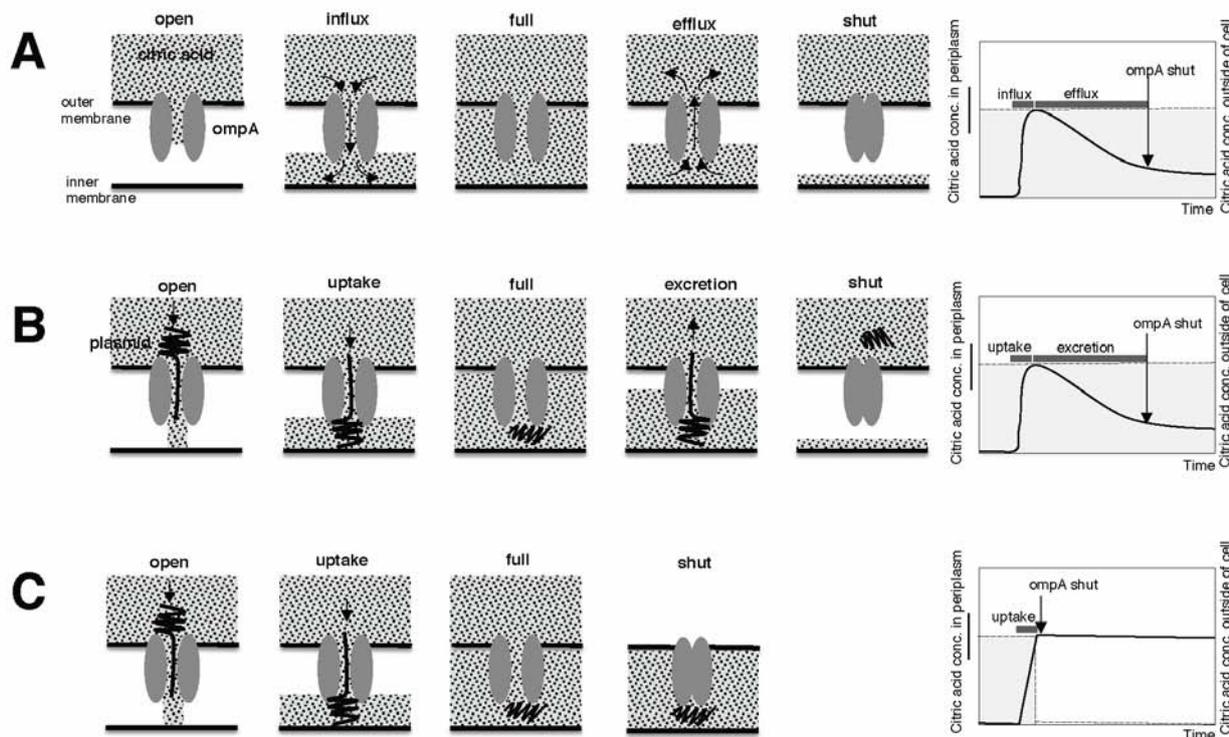


Fig. 6. Schematic representation of predicted mechanism of acid stimulus-triggered transformation.

A: OmpA-related model for influx and efflux of citric acid (bi-directional citric acid transporter). The graph schematically indicates the extracellular (dotted line) (constant at 10 mM) and periplasmic (black line) citric acid concentrations. When *E. coli* is exposed to citric acid, inflow of citric acid through OmpA channel occurs. Periplasmic citric acid concentration reaches a maximum value (influx, full), after which efflux begins (efflux). Decrease of citric acid concentration in the periplasm encourages the OmpA channel to close (shut).

B: OmpA-related model for uptake and excretion of plasmid DNA. When *E. coli* is exposed to citric acid, citric acid enters the periplasm through the opened OmpA channel (open). Influx of citric acid into the periplasm (uptake) is coupled with that of the plasmid. Periplasmic citric acid concentration reaches a maximum value (full), after which efflux of citric acid begins. The excretion of plasmid coupled with the efflux of citric acid (excretion) occurs. Decrease of citric acid concentration in the periplasm encourages the OmpA channel to close (shut).

C: Predicted model for acid stimulus-triggered transformation. When *E. coli* is exposed to citric acid, citric acid enters the periplasm through the opened OmpA channel (open). Plasmid coupled with citric acid is symported into the periplasm (uptake, full). When *E. coli* is immediately removed from citric acid exposure (shut) (e.g., by plating to LB agar), citric acid no longer enters the periplasm due to closing of the barrier by shutting of the OmpA gate. Plasmid DNA subsequently permeates the inner membrane to gain entry to the cytoplasm.

acid concentration in the periplasm will then result in the shutting of OmpA. The influx of citric acid into the periplasmic space will then no longer be facilitated through OmpA.

Fig. 6B shows the predicted mechanism in terms of uptake and excretion of exogenous plasmid on the basis of the events portrayed in Fig. 6A. *E. coli* that has been exposed to citric acid will transport plasmid (coupled with citric acid) from the extracellular environment into the periplasm through the OmpA channel, which opens upon the sensing of citric acid exposure. However, when the concentration of citric acid within the periplasm increases over a threshold, *E. coli* will excrete plasmid coupled with citric acid. Decrease of citric acid concentration within the periplasm will render the OmpA channel closed, resulting in plasmid excretion into the extracellular space.

Fig. 6C shows a model of the establishment of acid stimulus-triggered transformation. When *E. coli* is exposed to a citric acid solution containing the plasmid, *E. coli* incorporates the plasmid in concert with the influx of citric acid through the opened OmpA channel. If *E. coli* cells are immediately transferred to an LB plate, the citric acid will be removed from the external environment of the bacterial cell (neutralized). The OmpA channel will close and *E. coli* will no longer excrete plasmid. Using a permeation mechanism present in the inner membrane, the plasmid will move into the cytoplasm, resulting in transformation. Thus, OmpA would serve as a bi-directional plasmid/citric acid transporter.

We have confirmed that acid stimulus-triggered transformation by plasmid DNA occurs not only in *E. coli* but also in *Pseudomonas* spp. and *Klebsiella* spp. (data not shown). Therefore, acid stimulus-triggered transformation appears to be a phenomenon conserved among Gram-negative bacteria. To our knowledge, neither the direct stimulation of OmpA by citric acid nor the interaction of OmpA and plasmid DNA have been described before.

As with OmpA-deficient strains, isolates harboring mutations in *cit-3*, *cit-4*, or *ompR* exhibited decreased efficiency of acid-stimulus-triggered transformation. Further studies on the interactions between Cit-3, Cit-4, OmpR, and plasmid DNA will be needed to

better understand the process of acid stimulus-triggered transformation.

CONCLUSION

The novel phenomenon in which *Escherichia coli* cells uptake exogenous plasmid DNA by exposure to low concentration of citric acid and transform to antibiotic resistance was found. The cells transformed to ampicillin resistance emerged at 10^4 cells per microgram of pUC18 DNA. Acid stimulus-triggered acquisition of competency in *Escherichia coli* was caused not only by citric acid, but also lactic acid, succinic acid, and malic acid. The transformation efficiency of OmpA disruptant decreased to 5% of that of wild type, suggesting that OmpA is closely related to acid stimulus-triggered acquisition of competency in *E. coli*.

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CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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