Sensitivity of *Escherichia coli* to Viral Nucleic Acid, XII. Ca^{2+}- or Ba^{2+}-Facilitated Transfection of Cell Envelope Mutants

Akira Taketo

Department of Biochemistry, School of Medicine, Kanazawa University, Kanazawa

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Using various envelope mutants of *Escherichia coli*, the relationship between cell surface structure and the Ca^{2+}- or Ba^{2+}-dependent competence for transfection was investigated. In contrast with rough strains, smooth bacteria treated with Ca^{2+} or Ba^{2+} were incompetent for the transfection by ØA RF. In *E. coli* K12 D21 derivatives, Ca^{2+}-dependent competence remarkably increased by *lpsA1* mutation and the highest level of competence was attained by further deficiency in glucose units of the LPS. Upon treatment with $BaCl_2$, strain D21 and its *lpsA1* mutant became highly competent for ØA RF. The effect of Ba^{2+} was, however, feeble for *lpsA1* mutants further deficient in heptose units and/or glucose units. Among different LPS mutants of *E. coli* B, variation of the Ca^{2+}- or Ba^{2+}-dependent competence was relatively small and even the competence of strain BB12, whose LPS core contained only two KDO units, was nearly equal to that of wild type bacteria. However, the level of cellular competence induced by Ba^{2+} was not allways parallel to that induced by Ca^{2+}. In mutants deficient in outer membrane protein I, either Ca^{2+}- or Ba^{2+}-dependent competence increased several-fold, whereas in mutants devoid of outer membrane II*, the competence decreased considerably. Unlike nucleoside transport, the uptake of DNA was not affected by *tsx* mutation.

Introduction

Upon treatment with Ca^{2+} or Ba^{2+}, cells of Gram-negative bacilli such as *E. coli*, *Aerobacter aerogenes* or *Salmonella typhimurium* develop competence for transfecting DNA [1–3]. Unlike spheroplast formation, treatment with Ca^{2+} or Ba^{2+} is very simple and by far more physiological as evidenced by retention of the cellular viability, which is a major premise for DNA transformation. The Ca^{2+}-dependent transfection too is, however, affected by various external and internal factors and conditions [4–6]. The situation may be similar as to the Ba^{2+}-dependent transfection system. Our previous work suggested the importance of the cell surface structure in the Ca^{2+}-dependent DNA-uptake process [4, 5]. In order to clarify the mechanism as well as to facilitate practical use of the systems, the role of surface LPS and outer membrane proteins in the Ca^{2+}- or Ba^{2+}-dependent transfection has been investigated, taking advantage of envelope mutants of *E. coli*.

Abbreviations: RF, double-stranded replicative form of DNA; LPS, lipopolysaccharide; KDO, 2-keto-3-deoxy-octonate; PFU, plaque-forming unit; R, rough.

Requests for reprints should be sent to Dr. Akira Taketo, Department of Biochemistry, School of Medicine, Kanazawa University, *Kanazawa*, Ishikawa 920, Japan.

Materials and Methods

Bacteria

E. coli K12 D21 and its LPS mutants D21e7, D21f1, D21f2 [7] together with K12 CR34 and GR467 [8] were kindly provided by Dr. H. G. Boman. Strain BB and its LPS mutants BB1, BB2, BB4, BB5, BB7, BB9, BB12 and BB20 [9] as well as *E. coli* F492 (smooth form) and F470 (rough mutant) [10] were generous gifts from Dr. K. Jann. Strain P400 and its outer membrane mutants P530 (deficient in protein I), P530-1gII (deficient in protein I and II*) and P530-3bII (deficient in protein II*) [11] were kindly donated from Dr. U. Henning. Outer membrane mutants P407 *tsx*, P460 *con* and P1731 *con tsx* [12] were generously provided by Dr. P. A. Manning.

Viral DNA and transfection

The RF DNA of ØA was prepared as described previously [13]. The RF, when preserved at 4 °C, fully retained its infectivity extending over 4 years. For preparation of the competent cells, bacteria were grown at 37 °C in nutrient broth with shaking and when A_{660} of the culture (measured with Bausch & Lomb Spectronic 20) reached 0.7 or 0.4 (in the case of poorly growing mutants), chilled in ice-water. The cells collected by centrifugation were made competent by treating with chilled 0.05 M $CaCl_2$ or 0.1 M $BaCl_2$ and then infected with ØA RF, as described previously [3, 14].

Results

Competence of smooth form

In the experiment presented in Table I, a smooth strain F492 (08 K27$^-$: H$^-$) and its rough mutant F470 were treated with $CaCl_2$ or $BaCl_2$ and their competence for ØA RF was tested. Upon treatment with Ca^{2+} or Ba^{2+}, the smooth bacteria did not develop any competence for the RF, whereas the isogenic rough mutant became competent by the treatments. For this rough mutant, Ba^{2+} was superior to Ca^{2+} but the competence attained was very low (less than 5% of strain C). At any rate, it is clear that O-specific polysaccharide chains in LPS are obstructive to the competence development.

Table I. Competence of a smooth form as compared with its rough mutant. The bacteria were treated with and suspended in chilled 0.05 M $CaCl_2$ or 0.1 M $BaCl_2$ at a density of A_{660} =15. The suspension (0.1 ml) was mixed at 0 °C with 0.05 ml of ØA RF (0.02 A_{260} unit). After 20 min, the mixture was diluted and plated with the indicator strain C.

Strain	Competence (transfectants/ml)	
	Ca^{2+}-dependent	Ba^{2+}-dependent
F492 (smooth)	$<1.0\times10^1$	$<1.0\times10^1$
F470 (rough)	1.5×10^3	9.5×10^3

Competence of LPS mutants from K12

Involvement of the cell surface LPS in the Ca^{2+}-dependent competence was suggested previously [5]. In the present experiments, a series of K12 LPS mutants devoid of increasing parts of the core polysaccharide [7] has been tested for their Ca^{2+}- or Ba^{2+} induced competences. As shown in Table II, cells of D21 became considerably competent upon the Ba^{2+}-treatment, but Ca^{2+} was relatively insufficient for this strain. However, in strain D21e7 lacking galactose residue, a high level of competence was induced either by Ca^{2+} or Ba^{2+}. The highest competence was attained in a glucose-less LPS mutant D21f1 by the Ca^{2+}-treatment. On the other hand, the Ca^{2+}-dependent competence significantly decreased in strain D21f2 deficient further in heptose residue. By the Ca^{2+}-treatment, however, even this strain developed the competence thirteen-fold higher than that of wild-type D21. In the mutants devoid of glucose (D21f1) and heptose (D21f2), the Ba^{2+}-induced competence was rather low.

Table II. Competence of LPS mutants derived from *E. coli* K12. The bacteria were treated with chilled $CaCl_2$ or $BaCl_2$ and their competence were determined using a definite amount of ØA RF (0.02 A_{260} unit).

Strain	Competence (transfectants/ml)		
	Ca^{2+}-dependent	Ba^{2+}-dependent	Ba^{7+}/Ca^{2+}
D21	1.2×10^4(1) [a]	3.7×10^5(1)	31
D21e7	1.5×10^6(130)	1.4×10^6(3.8)	0.93
D21f1	9.6×10^6(800)	6.6×10^4(0.18)	0.0069
D21f2	1.5×10^5(13)	6.8×10^4(0.18)	0.45
CR34	1.1×10^5(1)	9.3×10^4(1)	0.85
GR467	2.9×10^5(2.6)	4.5×10^4(0.48)	0.16

[a] Relative titer.

Table II also includes the data on strain CR34 and its heptose-leaky mutant GR467 whose LPS is partially deficient in galactose and glucose as well as in heptose [8]. The level of the Ca^{2+}-dependent competence of the leaky mutant was not particularly different from that of its parental strain. Like D21, strain CR34 was a derivative of K12 and yet the competence level differed significantly from that of D21.

Competence of LPS mutants from B

Prehm *et al.* [9] have recently elucidated the structure of LPS from *E. coli* BB and substructures of cell wall defective mutants. Table III summarizes the experimental results on the competences of *E. coli* BB and its mutants with increasing structural defects in the cell wall LPS [9]. The Ca^{2+}-dependent competence was about three-fold high in BB1 (deficient in glucose units, branch heptose, phosphate and pyrophosphorylethanolamine), but considerably low in BB5, BB20, BB7 and BB4. The structures of BB1 and BB7 LPS differ from those of BB20 and BB9 LPS, respectively, only by the absence of pyrophosphorylethanolamine residues. The Ca^{2+}-dependent competences were widely different between the two pairs (BB1 : BB20, BB7 : BB9), suggesting that the presence of the pyrophosphorylethanolamine group was indifferent to the competence. In addition, the level of the competence did not correlate with the length of the core oligosaccharides. On the other hand, Ba^{2+}-dependent competence was fourteen-fold higher in BB5 (deficient in terminal glucose, branch heptose, phosphate and pyrophosphorylethanolamine) and four to three-fold higher in BB20, BB1, BB9 and BB7.

Table III. Competence of LPS mutants derived from *E. coli* B. The cellular competence was determined using 0.02 A_{260} unit of ØA RF.

Strain	Competence (transfectants/ml)		
	Ca^{2+}-dependent	Ba^{2+}-dependent	Ba^{2+}/Ca^{2+}
BB	4.7×10^5 (1) [a]	5.6×10^4 (1)	0.12
BB5	7.8×10^4 (0.17)	8.0×10^5 (14)	10
BB2	1.4×10^5 (0.30)	3.4×10^4 (0.61)	0.24
BB20	5.5×10^4 (0.12)	2.2×10^5 (3.9)	4.0
BB1	1.6×10^6 (3.4)	2.5×10^5 (4.5)	0.16
BB9	5.8×10^5 (1.2)	2.0×10^5 (3,6)	0.34
BB7	4.6×10^4 (0.098)	1.4×10^5 (2.5)	3.0
BB4	9.1×10^4 (0.19)	3.8×10^4 (0.68)	0.42
BB12	1.8×10^5 (0.38)	8.4×10^4 (1.5)	0.47

[a] Relative titer.

Table IV. Competence of outer membrane protein mutants. The cellular competence was determined using 0.02 A_{260} unit of ØA RF.

Strain	Competence (transfectants/ml)		
	Ca^{2+}-dependent	Ba^{2+}-dependent	Ba^{2+}/Ca^{2+}
P400	2.1×10^5 (1) [a]	2.4×10^5 (1)	1.1
P530	7.8×10^5 (3.7)	8.0×10^5 (3.3)	1.0
P530-1gII	4.0×10^4 (0.19)	5.3×10^3 (0.022)	0.13
P530-3bII	2.7×10^4 (0.13)	5.0×10^3 (0.021)	0.19
P407	1.8×10^5 (0.86)	1.2×10^5 (0.50)	0.67
P460	1.8×10^4 (0.086)	2.5×10^3 (0.010)	0.14
P1731	2.0×10^4 (0.095)	2.0×10^3 (0.0083)	0.10
JF404	3.8×10^5 (1)	3.5×10^6 (1)	9.2
JF404-2a	9.0×10^3 (0.024)	1.3×10^5 (0.037)	14

[a] Relative titer.

as compared with strain BB. The order of the Ba^{2+}-dependent competences of pyrophosphorylethanolamine-deficient mutants was BB5 > BB1 > BB7 > BB4 and was apparently parallel to the length of the core oligosaccharides. In general, however, variation of the Ca^{2+}- or Ba^{2+}-dependent competences was not so remarkable among the nine strains, and even in the deepest rough mutant BB12 (having only two KDO units) the competence level amounted to nearly 40% (Ca^{2+}-system) and to 150% (Ba^{2+}-system) of wild-type strain BB. In BB derivatives, the order of the competences induced by Ca^{2+} considerably deviated from that induced by Ba^{2+}.

Competence of outer membrane protein mutants

The Ca^{2+}-dependent transfection was inhibited by cell envelope preparations and the inhibitory effect of cell envelope fragments was reduced by a pretreatment with pronase [4]. It seems probable that, besides LPS, some envelope proteins are involved in the Ca^{2+}- or Ba^{2+}-dependent interaction with the transfecting DNA. Therefore, the role of the outer membrane protein in the competence development was investigated using several mutants recently characterized [11, 12]. Upon treatment with Ca^{2+} or Ba^{2+}, strain P530 (deficient in outer membrane protein I) developed competence four to three times higher than that of the wild-type strain P400 (Table IV). On the other hand, the competence of JF404-2a (devoid of outer membrane protein II*) was lower than that of the parental strain JF404: the Ca^{2+}-dependent competence of the mutant was 2.4% and the Ba^{2+}-dependent competence was only 3.7%. Similarly, both Ca^{2+}- and Ba^{2+}-dependent competences were considerably decreased in strain P460 *con* deficient in protein 3A (which was thought to be identical with protein II* [15]). Strains P530-1gII and P530-3bII are phage TuII*-resistant mutants lacking both proteins I and II* [11]. The Ca^{2+}-dependent competence of these mutants was 19 and 13%, resp., whereas the Ba^{2+}-dependent competence was about 2%, as compared to wild-type strain P400. In these mutants, the competence-enhancing effect of protein I deficiency was suppressed by the counteracting effect of protein II* deficiency. On the other hand, differences of the competence level were only marginal between P400 and P407 *tsx* as well as between P460 *con* and P1731 *con tsx*, suggesting dispensability of *tsx*-protein for the competence development.

Discussion

The results presented above have demonstrated the importance of the surface LPS and proteins in development of the cellular competence for transfecting DNA. In smooth form *E. coli*, presence of O-specific polysaccharide chain in LPS thoroughly prevented development of the Ca^{2+}- or Ba^{2+}-dependent competence. Conventional strains used in transfection experiments (such as K12, C and B) are rough mutants and competences of these bacteria are also affected by structure of R-core. Thus, in K12 D21 derivatives, the Ca^{2+}-dependent competence was enhanced by deficiency in galactose residues (*1psA* mutation) and more efficiently by the absence of glucose units (D21f1). Further defect in LPS lowered the level of the competence in D21f2, suggesting that the presence of heptose

group(s) was profitable to the competence development. LPS of strain D21f2 contains only KDO and lipid A [16]. In this mutant, however, the Ca^{2+}-dependent competence was still 13 times higher than in the wild-type cells (D21). The phosphorus content in LPS from D21e7, D21f1 and D21f2 is two-thirds of the normal values [17]. Since Ca^{2+}-dependent transfection is extremely sensitive to phosphate [5], increased competence in the LPS mutants might partly be due to the reduced phosphorus content. However, different competence level among the three LPS mutants is inexplicable by the phosphate content alone. Among the derivatives of BB, the Ca^{2+}-dependent competence was relatively high in BB1 and glucose groups were absent in this strain as well. Unlike K12 D21, strain BB lacks galactose residue in its LPS and this may possibly be related to the higher Ca^{2+}-dependent competence in BB. In BB derivatives, deficiency of glucose or heptose units in LPS caused somewhat tangled effects on the competence development. However, it is noteworthy that even strain BB12, whose LPS contains only two KDO units and lipid A, developed the Ca^{2+}- or Ba^{2+}-dependent competences comparable with those of wild-type BB. It is therefore evident that R-core components other than KDO are dispensable for development of the basal level of the competence. To elucidate the role of KDO and lipid A in the competence development, isolation of the mutants defective in these units is prerequisite.

From the results presented above, it is clear that sugar residues in LPS affect the competence development. Consequently, the effect of sugar was tested in the Ca^{2+}-dependent transfection system. In strain C, galactose or sucrose, when added at 0.4 to 0.5 M, reduced yield of the transfectants. At 0.1 to 0.2 M, these sugars caused significant increase in the transfection efficiency, possibly by nonspecific protection of the recipient cells (data not shown).

Efficiency of the Ca^{2+}-dependent transfection is influenced by the composition of outer membrane proteins. Thus, deficiency in protein I caused 3 to 4-fold increase in the transfection efficiency, whereas mutants missing protein II* (or *ompA* protein) exhibited considerably decreased competence for RF DNA. More recently, Hantke has reported that cellular uptake of nucleosides is severely impaired in *tsx* mutants [18]. The competence for DNA transfection was, however, unaffected by the *tsx* mutation, suggesting that *tsx* protein is not the gate or channel for penetration of polynucleotides.

Properties of the cellular competence induced by Ba^{2+} are mostly in common with those induced by Ca^{2+} [3]. Ba^{2+} was, like Ca^{2+}, quite inefficient for smooth bacteria. Similarly, the Ba^{2+}-dependent competence too was low in protein II*-deficient or *ompA* mutants (JF404-2a and P460) and high in a galactose-less strain (D21e7) as well as in protein I-deficient cells (P530). Nevertheless, the ratios of the Ba^{2+}- and the Ca^{2+}-dependent competence varied considerably in other strains. Thus, the Ba^{2+}-dependent competence was considerably low in D21f1, whereas the level of Ca^{2+}-dependent competence was maximal in this glucose-deficient mutant. In such strains as D21, BB5, JF404 and JF404-2a, Ba^{2+} was far more efficient than Ca^{2+}. Structural features of the cell surface responsible for these differences are presently unknown. The two ions might differently affect some processes other than DNA uptake (restriction, formation of initiation complex etc.).

The mechanism of Ca^{2+}- or Ba^{2+}-dependent uptake of DNA is still unknown. Our working hypothesis postulates that $Ca^{2+}(Ba^{2+})$-induced crystallization of membrane phospholipids distorts conformation of envelope protein subunits and thereby allows DNA penetration. Neutralization of negative charge on cell surface is probably another important effect of Ca^{2+} or Ba^{2+}. LPS and other membrane proteins may affect the cellular competence through topological modulation of the putative channel for DNA. Further genetical analysis and biochemical characterization of cell envelope may afford useful information on this puzzling subject.

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