Genetic Competence in *Escherichia coli* Requires Poly- β -Hydroxybutyrate/Calcium Polyphosphate Membrane Complexes and Certain Divalent Cations

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In earlier studies of genetic competence in *Escherichia coli* induced with calcium-containing buffers, a strong correlation was found between transformation efficiency and the formation of poly- β -hydroxybutyrate/calcium polyphosphate (PHB/Ca²⁺/PP_i) complexes in the plasma membranes. In this study, we replaced Ca²⁺ with one of a number of other cations—monovalent, divalent, and trivalent—and found significant numbers of transformants (transformation efficiency, >10⁵/μg of pBR322 DNA) only when the cells had high levels of PHB/Ca²⁺/PP_i and the medium contained at least one of the divalent cations Ca²⁺, Mn²⁺, Sr²⁺, or Mg²⁺. Cells with high levels of the complexes were not competent when the medium did not contain these cations, but the cations were also ineffectual when the cells had few complexes. Surprisingly, Mn, Sr, and Mg were not incorporated into the complexes in place of Ca. These results indicate that PHB/Ca²⁺/PP_i complexes and the above-mentioned divalent cations each have essential but disparate roles in genetic competence. Moreover, the strong selectivity of PHB/PP_i for Ca²⁺ suggests the binding sites in the complexes are ionophoretic.

Transformation of bacterial cells with plasmid or chromosomal DNA has become a routine procedure, yet the mechanism by which the hydrophilic DNA molecule is transported through the plasma membrane is still unknown. Reusch and Sadoff (14) suggested that complexes of poly- β -hydroxybutyrate/calcium polyphosphate (PHB/Ca²⁺/PP_i), which are formed in the cytoplasmic membranes of *Escherichia coli* (12) when they are made genetically competent by suspension in cold calcium-containing buffers, facilitate the transport of DNA through the plasma membrane. The membrane complexes are also formed in *Azotobacter vinelandii* and *Bacillus subtilis* when competence is induced by physiological means (13). The presence of PHB/Ca²⁺/PP_i in membranes may be discerned with the aid of the hydrophobic probe *N*-phenyl-1-naphthylamine (NPN), which undergoes a sharp and irreversible increase in fluorescence (with a peak at ca. 56°C) when the complexes dissociate (12, 13). Freeze fracture electron micrographs of *E. coli* and *A. vinelandii* showed alterations in the plasma membrane structures of these bacteria that correlate with their incorporation of PHB (11).

Typical thermotropic fluorescence spectra of NPN in *E. coli* DH5 α log-phase cells cultured in SOB medium (2% Bacto Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl) (4) are shown in Fig. 1. The small fluorescence peak at 56°C indicates low concentrations of PHB/Ca²⁺/PP_i. Suspension of the cells in cold competence-inducing buffer TFB (4), which is 10 mM Ca²⁺, 45 mM Mn²⁺, and 100 mM K⁺ in 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid], pH 6.3, resulted in a 10-fold increase in fluorescence. This is consistent with earlier studies with other strains of *E. coli* (12, 14) and correlates with a 50- to 100-fold increase in the concentration of membrane PHB/Ca²⁺/PP_i. The transformation efficiencies of the log-phase and competent cells correlated with the intensities of the fluorescence peak; the log-phase cells had very low transformation efficiencies, and suspension in cold TFB buffer effected a 4,000-fold increase in transformability (Table 1).

The importance of Ca²⁺ for inducing competence in *E. coli* is well established (4, 9); however, certain other divalent cations may substitute for Ca²⁺ with varying degrees of success, namely, Mn²⁺, Mg²⁺, Sr²⁺, and Ba²⁺ (5, 17). Since most of these cations have similar coordination geometries, we surmised that they may serve as substitutes for Ca²⁺ in the membrane complexes. When log-phase cells were suspended in MES, pH 6.3, containing the chloride salt (50 mM) of one of the above divalent cations, we found that they enhanced the formation of PHB/PP_i complexes, with Ba²⁺ clearly the least effective (Fig. 2A). Ba²⁺ had no effect on competence, but transformation efficiencies for the other cations were all above 10⁵/μg of pBR322 DNA, in the order Mn > Ca > Sr = Mg (Table 1).

The effects of other divalent cations on PHB/Ca²⁺/PP_i and on competence were examined in the same manner. The broad fluorescence curves of Ni²⁺ and Co²⁺ suggest that the cell membranes contained some poorly formed complexes mixed with free PHB, but Zn²⁺, Cu²⁺, Cd²⁺, and Eu²⁺ were completely ineffective in producing the membrane complex (Fig. 2B). Only Ni²⁺ increased competence and only marginally. The others produced few to no transformants, and the cells had markedly reduced viability (Table 1), but this was not the sole cause for loss of competence, since the addition of small amounts of Cu²⁺ (≤ 5 mM) to TFB medium clearly reduced the levels of PHB/PP_i and transformation efficiency without affecting viability (Table 2).

The trivalent cations Al³⁺ and La³⁺, at 50 mM in MES medium, completely inhibited formation of PHB/PP_i, competence, and viability (Fig. 2C; Table 1). Again, the loss of viability was not the sole cause of noncompetence. The addition of Al³⁺ or La³⁺ to TFB medium at viable concentrations— ≤ 1 mM AlCl₃ or ≤ 0.1 mM LaCl₃, respectively—had a pronounced inhibitory effect on complex formation and transformability despite the high concentrations of Ca²⁺ and Mg²⁺ in the medium (Table 2).

When log-phase cells were suspended in ice-cold MES buffer or MES with 50 mM alkali metal salts, we observed fluorescence peaks with up to 83% of the intensity of those

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FIG. 1. Thermotropic fluorescence spectra of the membrane probe NPN in *E. coli* DH5 α whole cells. NPN was added to the cold cell culture at a final concentration of 2×10^{-6} M, and the fluorescence was measured continuously as the temperature was increased by $1.5^\circ\text{C}/\text{min}$. Excitation was at 340 nm; emission was at 420 nm. Fluorescence in the absence of NPN was not significant; the baseline fluorescence level of NPN in the medium was subtracted. Curves show relative fluorescence intensities on the same scale. SOB, cells in log-phase growth (A_{550} , 0.4) in SOB medium; SOB $\times$ 10, fluorescence multiplied by 10; MES, cells described above collected by centrifugation ($750 \times g$) at 4°C and resuspended in 1/3 volumes of ice-cold 10 mM MES, pH 6.3, for 30 min; TFB, cells described above collected and resuspended in ice-cold TFB medium (10 mM MES [pH 6.3], 100 mM KCl, 10 mM CaCl_2 , 45 mM MnCl_2) for 30 min.

achieved in TFB medium (Fig. 1 and Fig. 2D). Yet notwithstanding the relatively high concentration of PHB/PP_i in the membranes, the cells were not transformable (Table 1). This unexpected result caused us to reexamine the relationship between the alkaline earth cations and the membrane complexes.

It proved impossible to obtain cells completely free of the membrane complexes in the presence of divalent cations. The complexes can be removed by washing cells with EDTA or phosphate buffers, and this abolishes competence (12) but also removes divalent cations. However, cells in SOB medium have relatively low concentrations of PHB/PP_i, so we examined the effects of varying the Mg^{2+} concentration in this medium. We found that cells cultured to mid-log phase in medium containing all the constituents of SOB except Mg^{2+} and then resuspended in 1/3 volumes of this medium (ice-cold) (to the same density as in MES) had no detectable PHB/PP_i and were not competent. Cells cultured in complete SOB medium (which contains 20 mM Mg^{2+}) and resuspended in the same way in this medium had low levels of PHB/PP_i and low but measurable competence. Resuspending these cells in SOB–50 mM Mg^{2+} effected small increases in the number of complexes and in competence, but transformation efficiency was still 10-fold less than in MES–50 mM Mg^{2+} , even though viability was 10-fold greater (Table 1). Clearly, the Mg^{2+} concentration was not the sole factor influencing complex formation or competence. In this study, the level of membrane complexes was the best predictor of competence.

To determine whether the effective divalent cations (Mn^{2+} , Sr^{2+} , and Mg^{2+}) acted by replacing Ca^{2+} in the membrane complexes, and to establish the selectivity order, we compared the cation composition of the complexes with that of the buff-

TABLE 1. Effects of salts on formation of PHB/PP_i membrane complexes and development of competence in *E. coli* DH5 α

Medium	Cation(s)	Concn (mM)	T_f ($^\circ\text{C} \pm 0.5^\circ\text{C}$) ^a	Fluorescence intensity (% of maximum $\pm 5\%$) ^b	Viability (log no. of cells/ml ± 0.3) ^c	Transformation efficiency ^d
MES ^e	K^+ , Mn^{2+} , Ca^{2+}	100, 45, 10	57	100	8	7.5 (8)
SOB ^f	Mg^{2+}	20	56	10	9	3.5 (5)
SOB ^g	Mg^{2+}	20	56	24	9	3.7 (3)
SOB	Mg^{2+}	50	56	29	9	4.4 (3)
MES	None	0	55	73	8.5	<1 (2)
MES	Li^+	50	54	71	8.5	<1 (2)
MES	Na^+	50	54	83	8.5	<1 (2)
MES	K^+	50	55	71	8.5	<1 (2)
MES	Rb^+	50	53	71	8.5	<1 (2)
MES	Mg^{2+}	50	56	93	8	5.3 (8)
MES	Ca^{2+}	50	57	93	8	5.8 (6)
MES	Sr^{2+}	50	56	93	8	5.3 (4)
MES	Ba^{2+}	50	57	70	8	3.5 (4)
MES	Mn^{2+}	50	56	83	8	6.4 (6)
MES	Ni^{2+}	50	52	50	7	3.9 (4)
MES	Co^{2+}	50	54	51	5	2.0 (3)
MES	Zn^{2+}	50	— ^h	—	3	<2 (3)
MES	Cd^{2+}	50	—	—	<2	<1 (2)
MES	Eu^{2+}	50	—	—	<2	<1 (2)
MES	Cu^{2+}	50	—	—	<2	<1 (2)
MES	Al^{3+}	50	—	—	<2	<1 (2)
MES	La^{3+}	50	—	—	<2	<1 (2)

^a T_f , temperature of peak fluorescence of NPN.

^b Magnitude of fluorescence peak as percentage of maximum in TFB medium.

^c After 30 min in the ice-cold salt medium, cells were pelleted and resuspended in SOB plus 20 mM glucose and cultured for 1 h at 37°C . Viability and competence were then determined by plating the cells on the appropriate medium.

^d Measured as log number of transformants per μg of pBR322 DNA ± 0.3 . The numbers in parentheses are the numbers of trials.

^e This composition is TFB medium.

^f Log-phase cells in SOB medium, A_{550} of 0.4.

^g Cells described in footnote f pelleted and resuspended in 1/3 volumes of ice-cold SOB medium.

^h —, no peak.

FIG. 2. Thermotropic fluorescence spectra of NPN in *E. coli* DH5α (as in Fig. 1). Measurements were made after log-phase cells were suspended for 30 min in ice-cold MES, pH 6.3, containing the chloride salt (50 mM) of the listed cations. All curves show relative fluorescence intensities on the same scale.

ers. The cells, made competent in medium containing a single one of these divalent cations, were washed with methanol and acetone to extract water and lipids, and the dry cell pellet was then extracted with dry chloroform at 4°C to recover PHB/ M^{2+}/PP_i , where M^{2+} may be any divalent cation. The extracts were chromatographed on a high-pressure liquid chromatography nonaqueous gel permeation column, eluent fractions were screened for PHB by immunoassay (15) and chemical assay (7), and the divalent cation composition was determined by graphite furnace atomic absorption spectrometry (Varian SpectraAA-300/400 Zeeman). Fractions containing PHB were also analyzed for PP_i by chemical assay (6) and by enzymatic assay with polyphosphate kinase (1). The peak levels of PHB eluted in a fraction in the molecular weight range $17,000 \pm 4,000$, as estimated by comparison with polyisoprene and synthetic PHB standards, and this fraction also contained peak amounts of PP_i and Ca^{2+} . Ca^{2+} was by far the predominant cation found, regardless of the cation composition of the com-

petence-inducing buffer. Concentrations of Mg^{2+} , Sr^{2+} , and Ba^{2+} were below detection levels. Only the Mn^{2+} concentration was measurable, but at levels less than 3% of that of Ca^{2+} (Table 3).

These studies indicate that a significant increase in transformation efficiency, i.e., $>10^5$ transformants per μg of DNA, is achieved when certain divalent cations (Ca, Mn, Sr, or Mg) are added to cells with elevated levels of PHB/ Ca^{2+}/PP_i complexes in the plasma membranes (Table 1). For *E. coli* DH5α, the order of effectiveness for complex formation was $Ca = Sr = Mg > Mn$, and that for competence was $Mn > Ca > Sr = Mg$; the latter ranking differs slightly from the order for strain DH1 reported by Hanahan, $Mn > Ca \sim Sr \gg Mg$ (4), but this may be attributed to variations in the responses of individual strains to specific salts, perhaps due to differences in the cell envelope (5). These differences may also explain the inability of Ba^{2+} to increase competence in DH5α, despite its reported ability

TABLE 2. Effects of Cu^{2+} , La^{3+} , and Al^{3+} on viability and competence of *E. coli* DH5 α

Salt and concn (mM) in TFB	Viability (log no. of cells/ml)	Transformation efficiency ^a
TFB (no salt)	8	7.3
CuCl_2		
0.2	8	7.1
0.5	8	6.9
1.0	8	6.2
5.0	8	<1
AlCl_3		
0.2	8	6.4
0.5	8	5.8
1.0	8	5.6
5.0	8	<1
LaCl_3		
0.05	7	6.5
0.1	6	4.8
0.2	6	3.7
0.5	6	<1

^a Measured as log number of transformants per μg of DNA.

to do so in other strains (5, 17). The correlation between competence and complex formation is strong for a given cation(s) (12) but less cogent when comparing the results obtained with different cations (Table 1); this is expected since cations—in particular, multivalent cations—have profound specific effects on membrane structure and enzyme activity, which may in turn influence the efficiency of plasmid transfer and expression.

The data also suggest that $\text{PHB}/\text{Ca}^{2+}/\text{PP}_i$ complexes and the above-mentioned divalent cations each have essential but disparate roles in transformation. The independent need for the divalent cations was revealed by the noncompetence of cells in MES buffer with or without alkali metal cations, despite high levels of the membrane complexes (Table 1). The insufficiency of the divalent cations was illustrated by the marked difference in the competence of cells in SOB and MES with equal concentrations of Mg^{2+} (Table 1) and by the inhibitory effects on competence of Cu^{2+} , La^{3+} and Al^{3+} when added in low concentrations to buffers containing Mn^{2+} and Ca^{2+} (Table 2). The inference of separate roles is also supported by the finding that the divalent cations are not incorporated into the complexes in place of Ca^{2+} (Table 3). In summary, cells with $\text{PHB}/\text{Ca}^{2+}/\text{PP}_i$ complexes were not always competent, but competent cells always had high levels of $\text{PHB}/\text{Ca}^{2+}/\text{PP}_i$ complexes; and cells exposed to Ca^{2+} , Mn^{2+} , Sr^{2+} or Mg^{2+} were

TABLE 3. Cation selectivity of PHB/PP_i : relationship between the cation compositions of the competence-inducing buffer and of the PHB/PP_i membrane complex

Buffer		PHB/PP_i	
Cation(s)	$\text{Ca}^{2+}:\text{M}^{2+}$	Cation(s)	$\text{Ca}^{2+}:\text{M}^{2+}$
Ca^{2+} , Mn^{2+}	1:4.5	Ca^{2+} , Mn^{2+}	>30:1
Mg^{2+}	0:100	Ca^{2+}	>500:1
Ca^{2+}	1:1	Ca^{2+}	1:1
Sr^{2+}	0:100	Ca^{2+}	>100:1
Ba^{2+}	0:100	Ca^{2+}	>100:1
Mn^{2+}	0:100	Ca^{2+} , Mn^{2+}	>30:1

not always competent, but cells were not competent unless one of these divalent cations was present.

The complexes are postulated to form channels or defects in the lipid bilayer of the plasma membrane which facilitate DNA transfer. While this is an essential role in transformation, it is only one of many critical steps in this multifactorial process, and it should not be surprising that there could be many such channels in the plasma membrane without the cells forming transformants. DNA must also penetrate the outer membrane to reach the periplasm or the Bayer adhesion sites (2), and many events separate the entry of DNA into the cytoplasm from its expression. The divalent cations, like the complexes, are required during the process of DNA binding; hence, we may speculate that they increase the permeability of the outer membrane and/or bind DNA to PP_i at the mouth of the channel complex by cross-linking the phosphate groups of the two polymers. They also may furnish Ca^{2+} for the complexes indirectly by displacing it from less stringent cellular binding sites.

The results also provide us with some unexpected insight into the geometry of the Ca^{2+} binding sites in PHB/PP_i . The remarkably high selectivity for Ca^{2+} over cations of the same charge and coordination geometry strongly suggests that the cation binding sites in the membrane complexes are ionophoretic (10, 18). Only multidentate ligands are capable of selecting specific cations with high efficiency (8, 16). Although PP_i is well known for its ability to sequester divalent cations in the presence of high concentrations of monovalent cations (3), it does not discriminate well among divalent cations, and this suggests that PHB participates in the selection of Ca^{2+} . The coordination preferences of Ca^{2+} are well known, and from them we may infer that the cation binding sites of PHB/PP_i contain seven or eight oxygen ligands in irregular geometry, forming an equilibrium cage with a radius of approximately 0.1 nm. This is consistent with the model for the complexes proposed by Reusch and Sadoff (14).

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