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Rapid communication

A simple method to provide a shuttling plasmid for delivery to other host ascertained by prolonged stability of extracellular plasmid DNA released from *Escherichia coli* K12 *endA* mutant, deficient in major endonuclease

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Escherichia coli **lyses by lambda phage propagation. Circular plasmid DNA was present during *E. coli* lysis as an extracellular plasmid DNA (excpDNA) that was stable enough to transform coexisting competent *Bacillus subtilis* cells. Detailed investigations unveiled that excpDNA is transient in both quality and quantity, with stability lasting no more than several hours. A survey using *E. coli* lambda lysogens with various genetic backgrounds demonstrated that the loss of Endonuclease I (*ΔendA*::*kan*) conferred extraordinary stability upon excpDNA for as long as 48 h. Studies on *endA* mutants suggested that excpDNA remained localized in cell debris, in contrast to *E. coli* genome DNA, which diffused into medium at an early point in lysis. Lambda lysogens constructed on *endA recA* mutants are presented for potential pipelines in delivery to other competent proficient microbes.**

Keywords: *B. subtilis*/*endA*/extracellular nucleic acids/lambda lysogen/transformation.

Abbreviations: DNase, deoxyribonuclease; excpDNA, extracellular plasmid DNA.

Plasmids employed by *Escherichia coli* host-vector systems have allowed elaborate DNA manipulations, starting from cloning to alteration for delivery to another host (*1*). In the delivery, it was believed to be necessary to prepare biochemically purified DNA before use. Along with our investigation of the preparation of lambda DNA to transform *Bacillus subtilis* 168 competent cells (*2*), plasmids released from lysing *E. coli* upon lambda induction were fortuitously demonstrated to be stable (*3*, *4*). Thereafter, plasmid DNA stable enough to transform co-existing competent *B. subtilis* in the medium was called extracellular plasmid DNA (excpDNA). Besides scenarios involving conceptual similarity to naturally occurring horizontal gene transfer, *E. coli* was also found to have the potential to provide stable excpDNA for delivery to species other than *B. subtilis* (*5*). The underlying significance of this finding appeared to be that the lambda induction was to pose to the non-permissive temperature and that there is no need for a biochemical reagent to prepare excpDNA whose size extended to inserts of a BAC vector-borne vector as large as 100 kb (*3*, *4*). Although, the inherent status of the excpDNA in lysing *E. coli* remained provisional, stability and quality of excpDNA are critical to gene delivery systems to wide ranges of other hosts. As our discovery was based on limited *E. coli* strains as listed in Table I, more lambda lysogenic *E. coli* mutants including *recA* should be constructed to offer broader and more general practical applications.

The λgt11[*lac*5 nin5 *c*I857 *S*(am)100] lambda phage did not propagate in *E. coli* strains other than those carrying the su^+ allele due to an amber mutation in the *S* gene (*6*). λgt11 was converted to S^+ by *in vivo* recombination with the λpirR6K (imm434 S^+) phage through co-infection with LE392 (*supE supF* λ^-) and through selection from a plaque on a λpirR6K lysogenic strain (λimm434 su^-). The isolated phage cI857 S^+ formed turbid plaques on BW25113 (λ^- su^-) cells at 30°C, and a lysogenic strain was purified from the centre of the plaque. Strain MIC1104 grew normally on an Luria-Bertani (LB) plate when incubated at 30°C but not when incubated at 39°C.

MIC1104 was transformed by binary plasmid pGETSGFP, which replicates in both *E. coli* and *B. subtilis* (*3*) as illustrated in Fig. 1, *via* normal Ca^{2+}-mediated transformation. The complete nucleotide sequence is available upon request. Transformant MIC1104(p) was selected by ampicillin at 50 μg/ml. Competent *B. subtilis* 1A1 was prepared according to the method reported previously (*7*), stocked with 15% glycerol (v/v) at −80°C and thawed before use. Residual transformation efficiency was confirmed to be unaltered by using either of two of our laboratory's standard plasmids, pHY300PLK (TcR) or pUB110 (KmR), selected by tetracycline at 10 μg/ml or by kanamycin at 25 μg/ml, respectively.

MIC1104(p) started lysis at an elevated temperature as previously described (*3*, *4*). To assure reproducible results in this study, lambda induction was performed at 39°C, not 37°C as previously used. Briefly, the preculture that was made after shaking for 15–17 h was diluted in 50 ml pre-warmed LB, 0.5% (v/v), in a 250-ml conical flask. Growth was assayed by

Table I. Bacterial strains and plasmids.

Bacterial strains	Relevant genotypes	Antibiotic selection	Sources
E. coli			
LE392	F$^-$*supE*44 *supF*58 *lacY*1 *or del(lacIZY)*6 *trpR*55 *galK*2 *galT*22 *metB*1 *hsdR*514(rK$^-$ mK$^+$)		(*3*)
MIC128	Lysogenic LE392 by λgt11		(*3*)
BW25113	*Δ*(*araD-araB*)567 *ΔlacZ*4787(::*rrnB*-3) Δ(*rhaD-rhaB*)568, *hsd*R514	Laboratory stock	
JW2912-L	BW25113 plus *ΔendA*::*kan*	Km	(*9*)
JW2912-S	BW25113 plus *ΔendA*::*kan*	Km	(*9*)
MIC1104	Lysogenic BW25113 of λgt*c*I857 S^+		This study
MIC1105	Lysogenic JW2912-L of λgt*c*I857 S^+		This study
MIC1106	Lysogenic JW2912-S of λgt*c*I857 S^+		This study
MIC1104(p)	MIC1104 transformed by pGETSGFP	Ap	This study
MIC1105(p)	MIC1105 transformed by pGETSGFP	Ap	This study
MIC1106(p)	MIC1106 transformed by pGETSGFP	Ap	This study
JA221	F$^-$ *hsd*R *hsd*M$^+$ *trpE*5 *leuB*6 *lacY recA*1 λ$^-$	Laboratory stock	
DH5α	F$^-$ Φ80*dlacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deo*R *recA*1 *endA*1 *hsd*R17(rK$^-$, mK$^+$) phoA supE44 thi-1 gyrA96 relA1 λ$^-$	Laboratory stock	
DH10B	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) Φ80*dlacZ*ΔM15 Δ*lacX*74 *recA*1 *endA*1 *araD*139 Δ(*ara leu*)7697 *galU galK rpsL nupG* λ$^-$	Laboratory stock	
HST08	F$^-$ *endA*1 *supE*44 *thi*-1 *recA*1 *relA*1 *gyrA*96 *phoA* Φ80*dlacZ*ΔM15 Δ(*lacZYA-argF*)U169 Δ(*mrr-hsd*RMS-*mcrBC*) Δ*mcrA* λ$^-$	Laboratory stock	
MIC8511	Lysogenic JA221 of λgt*c*I857 S^+		This study
MIC8510	Lysogenic DH5a of λgt*c*I857 S^+		This study
MIC8497	Lysogenic DH10B of λgt*c*I857 S^+		This study
MIC8499	Lysogenic HST08 of λgt*c*I857 S^+		This study
B. subtilis			
1A1	*trpC2*		(*2*)

Ap, ampicillin resistance; Km, kanamycin resistance.

measuring the OD600. After 5 h of cultivation with shaking at 120 rpm in a water bath at 30°C, lambda was induced by being exposed to heating at 39°C to inactivate the labile CI857 repressor, and the aliquot (500 µl) at the time interval shown in Fig. 1 was mixed with 25 µl *B. subtilis* 1A1 competent cells from frozen stocks. After shaking for 60 min at 37°C, all the solution was spread onto a selection plate and incubated at 30°C. The colonies formed were scored the next day.

Figure 1 shows the stability of pGETSGFP as excpDNA measured by the frequency of *B. subtilis* transformants. After increasing for the first 2 h, probably due to the increasing number of lysing *E. coli* cells, this frequency started to decrease rapidly to zero after three more hours. No colonies formed in the presence of deoxyribonuclease (DNase) I (at 5 µg/ml) during transformation, similar to previous results (*3*, *4*). This observation clearly indicated for the first time that the excpDNA remained transient for ~4 h and suggested certain DNase(s) from the lysing host attributed to the degradation.

In *E. coli*, various DNase enzymes have been categorized into two types: exonucleases and endonucleases. Among them, Endonuclease I, encoded by the *endA* gene that makes double-strand breaks in duplex DNA, was considered the most abundant DNase activity from *E. coli* (*8*). The *ΔendA*::*kan* strain JW2912, which originated from the Keio Collection (*9*), showed two types of colonies with different sizes on LB plates, regardless of 25 µg/ml kanamaycin supplementation. The size difference was so clear that the large colony was renamed here JW2912-L and its lambda lysogen was named MIC1105. The small colony former was named JW2912-S, and MIC1106 was derived as a lambda lysogen. These two strains grew at similar rates in LB liquid medium and their pGETSGFP plasmid transformants MIC1105(p) and MIC1106(p) were subjected to an excpDNA production experiment in the same conditions as for the parental *endA*$^+$ strain MIC1104(p).

The stability of excpDNA from these *endA* mutants measured similarly was surprisingly high and long lasting compared with the parental *endA*$^+$ strain. As indicated in Fig. 1, the number of *B. subtilis* transformants peaked at 5 h, and the level remained unaltered even after 30 h. To observe stable excpDNA, lysates of both strains were biochemically analysed. A 300-µl aliquot of each lysate was centrifuged for 5 min at 5,000 rpm on a TOMY MX301 apparatus. The precipitate and supernatant were separately posed to the alkali-SDS plasmid isolation method, and DNA prepared by ethanol precipitation was dissolved into 25 µl Tris-EDTA (TE) buffer. Five microliters was diluted in 10 µl EcoRV digestion reaction and analysed on regular gel electrophoresis analyses.

The kinetics of DNA recovery depicted in Fig. 2 unveiled remarkable effects of Endonuclease I on excpDNA and on the *E. coli* genome as well. The appearance of these two DNAs from the *endA*$^+$ strain

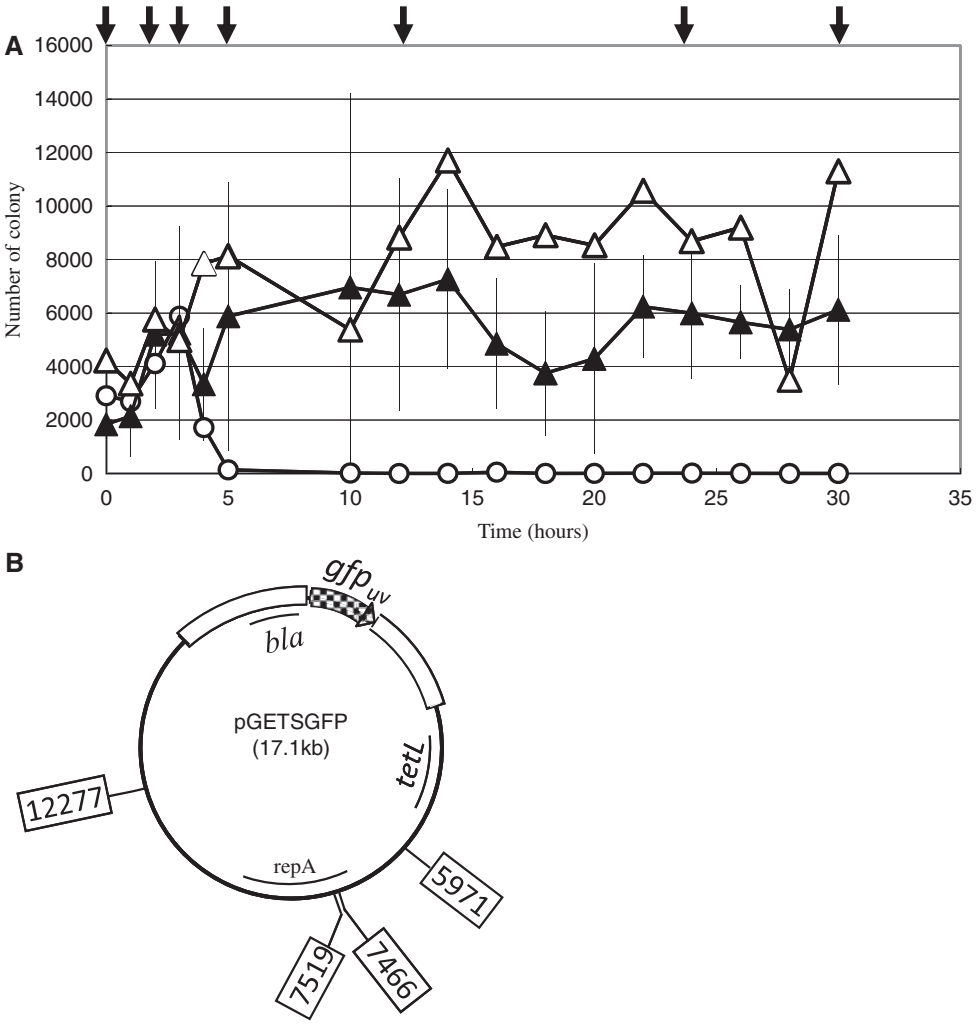

Fig. 1 Kinetics of *E. coli* lysis and excpDNA formation after lambda induction. (**A**) Growth of lambda lysogen measured by OD^{600} in LB medium at 30°C and after a temperature shiftup was similar to that referred to in Fig. 1 of ref. (*3*) or Fig. 4 of ref. (*4*). The lysate from endA+ MIC1104(p) (○) shows a peak in the number of B. subtilis transformants at 3 h after induction, in contrast to those from endA-deficient mutants MIC1105(p) (▲) and MIC1106(p) (△). Error bars by three independent measures for MIC1106(p), and average figures by two independent experiments, are shown for the other two. DNA isolated at the times indicated by vertical arrows is analysed in Fig. 2. (**B**) pGETSGFP is shown with four EcoRV sites and antibiotic-resistance genes; *bla*, beta-lactamase gene for *E. coli* selection; *tetL*, tetracycline-resistance determinant gene for *B. subtilis*; *repA* for replication in *B. subtilis*.

MIC1104(p) was transient up to 3 h after the temperature shift for lambda induction, in consistent with the transient *B. subtilis* transformation measures in Fig. 1. In contrast, excpDNA from the *ΔendA*::*kan* strain MIC1106(p) suffered no degradation even after 30 h at 39°C. Very similar data from MIC1105(p) are not shown. The structural stability was well consistent with the unaltered ability to give *B. subtilis* transformants and brought about simply by the complete loss of host Endonuclease I. Furthermore, high molecular weight genomic DNA bands were recovered from the supernatant and gave rise to certain discrete bands on EcoRV digestion. These observations strongly indicated that most of the *E. coli* genome was dispersed outside of the debris and, therefore, was recovered in the supernatant, remaining less damaged during *ΔendA*::*kan* mutant lysis. The prolonged stability and major location of excpDNA and *E. coli* genome DNA prompted us to construct other lambda lysogens for *E. coli* with the *endA*1 and *recA*1 mutants listed in Table I as stable excpDNA producers for versatile use. All the derivative strains from endA1 mutants listed in Table I showed prolonged stability similar to those from *ΔendA*::*kan* mutants, albeit the colony size variation of the *ΔendA*::*kan* mutants from the Keio collection remained uncertain.

A similar long-life excpDNA carrying a BAC vector-borne large insert in *recA*1 *endA*1 strains (data not shown) may offer a simple but primary pipeline to expedite large DNA handling discipline in *B. subtilis*-based BGM vector systems (*10–14*). The use of a shuttling plasmid between *E. coli* and microbes other than *B. subtilis* is being investigated.

Conflict of interest
None declared.

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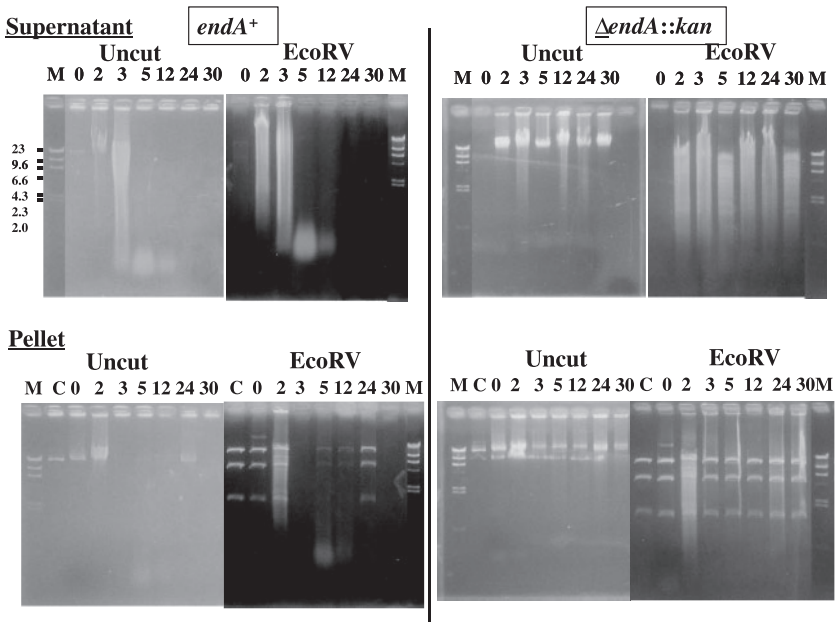

Fig. 2 Prolonged excpDNA from lysate of *endA* mutant. DNA isolated from MIC1104(p) (*endA*$^+$; left) and MIC1106(p) (*ΔendA*::*kan*; right) at indicated times is shown in Fig. 1 [supernatant (top) and debris (bottom)]. Undigested and EcoRV digestion suggested that plasmid DNA was recovered mostly from debris and *E. coli* genome DNA was recovered mostly from supernatant in the lysate. Both DNAs were extremely stabilized by the loss of the *endA* product, consistent with the prolonged excpDNA presence indicated in Fig. 1. Lane M included a size marker, lambda DNA fragmented by HindIII digestion indicated in the top left with their sizes. Lane C (bottom only) included the pGETSGFP plasmid shown in Fig. 1 purified from non-lysogenic *E. coli*. The plasmid seen from the *endA*$^+$ pellet at 24 h originated from certain *E. coli* fractions that escaped from lambda propagation and grown at these time periods. They gave no transformants (Fig. 1) and were not regarded as excpDNA.

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