Growth Inhibition of *Escherichia coli* by E Colicin Plasmids

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Plasmids were isolated from E colicinogenic strains and transformed into prototrophic *Escherichia coli* K12 strain DB364. Screening of E colicinogenic transformants for growth on defined medium revealed an apparent amino acid auxotrophy mediated by E4 and, to a lesser extent, E7 colicin plasmids. The auxotrophy was further investigated in E4 colicinogenic strains. From such auxotrophic transformants, denoted Pmi+ (plasmid-mediated inhibition of growth), Pmi− variants were obtained at a frequency of $3 \times 10^{-4}$ per bacterium. Plasmid loss was not detected among Pmi− clones. Isolation of E4 colicin plasmids from Pmi− clones and retransformation of strain DB364 with these plasmids showed that 40% of the plasmids were unable to inhibit growth of DB364 and were inferred to have alterations in an E4 colicin plasmid gene termed *pmi*. All such plasmids were indistinguishable from native E4 colicin plasmids, with respect to colicin immunity, colicin production and excretion, and sensitivity to lysis by mitomycin C. Experiments examining the nutritional basis of the plasmid-mediated auxotrophy indicated that at least seven amino acids, isoleucine, leucine, valine, arginine, methionine, serine and glycine, were involved in the auxotrophy. However, supplementation with only these seven amino acids did not completely restore growth. Assays of the activities of enzymes involved in amino acid biosynthesis in colicinogenic and non-colicinogenic strains under repressing and derepressing growth conditions suggested that E4 colicin plasmids did not repress synthesis of the implicated amino acids.

INTRODUCTION

The E colicins are highly specific bactericidal proteins which are classified by their reduced activity against a class of *Escherichia coli* mutants lacking the outer membrane protein which acts as the receptor for vitamin B12, E colicins and phage BF23 (Di Masi *et al.*, 1973; Davies & Reeves, 1975). The E colicins, represented by immunity types E1, E2 and E3, have been extensively studied. Several additional E colicins have been described: colicins E4, E5, E6 and E7 by Horák (1975) and Males & Stocker (1980, 1982), and most recently colicins E8 and E9 (Cooper & James, 1984). Through tests of serological cross-reactivity amongst E colicins, assays of shared colicin receptor activity, and recombination between different E colicin plasmids and E3 colicin plasmids having N-terminal colicin mutations to restore colicinogeny, Mock & Pugsley (1982) suggested that considerable homology exists amongst the E colicins, and that their plasmid determinants share a common evolutionary origin. This view is supported by observed similarities in bactericidal activities for colicins E2 and E7, and those of colicins E3 through E6, as well as by the shared sequence homology and similarities in patterns of maxicell-derived proteins encoded by E colicin plasmids (D. P. Brunner & E. A. Galella, unpublished data).

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Abbreviations: AHAS, acetohydroxy acid synthase; OTC, ornithine carbamoyltransferase.

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While studying the modes of action of colicins E4, E5, E6 and E7 and their plasmid determinants, we encountered an unexpected phenomenon involving an E4 colicin plasmid, pColE4-K365. Transformation of prototrophic *E. coli* K12 strain DB364 with pColE4-K365 resulted in colicinogenic transformants which were no longer able to grow on unsupplemented defined medium. Strain DB364 was converted from prototrophy to apparent auxotrophy. This novel effect suggested that pColE4-K365 encoded a product which perturbed cellular metabolism and, consequently, cell growth. We investigated the distribution of this effect among *E coli* plasmids and the nutritional basis of the apparent auxotrophy.

**METHODS**

**Bacterial strains**. Table 1 lists the bacterial strains used and the plasmids that they carry.

**Media**. The complex media used were nutrient broth no. 2 (CM67, Oxoid) and blood agar base (CM55, Oxoid). The defined medium was CY medium, based upon the formula of Davis minimal medium (Davis & Mingioli, 1950) with glycerol (5 ml l⁻¹) as energy source and prepared by mixing 3 vols distilled water with 1 vol. C-salts consisting of (g l⁻¹): trisodium citrate, 1.8; K₂HPO₄, 28.0; KH₂PO₄, 8.0; (NH₄)₂SO₄, 40; MgSO₄·7H₂O, 0.4. CY medium was solidified with 1.5% (w/v) granulated agar (Difco).

**DNA-mediated transformations**. Plasmids were isolated from colicinogenic clones essentially by the method of Clewell & Helinski (1969), as described by Timmis et al. (1978). Transformations with purified plasmid DNA were accomplished as described by Cohen et al. (1972), with selection for specific colicin immunity and screening for colicin production. As a final phenotypic test, transformants were streaked onto defined medium, incubated and examined for growth at 48 h. DB364, a prototrophic *E. coli* K12 strain, was also streaked onto defined medium as a control. This strain produced heavy growth at the site of inoculation and numerous colonies along the streak.

**Amino acid broth experiments**. DB314 was grown in 25 ml complex medium to a cell density of 5 x 10⁸ cells ml⁻¹, harvested by centrifugation (3400 g, 15 min, room temperature), the supernatant decanted, and the bacteria washed with 10 ml of unsupplemented defined medium. After a second centrifugation, bacteria were resuspended in 5 ml defined medium, thus concentrating the cells fivefold. A series of 125 ml Erlenmeyer flasks were prepared, containing 40 ml defined medium with or without amino acid supplements. Amino acids were prepared, either in pools or individually, as 1% solutions, and were sterilized by membrane filtration (0.45 µm pore size). Amino acid pools were added (final concentration 50 µg ml⁻¹ each amino acid) and the volume of liquid in each flask adjusted to 42 ml with sterile water. One flask containing all 20 L-amino acids served as a positive control. A second flask remained unsupplemented as a negative control. Each flask was inoculated with approximately 5 x 10⁸ bacteria which had been washed and concentrated as described above.

**Amino acid plate assays**. The assay of Zubrzycki et al. (1969) was used to screen for nutritional requirements of multiple auxotrophs. Amino acid solutions were prepared as described above. A 10 ml culture of DB314 was grown in complex medium at 37 °C overnight, harvested and resuspended in 5 ml defined medium. A sample of cells (0.2 ml) was used to inoculate 40 ml defined medium with agar (0.6% agar) at a density of 4 x 10⁸ cells ml⁻¹. Following inoculation, the molten soft agar was poured over a defined agar plate and allowed to solidify. Samples (10 µl) of various amino acid solutions were spotted onto the surface of the agar approximately 25 mm apart. When the solutions had soaked into the soft agar, the plates were incubated for 24 h at 37 °C, and examined for growth either within or between the drops. Significant growth indicated that an amino acid(s) in the drop was required. Systematic removal of single amino acids from such pools, followed by re-testing as above, allowed for identification of the growth promoting pool member.
Growth inhibition by E.colicin plasmids

Table 2. Growth response of E.colicinogenic transformants of prototrophic strain DB364

<table>
<thead>
<tr>
<th>Colicin plasmid</th>
<th>Growth inhibition*</th>
<th>Plating efficiency†</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1-P33</td>
<td>−</td>
<td>0.82</td>
</tr>
<tr>
<td>E1-N10-S1</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>E1-30</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>E1-K30</td>
<td>−</td>
<td>0.84</td>
</tr>
<tr>
<td>E1-CA62</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>E1-K53</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>E2-P9</td>
<td>−</td>
<td>0.90</td>
</tr>
<tr>
<td>E3-CA38</td>
<td>−</td>
<td>0.90</td>
</tr>
<tr>
<td>E4-K365</td>
<td>+</td>
<td>0.0003 (0.002, 0.003)</td>
</tr>
<tr>
<td>E4-CT9</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>E4-P33</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>E4-962</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>E5-099</td>
<td>−</td>
<td>1.07</td>
</tr>
<tr>
<td>E6-Ind8</td>
<td>−</td>
<td>0.79</td>
</tr>
<tr>
<td>E6-CT14</td>
<td>−</td>
<td>0.95</td>
</tr>
<tr>
<td>E7-K317</td>
<td>±</td>
<td>0.0004 (0.74, 0.84)</td>
</tr>
</tbody>
</table>

ND, Not determined.

* As defined by observation of ability to form isolated colonies following streaking on defined medium.
† Plating efficiency = c.f.u. on defined medium/c.f.u. on complex medium. Values are means of at least three independent experiments, following 48 h incubation at 37 °C. Plating efficiencies within parentheses represent data following incubation of samples on defined media for 96 h and 144 h, respectively.

Enzyme assays. Cultures were incubated with shaking in 200 ml complex medium at 37 °C to a cell density of 5 × 10⁸ cells ml⁻¹, harvested by centrifugation (9630 g, 10 min, 4 °C), washed in unsupplemented defined medium and resuspended in 200 ml of defined or complex medium. After 90 min incubation, cultures were harvested, washed and resuspended as described by Smith et al. (1979). The cell suspension was disrupted by passage through a French pressure cell (American Instrument Co., Inc.) at approximately 12000 lbf in⁻² (82.8 MPa). Cell debris was removed by centrifugation (17000 g, 25 min, 4 °C), and the crude extract assayed for enzymic activities. Acetohydroxy acid synthase (EC 4.1.3.18) (AHAS) activity was assayed according to the method of Smith et al. (1979). Ornithine carbamoyltransferase (EC 2.1.3.3) (OTC) activity was assayed according to the method of Shinners & Catlin (1978). Specific activities are expressed as μmol product min⁻¹ (g protein)⁻¹. Protein was quantified by the Lowry method, using bovine serum albumin (fraction V) as the protein standard.

RESULTS

Inhibition of growth by E.colicin plasmids

Plasmids were isolated from E.colicin strains and used to transform prototrophic E.coli K12 strain DB364. Screening of colicinogenic transformants for growth, defined as the ability to form colonies on defined medium, revealed that growth was severely inhibited following introduction of any of the E4 colicin plasmids (pColE4-K365, pColE4-CT9, pColE4-962 and pColE4-P33) tested (Table 2). Growth appeared normal in all respects on complex medium. E4 colicin plasmids were able to inhibit growth of prototrophic strain DB364 by converting it to an apparent auxotroph; such clones were denoted Pmi+ (for plasmid-mediated inhibition of growth). To quantify the magnitude of growth inhibition and determine its distribution amongst E.colicin plasmids, E.colicinogenic transformants of each immunity type were grown in complex medium (to avoid nutritional selection pressure), and their plating efficiencies determined (c.f.u. on unsupplemented defined medium/c.f.u. on complex medium) (Table 2). In contrast to E4 and, to a lesser extent, E7 colicinogenic clones, DB364 transformants bearing E1, E2, E3, E5 and E6 colicin plasmids showed no growth inhibition, with plating efficiencies of approximately 0.90 after 48 h incubation. E7 colicinogenic clones grew poorly on defined medium, reaching plating efficiencies of 0.0004 at 48 h and requiring in excess of 96 h to reach plating efficiencies.
Fig. 1. DB314 culture viability and plating efficiency. Strain DB314 was cultured in 40 ml defined medium either supplemented with all 20 L-amino acids or unsupplemented. Culture samples were plated on complex agar to determine the total culture viability and on defined agar to compute the plating efficiency (see footnote to Table 2). ●, Culture viability (c.f.u. ml⁻¹) for a culture grown in supplemented defined medium and plated on complex agar; ○, culture viability (c.f.u. ml⁻¹) for a culture grown in unsupplemented defined medium and plated on complex agar; □, plating efficiency for a culture grown in supplemented defined medium; △, plating efficiency for a culture grown in unsupplemented defined medium.

Fig. 2. Growth of DB314 in defined medium supplemented with amino acids. Strain DB314 was grown in complex medium, washed, resuspended in defined medium and inoculated into flasks containing defined medium supplemented with various combinations of amino acids. Growth was monitored spectrophotometrically at OD₅₅₀ at various times (Gilford model 222A). ●, All amino acids included; ○, no leucine; □, no valine; △, no methionine; ▲, no arginine; ○, no valine or isoleucine.

obtained by other E colicinogenic clones in 48 h. E4 colicinogenic strains showed the greatest growth disturbance, with plating efficiencies of 0.0003 at 48 h. The plating efficiencies of E4 colicinogenic strains increased to only 0.002 at 96 h and did not increase any further with incubation for 144 h. Removal of sodium citrate from the CY defined medium and/or substitution of glucose (0.5%) for glycerol had no effect on the growth inhibition mediated by E4 colicin plasmids. Only E4 and E7 colicin plasmids inhibited growth of strain DB364. The shared growth inhibitory capacity of E4 and E7 colicin plasmids was not unexpected, based on data suggesting a common, but distantly separated, evolutionary origin for E4 and E7 plasmids (D. P. Brunner & M. A. Bott, unpublished data). The plasmid-mediated auxotrophy was further investigated in E4 colicinogenic strains.

Supplementation of defined medium with vitamin-free hydrolysed casein or all 20 L-amino acids alleviated the apparent plasmid-mediated auxotrophy. Without amino acid supplementation, growth was not observed in liquid defined medium until incubation times exceeded 20 h (data not shown). This delayed growth response by E4 colicinogenic cultures in defined medium was interpreted to have resulted from growth of a minor member of the inoculum population able to reverse the apparent auxotrophy (present in the population at a frequency of 0.0003, see
Growth inhibition by *E. coli* plasmids

above); growth was detectable only after 20 h due to nutritional selection. The major proportion of the population, comprised of auxotrophic bacteria, was unable to grow in defined medium.

Experiments were done to examine the growth response and plating efficiency of *E. coli* colicinogenic strain DB314 during incubation in unsupplemented and supplemented defined medium. Culture samples from flasks of defined medium supplemented with all twenty L-amino acids demonstrated a normal growth curve when plated on complex medium, and plating efficiencies which remained essentially constant at about 0.003 (Fig. 1). This observation would be predicted for two phenotypic classes growing together, at the same rate, in the absence of a selection pressure favouring 'outgrowth' of either phenotypic class.

Initially, the culture viability and plating efficiencies remained constant for culture samples from unsupplemented defined medium, as the majority of the population was unable to grow (Fig. 1). A decline in viability, amounting to an approximate loss of 85% of the inoculum viability, followed 350 to 400 min of nutrient deprivation (Fig. 1), and was attributed, in part, to cell lysis, as culture turbidities decreased approximately 75% during this period (data not shown). The decline in viability ceased and, after about 1000 min, culture viability began increasing. During the period between 400 and 1800 min, increasing plating efficiencies reflected the exponential outgrowth of the minor member of the inoculum population not inhibited by pColE4-K365; the major component of the inoculum population was reduced to 15% of its original viability and was unable to grow. The rate of change of the plating efficiencies approximated to the rate of exponential growth for the uninhibited prototrophic segregants (Fig. 1). The data clearly show two phenotypic classes were present: one inhibited by pColE4-K365 (auxotrophic), the other relieved of the inhibitory effect (prototrophic). The numerator of the ratio determining the plating efficiency represented the number of cells which were able to grow on defined medium (phenotypically Pmi-); this number increased exponentially. The denominator, on the other hand, was comprised of each of the two phenotypic classes, cells which were unable to grow in defined medium (auxotrophic or Pmi+) and cells (prototrophic or Pmi-) which increased exponentially. After approximately 1000 min, increases in total culture viability were detected, as well as a reduction in the slope of the plating efficiency increase. The Pmi- population at this time was sufficiently large (equal to or slightly larger than the static Pmi+ population) and contributed significantly to the total culture viability. Accordingly, the rate of change of the plating efficiency declined until the static population became an insignificant minority. At this time the plating efficiency ceased to increase; the numerator and denominator of the ratio were essentially the same, since the culture was comprised of essentially all Pmi- members. To test this interpretation further, a sample from the unsupplemented culture flask was removed at 1800 min and subcultured into a flask containing unsupplemented defined medium; rapid onset of growth with no apparent lag was observed (data not shown).

Plasmid loss (loss of colicinogeny) was not detected amongst Pmi- clones, and was, accordingly, not the explanation for the reversion to prototrophy. *E. coli* colicin plasmids isolated from Pmi- clones and used to re-transform strain DB364 showed that approximately 60% of the plasmids retained their ability to inhibit growth of DB364. The remaining 40% of plasmids were unable to inhibit growth, yet were indistinguishable from native *E. coli* colicin plasmids, with respect to colicin immunity, colicin production and excretion, and sensitivity to lysis by mitomycin C. Furthermore, inactivation of the *E. coli* colicin and lysis genes by Tn5 mutagenesis did not abolish the ability of pColE4-K365 to inhibit growth (D. P. Brunner & E. A. Galella, unpublished data). We inferred that the group of plasmids unable to mediate the auxotrophy arose through alteration of a unique *E. coli* colicin plasmid gene which we have designated pmi. The nature of the plasmid alteration, occurring at a frequency of $1.2 \times 10^{-4}$ per bacterium, is under investigation.

**Effects of amino acid availability on growth**

Plasmid-mediated inhibition of growth by *E. coli* colicin plasmids involved an apparent amino acid auxotrophy. However, no single or double amino acid supplementations allowed growth. To investigate the amino acid auxotrophy, *E. coli* colicinogenic clones were grown in complex
medium (to avoid nutritional selection pressure) and shifted into defined medium supplemented with different combinations of 19 of 20 amino acids. Removal of most individual amino acids had no adverse effects (data not shown). However, for a few amino acids, a perturbation of growth was seen upon amino acid removal (Fig. 2). Removal of isoleucine, leucine, valine, arginine, or methionine resulted in increased lags prior to growth, increased generation times and/or reduced final cell yields. The effects of removal of isoleucine were inferred from comparison of a culture without isoleucine and valine to one without valine, as *E. coli* shows valine-sensitivity in the absence of isoleucine (Umbarger & Freundlich, 1965; De Felice et al., 1974). The reproducible differences implicated at least five amino acids as critical for growth of E4 colicinogenic (Pmi*) clones. However, supplementation of defined medium with only these five amino acids was not sufficient to allow normal growth (data not shown). An assay for testing multiple amino acid requirements was employed to examine further the nature of the apparent amino acid auxotrophy (Zubrzycki et al., 1969). From pools which enhanced growth, amino acids were systematically removed and the effects upon growth monitored. Removal of amino acids from pools that promoted growth allowed for the identification of seven amino acids (five of which were previously implicated) that were able to enhance growth when added to defined medium (isoleucine, leucine, valine, arginine, methionine, serine and glycine). However, growth remained somewhat less than when supplemented with all 20 amino acids (Fig. 3).

The inability of E4 colicinogenic clones to grow in unsupplemented defined medium apparently involved the availability of at least seven amino acids for protein synthesis. To test whether the auxotrophic phenotype resulted from plasmid-mediated repression of synthesis of enzymes involved in amino acid biosynthesis under conditions normally resulting in derepression, we assayed the activity of two representative enzymes from colicinogenic and non-colicinogenic cultures under repressing and derepressing conditions. AHAS was assayed, due to its involvement in branched-chain amino acid biosynthesis and its responsibility for regulating carbon flow to valine and isoleucine. In addition, OTC was assayed, due to its involvement in the later stages of arginine biosynthesis.

Following incubation of strain DB364 and E4 colicinogenic strain DB314 under repressing and derepressing conditions, cultures were harvested and the specific activities of AHAS and OTC were determined. For each enzyme assayed, the specific activities were essentially identical between strains DB364 and DB314, when obtained from a given medium. AHAS activities for strains DB364 and DB314 from defined medium were 1.82 and 1.87 μmol min⁻¹ (g protein)⁻¹, respectively. These activities represented increases of 1.6-fold when compared with
activities of cultures from complex medium. OTC activities for strains DB364 and DB314 increased 14-fold under derepressing conditions (defined medium); enzyme activities for DB364 and DB314 from defined medium were 0.39 and 0.38 μmol min⁻¹ (g protein)⁻¹, respectively. There was no evidence for any plasmid-mediated effects upon the synthesis and activity of AHAS or OTC, two key enzymes involved in the synthesis of four of the seven amino acids implicated in the auxotrophic phenotype.

**DISCUSSION**

Following transformation with plasmid DNA from representatives of different E. coli immunity types, E4 and, to a lesser extent, E7 colicin plasmids adversely affected growth by mediating an apparent amino acid auxotrophy in *E. coli* K12 strain DB364. Examples of plasmids adversely affecting host cell growth have been reported. Antibiotic resistance plasmids can cause their hosts to grow more slowly (Nordström et al., 1977; Zünd & Lebek, 1980; Lebek & Zünd, 1981). As many as one-fourth of all wild *R* plasmids have been suggested to increase host generation time by more than 15% (Zünd & Lebek, 1980). Plasmids of incompatibility (Inc) groups P and N have also been reported to affect their hosts adversely. The IncP broad host range plasmid RK2 and each of fourteen other IncP plasmids tested were shown to carry *kil* genes which are potentially lethal for their hosts (Figurski & Helinski, 1979; Figurski et al., 1982). IncN plasmid pKM101, a derivative of R46, adversely affected the growth of *E. coli* K12 strain AB1157 upon plating on minimal glucose medium, as colonies exhibit a decreased growth rate (small colony morphology) due to effects on purine metabolism (Langer et al., 1981; Waleh & Stocker, 1981). The gene responsible for the small colony morphology, initially called *slo*, is now designated *kilA* (Winans & Walker, 1985).

The growth inhibitory effect observed with E. coli plasmids represents another example of plasmids adversely affecting host cell growth. The inability of E4 colicinogenic cultures to grow in defined medium involved the availability of at least seven amino acids for protein synthesis. These seven amino acids were initially proposed to represent those not synthesized under derepressing growth conditions due to the inhibitory function of a trans-acting product encoded by E4 colicin plasmids. Examination of the amino acids involved in the apparent auxotrophy showed that members of more than one biosynthetic family were affected. The involvement of different biosynthetic families suggested that, if the colicin plasmids did affect amino acid biosynthesis, a 'global' repression mechanism would have been involved. A colicin plasmid-encoded product may have inhibited amino acid biosynthesis in a global manner by acting in much the same way as that which has been demonstrated for the *trp* repressor (Johnson & Somerville, 1983; Bogosian & Somerville, 1983), or indirectly by interfering with levels of regulatory nucleotides which in turn affect gene expression (Yang et al., 1974; Stephens et al., 1975; Gallant, 1979; Adhya & Garges, 1982).

Assays of the activities of AHAS and OTC, two key enzymes involved in the synthesis of four of the seven amino acid implicated in the auxotrophic phenotype, showed that E4 colicin plasmids mediated their auxotrophy in strain DB364 by a mechanism other than repression of amino acid biosynthesis. As an alternative hypothesis, E4 colicin plasmids may have exerted their growth inhibitory effects by perturbing the accumulation and maintenance of amino acid pools in the cytoplasm, possibly due to the action of a plasmid-encoded protein on the cell permeability barrier. A model involving non-specific efflux from intracellular amino acid pools would explain the observed involvement of amino acids from different biosynthetic families.

Recent studies of several colicin plasmids, pColE1 (Sabik et al., 1983), pColE2 (Pugsley & Schwartz, 1983), pColE3 (Watson & Visentin, 1982; Jakes & Zinder, 1984) and pColN (Pugsley, 1984), as well as cloacin plasmid pCloDF13 (Oudega et al., 1982), have indicated the presence of a lysis gene, whose product alters cellular permeability and is involved in colicin release. However, production of the lysis protein was in itself not responsible for mediating the auxotrophy, as E. coli plasmids other than E4 (and E7), with intact lysis genes, did not inhibit growth of strain DB364. Furthermore, Tn5 mutagenesis of the pColE4-K365 lysis gene did not alter expression of the apparent amino acid auxotrophy (D. P. Brunner & E. A. Galella,
unpublished data). Conversely, a group of the E4 colicin plasmids isolated from Pmi− clones were found to have lost the ability to mediate the auxotrophy, but retained sensitivity to lysis by mitomycin C (an unaltered lysis gene), produced colicin and were immune. Hence, we propose the existence of a unique E4 colicin plasmid gene (pmi) which is responsible for mediating the apparent amino acid auxotrophy, and which is distinct from the lysis gene. Detailed analyses of pColE4-K365 and its mutant derivatives will allow for the genetic and biochemical characterization of the apparent amino acid auxotrophy, and an understanding of the mechanism by which pmi mediates its effects.

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