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# Calcium Requirement and Magnesium Stimulation of *Escherichia coli* L-Form Induction

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Calcium was found to be required for L-form penicillin induction of *Escherichia coli* K-12 W1485, and magnesium was found to be stimulatory.

Although Dienes observed *Escherichia coli* L-forms as early as 1939, the growth of *E. coli* L-forms in subcultures was not successful (1). Lederberg and St. Clair (4) and Landman, Altenbern, and Ginoza (3) were able to subculture *E. coli* L-forms repeatedly, but induction yields from normal rods were only moderate (10 to 50% L-form yield from rods). In preliminary work on induction of *E. coli* K-12 W1485, L-forms were obtained only sporadically, but eventually consistent but low yields were obtained just after the yearly cleaning of our distilled water system. Our distilled water system accumulated copious deposits of $CaCO_3$ which were present even in the condensing coils. Therefore, we tested the ability of $Ca^{2+}$ and $Mg^{2+}$ added to media made with fresh Ca-less distilled water to improve our L-form yields.

The basal medium contained beef heart infusion (Difco), 4%; sucrose, 20%; glucose, 0.8%; agar (Difco), 1.0%; and 10,000 units of penicillin G per ml. The *p*H of the medium was 6.7, and all experiments were at 37 C. *E. coli* rods were grown overnight in Difco Penassay broth; the cultures were then diluted in 0.1% peptone to about $5 \times 10^3$ cells per ml. Portions (0.1 ml) of the diluted cultures were then transferred to sterile plates and mixed manually with molten induction media (45 C). For each induction medium, four replicate plates containing penicillin and two plates containing no penicillin were poured. $Ca^{2+}$ and $Mg^{2+}$ salts were autoclaved separately and added to the

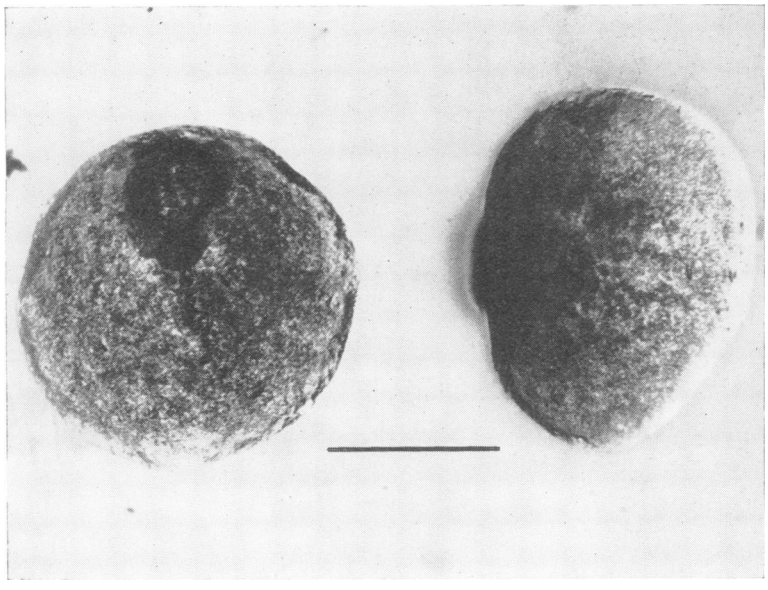

FIG. 1. *Morphology of a 19-hr-old E. coli K-12 W1485 L-form colony growing in the basal medium with 0.15% $CaCl_2$, 0.05% $MgSO_4 \cdot 7H_2O$, and 10,000 units of penicillin G/ml. Bar represents 0.1 mm.*

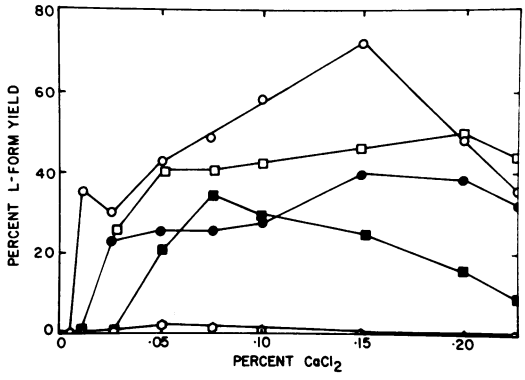


FIG. 2. *Effect of $CaCl_2$ and $MgSO_4$ on E. coli L-form yields. $MgSO_4 \cdot 7H_2$ at 0% (●); 0,05% (○); 0.2% (□); 0.4% (■); and 0.6% (◇).*

molten media at 45 C. L-form colonies counted had typical "fried egg" morphology (Fig. 1).

Figure 2 shows the effect of adding $CaCl_2$ and $MgSO_4$ to the induction media. $CaCl_2$ was absolutely required and could not be replaced by $MgSO_4$; media that contained all levels of $MgSO_4$ did not induce L-forms without added $CaC_2$. In Fig. 2 all curves go through the origin (0% yield with no added $CaCl_2$). The optimal concentration of added $CaCl_2$ was 0.15% and of added $MgSO_4 \cdot 7H_2O$ was 0.05%; concentrations above or below these added levels gave lower L-form yields.

$Mg^{2+}$ and $Ca^{2+}$ ions appeared to be the effective (active) ions, because it did not matter which anionic form of either was used except when both were chlorides (Table 1). Perhaps excess chloride inhibits L-form formation, but this was not tested with other cationic forms of chloride.

Landman et al. (3) showed a requirement for $MgCl_2$ which could be replaced by $MnCl_2$ and only slightly by $CaCl_2$. They did not test these ions together. $Mg^{2+}$ has been long known to stabilize protoplasts, and $Ca^{2+}$ can replace that requirement (5). Perhaps on the *E. coli* L-form membrane there are two distinct sites, one preferentially filled or more effectively filled by $Ca^{2+}$ than by $Mg^{2+}$. The shape of the curves in Fig. 2 suggest that, with increasing $Mg^{2+}$ concentration above 0.05%, $Mg^{2+}$ competes with $Ca^{2+}$ for the $Ca^{2+}$ site. Being less effective than $Ca^{2+}$, high $Mg^{2+}$ concentrations depress the L-form yield, but the peak is shifted to the left, indicating that $Ca^{2+}$ has a higher affinity for the site than does $Mg^{2+}$.

Table 2 shows that 10,000 units of penicillin G per ml was optimal and gave a maximal L-form yield of 72%. Landman et al. obtained maximal yields of about 50% as did Lederberg and St. Clair (3, 4), although Landman et al. (3) could obtain a 89% yield from $Mg^{2+}$-stablized protoplasts, but lost 51% of the cells in conversion to the protoplast. The high L-form yields from protoplast were probably due to selection of stable protoplasts.

It must be emphasized that the amounts of $Mg^{2+}$ and $Ca^{2+}$ indicated were only those added to the medium. Additional amounts were undoubtedly present as impurities in the medium. The $Ca^{2+}$ and $Mg^{2+}$ requirements were not determined, but we obtained similar results with two separate batches of Difco beef heart infusion (batch no. 497852 and 520298, the only ones tested). Moreover, the addition of $Ca^{2+}$ or $Mg^{2+}$

TABLE 1. *Effect of various combinations of anionic forms of calcium and magnesium on L-form induction*

| Added ion[a] | L-form count per 0.1 ml plated[b] | Rod count per 0.1 ml plated[b] |
|---|---|---|
| Nil | 0 | 521 |
| $MgCl_2$ alone | 0 | 468 |
| $MgSO_4$ alone | 0 | 493 |
| $CaCl_2$ alone | 303 | 441 |
| $Ca(NO_3)_2$ alone | 318 | 460 |
| $CaCl_2$ and $MgSO_4$ | 392 | 493 |
| $Ca(NO_3)_2$ and $MgSO_4$ | 389 | 547 |
| $Ca(NO_3)_2$ and $MgSO_4$ | 332 | 469 |
| $CaCl_2$ and $MgCl_2$ | 154 | 507 |

[a] $Mg^{2+}$ salts at 0.05%, and $Ca^{2+}$ salts at 0.15%.
[b] Average number of colonies from three plates; L-form counts from media containing 10,000 units of penicillin per ml; rod counts from the same media without penicillin.

TABLE 2. *Effect of penicillin G on L-form induction from E. coli rods*

| Penicillin G (units/ml) | Percent yield |
|---|---|
| 50 | 0 |
| 100 | 0 |
| 200 | 0 |
| 400 | 2.5 |
| 600 | 8 |
| 800 | 16.8 |
| 1,000 | 36 |
| 2,000 | 41 |
| 4,000 | 46 |
| 6,000 | 51 |
| 8,000 | 59 |
| 10,000 | 72 |
| 14,000 | 51 |
| 16,000 | 43 |
| 20,000 | 29 |

did not enable us to obtain stable L-forms. All L-forms we obtained were unstable, i.e., they reverted to rods in the absence of penicillin even though some had been carried through 21 subcultures. Stable *E. coli* L-forms have only been obtained from small mutants and not from normal rods (2).

We conclude that *E. coli* K-12 W1485 L-forms require $Ca^{2+}$ ions for induction and growth and that $Mg^{2+}$ ions are stimulatory.

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