Sensitivity of *Escherichia coli* to Viral Nucleic Acid. XVI. Temperature Conditions for Ca^{2+}-Dependent DNA Uptake in *Escherichia coli*

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Properties of Ca^{2+}- or Ba^{2+}-dependent transfection and transformation in *Escherichia coli* were examined, using ΦX174 replicative-form (RF) DNA and plasmid DNA. For the transfection and transformation, a heat pulse step was dispensable and the yield of transfectants was, in most *E. coli* strains, rather reduced by the heat treatment. The heat pulse step was also detrimental for the transformation of certain strains such as lipopolysaccharide mutants. The first stage of the DNA uptake process (formation of DNA · recipient cell complex) was dependent on low temperature and Ca^{2+} ion. A substantial amount of the complexed RF-DNA was released from the bacteria, by washing with a chilled Tris buffer. Although a RF-DNA · cell complex was formed even at 37 °C or in chilled 0.05 M $MgCl_2$, the complex did not yield transfectants.

Introduction

Although transfection and transformation are widely used in the analysis of genetic structure and function, the procedures are empirical and their efficiencies are low. In *E. coli*, various competent cellular preparations are available for transfection [1], whereas transformation of this Gram-negative bacterium depends exclusively on the $CaCl_2$ method which was originally devised for infection of lambdoid DNA [2]. The Ca^{2+}-dependent DNA infection process operationally consists of two phases: initial mixing of the competent bacteria with DNA in chilled $CaCl_2$ solution and subsequent heat pulse at 37 °C–42 °C. Regardless of extensive application to genetic engineering, little is known as to biochemical or biophysical meaning of the two phases.

In this report, the effect of heat pulse has been studied on Ca^{2+}- or Ba^{2+}-dependent transfection and transformation in various *E. coli* strains. In order to characterize the initial phase of the DNA uptake process, the formation and properties of the intermediate (DNA · recipient) complex have also been investigated.

Materials and Methods

Bacteria and infectious DNA

Escherichia coli BB and its lipopolysaccharide (LPS)-deficient strain BB12 [3] were generously provided by Dr. K. Jann. Strain C and its LPS mutants C61, C71, C23 and C23 · 1 [4] were kindly donated by Drs. U. Feige and S. Stirm. *E. coli* K12 JE5507 *lpo*$^+$ and JE5508 *lpo*$^-$ [5] were generous gifts from Dr. Y. Hirota. Replicative-form DNA of ΦX174 was used in transfection experiments, whereas plasmid pBR322 DNA and R6K DNA were employed in transformation.

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Transfection and Transformation

Bacteria were grown in a nutrient broth [6] at 37 °C, with shaking. At a density of about 3×10^8 cells/ml, the culture was chilled in ice water, collected by a centrifugation in the cold and drained (to minimize carry over of the medium). The cells were treated with ice-cold 0.05 M $CaCl_2$ or $BaCl_2$ as described previously [7, 8] and finally suspended in the $CaCl_2$ or $BaCl_2$ solution at a density of $OD_{660} = 15$. The competent cells were mixed with 1/2 volume of DNA in 0.05 M Tris · HCl (pH 7.5) at 0 °C and kept in the cold for 20 min. For heat pulse, small aliquots of the mixture were incubated at 37 °C or 42 °C for 2 min and then chilled at 0 °C.

For the assay of transfectants, the infected complexes were diluted with chilled 0.05 M $CaCl_2$ or 0.1 M $BaCl_2$ and plated with the indicator bacteria (strain C) by conventional agar-layer method. In certain cases, the diluted samples were smeared with the indicator cells using a glass triangle spreader. For assay of free phage by burst experiments, the infected complexes were diluted into

nutrient broth and shaken at 37 °C for 60 min. After chloroform treatment, the mixture was diluted and the progeny phage was titrated as usual.

To determine the number of transformants, the infected complex was diluted with chilled 0.05 M $CaCl_2$ (or 0.1 M $BaCl_2$) and plated with melted nutrient agar or smeared onto nutrient agar plate with a glass spreader. After about 10 min, 3 ml of melted nutrient agar were poured onto each plate and allowed to solidify at room temperature. The plates were incubated at 37 °C for 90 min and then covered with 3 ml of melted soft agar containing 0.25 mg/ml of penicillin G, to select transformants. Alternatively, the infected complex was diluted into nutrient broth and incubated at 37 °C for 90 min. After dilution, the bacteria were smeared onto nutrient agar plates containing 0.25 mg of penicillin G/ml, with a glass spreader or plateled with 3 ml of melted soft agar supplemented with 0.25 mg of penicillin G/ml. These plates were incubated further at 37 °C for 20–30 h and penicillin-resistant colonies were scored.

Results

Effect of growth phase on development of the Ca^{2+}-dependent competence

Cells of *E. coli* C were cultured in nutrient broth at 37 °C with shaking and the Ca^{2+}-dependent competence for transfecting DNA was determined periodically. As shown in Fig. 1, the cellular competence rapidly increased during early-log phase and reached its maximal level at mid-log phase. Preliminary experiments showed that the mid-log phase culture was suitable for Ca^{2+}-dependent transformation as well (data not shown). Usually, the competence thereafter declined towards stationary phase, although some cultures remained highly competent even at late-log to early stationary phase. Hereafter, bacteria harvested at mid-log phase were used in routine experiments of transfection and transformation.

Effect of heat pulse on the yield of transfectants in the Ca^{2+}-dependent system

As far as transfectants are titrated by double agar layer method, heat pulse is dispensable for Ca^{2+}- or Ba^{2+}-dependent infection of microvirid DNA [8, 9]. This method, however, leaves the possibility that

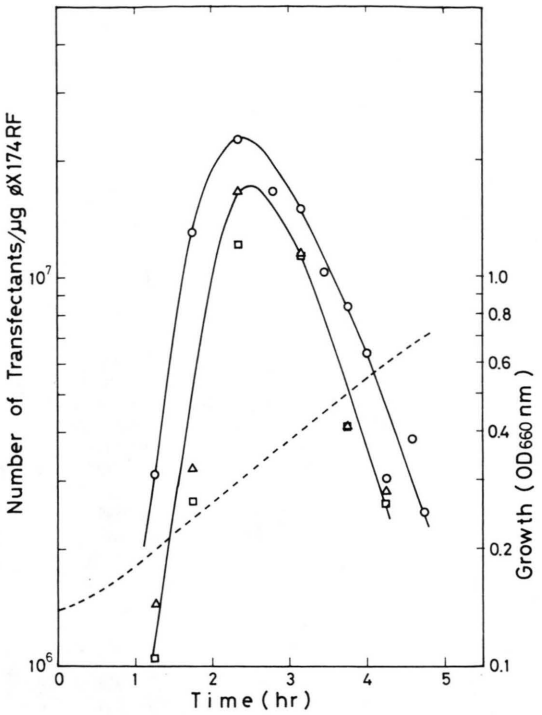

Fig. 1. Time course of development of competence for Ca^{2+}-dependent transfection in *E. coli* C. The bacteria were grown in nutrient broth at 37 °C, with shaking, and the competence for transfection with ΦX174 RF-DNA was periodically determined. - - - -, turbidity at 660 nm; ○, transfectants assayed without heat pulse; △, transfectants after 2 min-heat pulse at 37 °C; □, transfectants assayed after heat pulse at 42 °C.

the DNA-recipient cell complex suffers "heat-pulse" upon mixing with melted soft agar. In order to exclude this possibility, the effect of the heat pulse was compared by different assay methods. As shown in Table I, the heat pulse at 37 °C or 42 °C rather reduced the yield of transfectants in *E. coli* C, even when assayed by a spreading method. The plaque yield per se was not significantly different between the two assay methods. The yield of progeny phage determined by burst experiments was also low in the samples subjected to heat pulse.

In the absence of heat pulse, efficiency of Ca^{2+}- or Ba^{2+}-dependent transfection is considerably affected by the surface structure of recipient cells [10, 11]. Therefore, the effect of heat pulse on Ca^{2+}-dependent transfection was investigated with mutants deficient in surface LPS or lipoprotein. Although the yield of transfectants was relatively high in strain C71 (deficient in branch glucose) and C23

Table I. Plaque yield in *E. coli* cells transfected with ΦX174 RF-DNA. Competent cells in chilled 0.05 M $CaCl_2$ were mixed with ΦX174 RF-DNA and kept throughout at 0 °C (a) or subjected to 2 min-heat pulse at 37 °C (b) or 42 °C (c). After dilution with chilled 0.05 M $CaCl_2$, the number of infective centers (transfectants) was determined by double agar layer method or by spreading, as described in Materials and Methods. For burst experiments, the infected complex was diluted into nutrient broth and incubated at 37 °C for 60 min, with shaking. After chloroform treatment, free phage was titrated as usual.

Strain	Method	PFU/ml		
		0 °C[a]	37 °C[b]	42 °C[c]
C	double agar layer	4.5×10^5	1.4×10^5	1.8×10^5
C	spreading	5.7×10^5	9.4×10^4	1.3×10^5
C	burst	9.0×10^8	6.9×10^7	1.1×10^8
C61	double agar layer	2.0×10^5	5.4×10^4	3.7×10^4
C71	ditto	1.2×10^6	5.5×10^5	2.5×10^5
C23	ditto	1.1×10^6	4.5×10^5	2.8×10^5
C23 · 1	ditto	1.9×10^5	5.5×10^4	3.7×10^4
BB	ditto	1.1×10^4	1.2×10^4	9.1×10^3
BB12	ditto	1.9×10^4	5.4×10^3	2.7×10^2
K12C600·1	ditto	5.4×10^4	1.5×10^4	1.8×10^4
JE5507	ditto	1.8×10^3	3.9×10^2	9.0×10^2
JE5508	ditto	2.1×10^4	2.6×10^4	2.6×10^4

(deficient in branch heptose and terminal galactose), heat pulse was ineffective in the two LPS mutants as well as in C61 (deficient in branch heptose) and C23 · 1 (deficient in branch glucose and terminal and subterminal galactose residues). Upon heat pulse, yield of the transfectants was similarly reduced in BB12 (having only two 2-keto-3-deoxy-octonate units). Even for strain BB and K12 C600 · 1 the heat pulse was ineffective (Table I), however, somewhat effective for strain JE5508 *lpo*$^-$. As reported previously [11], this mutant was more competent than the parental strain JE5507 *lpo*$^+$.

Effect of heat pulse on the yield of transformants in Ca^{2+}-dependent system

In order to clarify whether a heat pulse is essential for Ca^{2+}-dependent transformation or not, a competent bacteria-pBR322 DNA mixture incubated at 0 °C was heated for 2 min at 37 °C or preserved throughout in ice-water. To allow phenotypic expression, the cells were transferred to nutrient broth or nutrient agar and transformants were selected by four different methods. As shown in Table II, the number of the transformants increased two-to four-fold, when the DNA recipient mixture was submitted to heat pulse and assayed by a spreading-agar overlay method. In a double agar layer method, however, the transformation was not significantly promoted by the heat pulse procedure. The heat pulse was also ineffective, when phenotypic expression was performed in liquid culture. Similarly, the heat treatment did not increase the yield of transformants in competent *E. coli* C cells infected with R6K plasmid DNA (data not shown). In various LPS mutants derived from *E. coli* C and BB, a heat pulse was detrimental for Ca^{2+}-dependent transformation as well as for transfection. Upon the heat treatment, the yield of transformants was increased two- to three-fold in K12 C600 · 1 and JE5508 *lpo*$^-$, but left essentially unchanged in BB and JE5507. These results demonstrate that a heat pulse is dispensable and sometimes obstructive for Ca^{2+}-dependent transformation of *E. coli* cells by plasmid DNA.

Effect of heat pulse on Ba^{2+}-dependent transfection and transformation

Cells of *E. coli* treated with chilled 0.1 M $BaCl_2$ are efficiently transfected with microvirid RF-DNA in the absence of heat pulse [8]. Upon treatment

Table II. Yield of transformants in *E. coli* treated with pBR322 DNA. Recipient cells in chilled 0.05 M $CaCl_2$ were mixed with pBR322 DNA and kept throughout at 0 °C (a), or subjected to 2 min-heat pulse at 37 °C (b) or 42 °C (c). The number of transformants was then determined as described in Materials and Methods.

Strain	Method	Transformants/ml		
		0 °C[a]	37 °C[b]	42 °C[c]
C	double agar layer	8.9×10^5	5.7×10^5	1.3×10^6
C	spreading and agar overlay	6.8×10^5	1.4×10^6	2.6×10^6
C	liquid culture and spreading	3.5×10^6	2.2×10^6	3.5×10^6
C	liquid culture and agar overlay	5.1×10^6	3.0×10^6	5.9×10^6
C61	liquid culture and spreading	2.8×10^4	2.9×10^3	3.6×10^3
C71	ditto	1.4×10^6	1.9×10^5	3.7×10^4
C23	ditto	2.2×10^5	1.9×10^4	1.2×10^4
C23 · 1	ditto	3.9×10^4	1.0×10^4	5.9×10^3
BB	ditto	8.0×10^2	1.4×10^3	7.8×10^2
BB12	ditto	3.8×10^3	5.0×10^2	1.0×10^2
JE5507	ditto	1.3×10^2	7.0×10^1	9.0×10^1
JE5508	ditto	1.7×10^3	2.2×10^3	3.0×10^3
K12C600·1	ditto	1.3×10^5	2.7×10^5	3.5×10^5

Table III. Effect of heat pulse on Ba^{2+}-dependent transfection and transformation. Competent cells of *E. coli* C in chilled 0.1 M $BaCl_2$ were mixed with ΦX174 RF-DNA or pBR322 DNA and kept throughout at 0 °C (a) or, after 20 min, subjected to 2 min-heat pulse at 37 °C (b) or 42 °C (c). For titration of transfectants, the mixture was diluted with chilled 0.1 M $BaCl_2$ and plated with the indicator bacteria. For assay of the transformants, each mixture was diluted into nutrient broth, incubated at 37 °C for 90 min and spreaded on nutrient agar plates containing penicillin.

Ba^{2+}-dependent system	Yield/ml		
	0 °C[a]	37 °C[b]	42 °C[c]
transfection	4.0×10^6	4.0×10^5	3.6×10^5
transformation	5.8×10^5	5.3×10^4	8.1×10^4

with chilled $BaCl_2$, *E. coli* C also developed competence for transformation with pBR322 DNA (Table III). Efficiencies of the Ba^{2+}-dependent transfection and transformation were, like those of Ca^{2+}-dependent systems, markedly reduced by heat pulse. Similar obstructive effect of heat pulse was observed in Ba^{2+}-dependent DNA infection of strain C/5 whose LPS was probably deficient at least in terminal galactose residue (data not shown).

Formation and properties of DNA/recipient cell complex

When the competent cells in chilled $CaCl_2$ were exposed to ΦX174 RF for 20 min and then centrifuged at 0 °C, the infectivity was exclusively found in the pellet fraction and less than 2% of the input infectivity remained in supernatant. In the absence of the recipient bacteria, the DNA in chilled $CaCl_2$ was never precipitated by centrifugation (Table IV). These results demonstrate that the DNA/recipient cell complex is formed in chilled $CaCl_2$, without heat pulse.

Transfectivity, however, was not proportional to the amount of DNA complexed to the recipient bacteria: thus plaque yield was distinctly higher in strain C than K12 C600 · 1 or JE5508, whereas the amount of free DNA remaining in the supernatant was nearly equal. Moreover, competence of *E. coli* BB was relatively low, though in-put DNA was thoroughly adsorbed by the strain. It seems noteworthy that when cells of *E. coli* C suspended in 0.05 M $CaCl_2$ and prewarmed to 37 °C were incubated with RF-DNA at the temperature, DNA was efficiently adsorbed by the bacteria but yield of

Table IV. Association of ΦX174 RF-DNA with *E. coli* under various conditions. Log phase cells harvested at $OD_{660} = 0.30$ were treated with 0.05 M $CaCl_2$ or $MgCl_2$ at 0 °C for 20 min and suspended, at $OD_{660} = 15$, in chilled 0.05 M $CaCl_2$ or $MgCl_2$. Each suspension was mixed with 1/2 volume of ΦX174 RF-DNA at 0° or 37 °C. After 20 min, a portion of the mixture was diluted with 0.05 M $CaCl_2$ or $MgCl_2$ (preequilibrated at specified temperature) and plated with indicator bacteria (strain C). The residual portion of the original mixture was centrifuged at 0° or 37 °C and ΦX174 RF-DNA remaining in the supernatant was titrated by Ca^{2+}-dependent transfection of *E. coli* C, in the absence of heat pulse.

Medium	Temp	Recipient	Infectivity [PFU/ml]	
[0.05 M]	[°C]	Bacteria	Original mixture	Supernatant
$CaCl_2$	0	None	4.3×10^5	4.6×10^5
$CaCl_2$	0	C	4.3×10^5	8.2×10^3
$CaCl_2$	0	BB	1.1×10^4	$< 2.9 \times 10^1$
$CaCl_2$	0	K12 C600 · 1	1.3×10^4	7.6×10^3
$CaCl_2$	0	JE5508	2.1×10^4	9.4×10^3
$CaCl_2$	37	C	3.8×10^1	2.0×10^3
$MgCl_2$	0	C	9.6×10^0	$< 2.9 \times 10^1$

Table V. Ca^{2+}-dependent association of ΦX174 RF-DNA with cells of *E. coli* C. Competent cells of *E. coli* C in chilled 0.05 M $CaCl_2$ were mixed with ΦX174 RF-DNA and kept at 0 °C for 20 min. Directly (Exp. I) or after heat pulse at 42 °C for 2 min and chilling (Exp. II), the mixture was centrifuged at $8000 \times g$ for 10 min at 0 °C and the "$CaCl_2$ supernatant" was removed. The pellet was suspended in chilled 0.05 M $CaCl_2$ and, after withdrawal of a small portion ("$CaCl_2$ pellet"), the infected cells were spun down and suspended in chilled 0.05 M Tris · HCl, pH 7.5. After 10 min at 0 °C, the suspension was centrifuged again and the "Tris supernatant" was removed. The sedimented bacteria were resuspended in chilled 0.05 M Tris, pH 7.5 and small portion was withdrawn for assay of infectivity ("Tris pellet"). Residual portion was centrifuged and the cells were washed with chilled 0.05 M Tris-0.005 M EDTA, pH 7.5 and finally suspended in chilled 0.05 M Tris · HCl, pH 7.5 ("Tris-EDTA pellet"). For assay of infectivity, each "pellet" fraction was diluted and plated with the indicator bacteria, whereas ΦX174 RF-DNA in "supernatant" fraction was titrated by Ca^{2+}-dependent transfection without heat pulse.

Fraction	Infectivity yield	
	Exp. I (without heat pulse)	Exp. II (with heat pulse)
Total	4.3×10^5 (100)[a]	1.4×10^5 (100)[a]
$CaCl_2$ supernatant	8.2×10^3 (1.9)	2.4×10^3 (1.7)
$CaCl_2$ pellet	2.9×10^5 (67)	1.2×10^5 (86)
Tris supernatant	1.7×10^5 (40)	4.1×10^4 (29)
Tris pellet	4.7×10^4 (11)	4.2×10^4 (30)
Tris-EDTA pellet	$< 4.3 \times 10^1$ (< 0.01)	$< 2.8 \times 10^1$ (< 0.02)

[a] %

transfectants was negligible low. If the mixture kept at 37 °C was chilled to 0 °C and then plated, a substantial amount of plaques was formed (data not shown). Although Mg^{2+} was ineffective for transfection, binding of RF-DNA to *E. coli* C efficiently occurred in chilled 0.05 M $MgCl_2$, as indicated by the disapearance of free DNA. When the Mg^{2+}-induced DNA · cell complex was washed with chilled Tris buffer, a small but significant amount of infective DNA was released (data not shown). These results, together with previous data [12, 13] suggest that both Ca^{2+} and low temperature are essential for proper binding of in-put DNA to a specific site on the recipient cell surface, and that the Ca^{2+}-mediated DNA · recipient complex, once formed, yields plaque under conventional incubation conditions (in nutrient media at 37 °C).

Several properties of the recipient cell (strain C) · RF-DNA complex were investigated, before and after heat-pulse. As shown in Table V, the DNA complexed to the recipient bacteria was not released by washing with chilled 0.05 M $CaCl_2$ but considerably liberated with chilled 0.05 M Tris · HCl, pH 7.5 (only 11–9% of input infectivity was deteced in the Tris-washed bacterial pellet). If the DNA-recipient cell complex was washed with 0.05 M Tris-0.005 M EDTA, pH 7.5, the infectivity was completely lost from the bacteria. Even after heat pulse, the DNA in the complex was substantially removed by the Tris treatment and about 30% of input infectivity remained in the washed cells. In Ca^{2+}-dependent DNA-binding capacity, early stationary phase cells of *E. coli* K12 C600 · 1 were not at all inferior to the log phase bacteria (Table VI). Although DNA bound to stationary phase cells was somewhat refractory to Tris washing, the plaque yield of the washed bacteria was still low.

Discussion

Although a heat pulse step is routinely included in Ca^{2+}-dependent transfection and transformation systems, microvirid DNA efficiently infects *E. coli* cells without the heat treatment. The heat pulse step is also dispensable for Ba^{2+}-dependent transfection of the viral DNA. The above results demonstrate that Ca^{2+}- or Ba^{2+}-dependent transformation of certain plasmid DNA (pBR322 and R6K) occurs in the absence of a heat pulse. In certain *E. coli* strains, especially in LPS mutants, the heat treatment rather decreases the yield of transfectants and transformants.

The present experiments indicate that, in chilled $CaCl_2$ solution, infecting DNA is weakly bound to the recipient cell surface. Thus, ΦX174 RF-DNA is sedimented with recipient cells by a low speed centrifugation and the bound DNA is substantially released by such a mild method as washing with 0.05 M Tris · HCl, pH 7.5. For the binding, Ca^{2+} (Ba^{2+}) and low temperature are necessary and sufficient, whereas the DNA · cell complex, diluted into growing media without heat pulse, yields progeny phages under normal temperature and nutritional conditions. Although binding of DNA to *E.*

Table VI. Ca^{2+}-dependent association of ΦX174 RF-DNA with cells of *E. coli* K12 C600 · 1. Cells of *E. coli* K12 C600 · 1, collected at $OD_{660} = 0.3$ (Exp. I) or 0.7 (Exp. II) and treated with chilled 0.05 M $CaCl_2$, were mixed with ΦX174 RF-DNA at 0 °C. After 20 min, the mixture was divided into two portions. One portion (A) was kept throughout at 0 °C, whereas the other portion (B) was subjected to heat pulse at 42 °C for 2 min and chilled in ice-water. Each mixture was fractionated by centrifugation and infectivity was assayed as in Table V.

Fraction	Relative infectivity			
	Exp. I		Exp. II	
	A	B	A	B
Total	100 (1.3×10^4)	100 (8.6×10^3)	100 (1.6×10^4)	100 (5.3×10^3)
$CaCl_2$ supernatant	1.8	3.0	0.27	0.55
$CaCl_2$ pellet	66	72	57	60
Tris supernatant	61	53	23	42
Tris pellet	4.5	10	5.7	13

coli occurs at 37 °C or in $MgCl_2$ solution, the DNA · cell complex is inactive in viral production. Even after treatment in chilled $CaCl_2$ solution, the Mg^{2+}-mediated DNA · cell complex remained noninfective. Evidently, an initial contact of DNA with the recipient surface is crucial for transfection and transformation.

In general, condition affecting Ca^{2+}(Ba^{2+})-dependent transfection of ΦX174 RF equally influenced transformation by pBR322 or R6K DNA. These results suggest that an essentially similar mechanism operates for the uptake of viral and plasmid DNA. For these DNA, *E. coli* C and its derivative strain C71 are superior recipients: efficiency in the Ca^{2+}-dependent system (without heat pulse) reached 5×10^7 transfectants/μg ΦX174 RF. This high efficiency, together with its restriction-deficient property, makes strain C an ideal recipient for foreign DNA. The competence of strain C might further be increased by *lpo*$^-$ mutation.

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