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Production of phenylalanine and organic acids by phospho*enol*pyruvate carboxylase-deficient mutants of *Escherichia coli*

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SUMMARY

Isogenic strains of *Escherichia coli* were grown aerobically in minimal medium in a 2-liter airlift fermentor to determine whether a *ppc* (phospho*enol*pyruvate carboxylase) mutation had the effect of directing glucose carbon into phenylalanine synthesis. Two host strains, YMC9 (ppc^+) and KB285 (ppc^-) were used, either with (Phe^c) or without (Phe^o) a plasmid which determines constitutive phenylalanine production. Carbon consumption and metabolic products were monitored. Phenylalanine production occurred only in strains carrying the Phe^c plasmid. ppc^- strains produced less cell mass and more acetate, pyruvate, and phenylalanine (in the Phe^c strains) than did isogenic ppc^+ strains. Lactate and ethanol production were not detected in any of the strains. Phe^c strains produced less acetate and pyruvate than their Phe^o homologs. Importantly, ppc^-/Phe^c produced at least six times as much phenylalanine (0.32 g phenylalanine/g dry weight cells) as ppc^+/Phe^c. Even in this case, however, phenylalanine was produced at ten-fold lower levels than acetate. Thus, although the ppc^- mutation stimulates phenylalanine production, it also stimulates the production of unwanted by-products such as acetate and pyruvate.

INTRODUCTION

We are investigating means by which carbon flow in microorganisms can be genetically and physiologically controlled so as to enhance the yield of metabolic products from substrate carbon. Our broad objective in this work was to direct carbon into phenylalanine (phe) production.

About 70% of the glucose used by aerobically growing *Escherichia coli* is metabolized via the Embden-Meyerhof-Parnas glycolytic pathway [4]. Glycolytic products are further metabolized in the tricarboxylic acid (TCA) cycle. Glucose metabolism serves both as an energy source and as a source of precursors for the synthesis of amino acids and other cell constituents. For example, phospho*enol*-

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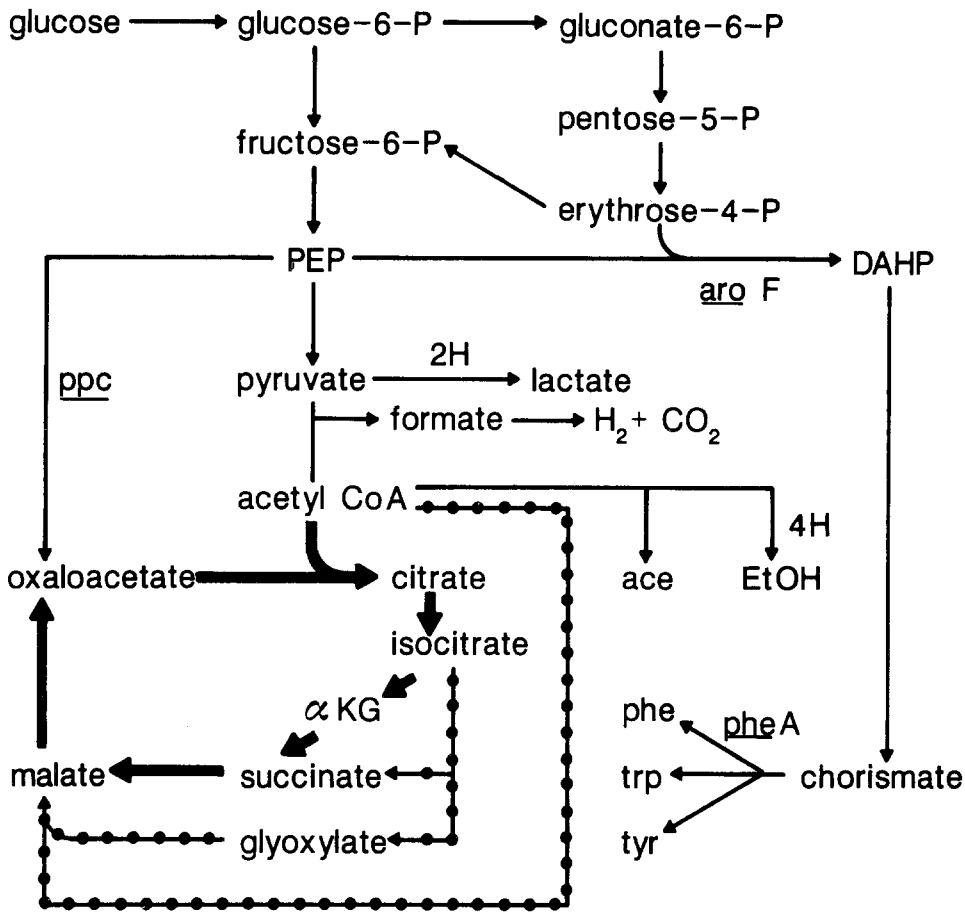

Fig. 1. Pathways of central carbon catabolism in *E. coli*, showing derivation of carbon skeletons for aromatic amino acid biosynthesis. The bold lines indicate the steps of the tricarboxylic acid (TCA) cycle; the dotted lines indicate the glyoxalate shunt. PEP, phospho*enol*pyruvate; DAHP, 3-deoxy-7-P-D-arabinoheptulose; ace, acetate; EtOH, ethanol; α-KG, α-ketoglutarate; phe, phenylalanine; trp, tryptophan; tyr, tyrosine.

pyruvate (PEP) is required for the synthesis of aromatic amino acids (Fig. 1).

The other 30% of glucose carbon is normally metabolized via the pentose phosphate pathway, which produces NADPH for anabolic processes. The pentose phosphate pathway also provides erythrose 4-phosphate for the biosynthesis of the aromatic amino acids (Fig. 1).

The TCA cycle requires a continual replenishment of four-carbon skeletons to replace those withdrawn for amino acid biosynthesis. In *E. coli*, this is accomplished by one of two anaplerotic pathways. The first converts PEP to oxaloacetate by the activity of PEP carboxylase, the product of the *ppc* gene [6]. The second converts two acetates to malate via the glyoxalate shunt. Since glycolytic intermediates inhibit the glyoxalate shunt, *ppc*$^-$ mutants cannot grow on glucose unless supplied with exogenous TCA cycle intermediates such as succinate. In the absence of glycolysis, however, *ppc* mutants grow normally on acetate.

We analyzed our cultures for end products commonly produced by *E. coli*. During aerobic growth on glucose, *E. coli* produces significant amounts of acetate [8]. When oxidative phosphorylation is limited, glycolytic carbon appears as the products of mixed acid fermentation. The specific balance of these products is determined by the oxidation-re-

duction balance within the cells: lactate, ethanol, and succinate require reducing equivalents for their formation, whereas acetate, pyruvate, formate, hydrogen, and carbon dioxide do not.

We hypothesized that a mutation in the *ppc* gene which disables the production of active PEP carboxylase should lead to an accumulation of the aromatic precursors PEP and erythrose 4-phosphate, and thereby to increased phenylalanine production. As revealed in our results, an increase in phenylalanine biosynthesis can only occur in cells which have been specifically enabled to produce phenylalanine in a constitutive manner by the inclusion of a plasmid (pKB702) carrrying key genes (*aro*F, and a feedback-resistant mutant of *phe*A) for phenylalanine biosynthesis. It was not the object of this work to explore and optimize those factors essential for constitutive phenylalanine biosynthesis; rather, we were interested in whether mutational inactivation of PEP carboxylase could increase phenylalanine production in strains enabled for phenylalanine synthesis. We further sought to understand the effects of the *ppc* mutation on the extent and spectrum of by-product formation.

MATERIALS AND METHODS

Media

O medium contained M9 salts [7] plus 5 g/l glucose and 0.001 g/l thiamine.

Goochee C (GOOC) medium was modified from Goochee et al. [5]. It contained, in g/l: NH_4Cl, 10.7; $CaCl_2 \cdot 2H_2O$, 0.13; $MgSO_4 \cdot 7H_2O$, 2.96; KH_2PO_4, 2.72; K_2HPO_4, 12.4; $FeCl_3 \cdot 6H_2O$, 0.16; $ZnSO_4 \cdot 7H_2O$, 0.03; $MnSO_4 \cdot H_2O$, 0.03; $NaMoO_4 \cdot 2H_2O$, 0.09; $CuSO_4 \cdot 5H_2O$, 0.094; $CoCl_2 \cdot 6H_2O$, 0.10; H_3BO_3, 0.03; KI, 0.07; $NiCl_2$, 0.05; EDTA, 5.69; thiamine, 0.001; glucose, 5.0.

When so indicated, the above media were supplemented with sodium succinate or succinic acid to a level of 0.8 g/l succinate ion, and/or ampicillin (amp) to a level of 50 mg/l.

Bacterial strains

Strains YMC9 [1] and KB285 and plasmid pKB702 were obtained from our collection. KB285 is a *ppc*$^-$ mutant of YMC9. It was obtained by mutagenesis of YMC9 with the mutator element *mud*I (Ap, *lac*) [3], enrichment for succinate-requiring mutants, and curing of the mutator element by incubation at 42°C. The tight linkage of our mutation to the *arg*ECBH locus confirmed that it was in *ppc*. pKB702 is a pBR322 derivative [2] which determines ampicillin resistance, and which carries *aro*F (the gene for tyrosine-repressible 3-deoxy-7-P-D-arabinoheptulose synthetase) and a feedback-resistant variant of *phe*A (which codes for chorismate mutase and prephenate dehydratase activities) (O'Connor and Backman, unpublished).

Culture conditions

For fermentation runs, strains were streaked on O agar plates which included succinate for *ppc*$^-$ strains and/or amp for plasmid-carrying strains, and incubated at 37°C for 48 h. Three to five isolated colonies were picked into 5 ml GOOC plus succinate and incubated at 37°C with shaking for 24–36 h. Fresh broth culture was inoculated to give about 10^7 cells per ml in 50–100 ml fresh GOOC plus succinate in a 500 ml flask, and incubated on a shaking platform (200 rpm) at 34°C, with sparging (0.1 l/min air) for 18–24 h. Using this culture, a 2-liter airlift fermentor (Bethesda Research Labs) containing GOOC plus succinate was inoculated to an initial cell density of 30 Klett units, and grown at 34°C. Using an air flow rate of 1.0 l/min, DO stayed above 75% at all times during all the fermentations. Concentrated NH_4OH was used to control pH between the limits of 6.8 and 7.1. Mazu DF60P (Mazer Chemicals, Inc., Gurnee, IL) antifoam was added manually as needed. Glucose and succinate were added periodically as needed until the Klett reading reached about 1000, at which point the feeding of glucose and succinate was stopped and the culture was allowed to enter stationary phase. The fermentation was terminated when the Klett reading started to fall.

Sampling protocol

15 ml of culture were aseptically removed and the culture turbidity was determined using a Klett-

Summerson colorimeter with a green filter. Two 5-ml aliquots were centrifuged and the supernatant was removed for an immediate glucose analysis. The remaining supernatant was stored frozen at −20°C for later analysis of other metabolites. The cell pellets were used for dry weight determination by 24-h drying in a vacuum oven at 60°C. For the last sample in each fermentation run, a suitable dilution was plated on nonselective O medium and incubated for 24–48 h at 37°C. 100 colonies were patched onto O plus amp plates and incubated for another 24 h to assay for presence of the plasmid. The percentage of colonies which grew on the patch plate represented a minimum value for plasmid stability, because the cells had undergone a minimum of 20 divisions during growth on the nonselective medium outside the fermenter.

Analytical methods

Glucose was analyzed with a glucose analyzer (Yellow Springs Instruments, model 27). A GOOC salts (without glucose) blank gave a background reading of 1.5 g/l glucose; therefore all readings were corrected by this amount.

Acetate and ethanol were assayed on a gas chromatograph (Perkin-Elmer Sigma 2000) equipped with a flame ionization detector and an integrator (Perkin-Elmer LCI-100). 1 ml culture supernatant was acidified with 17 μl 50% (v/v) H_2SO_4 and 3.5 μl were injected directly. The column was 6 ft × 2 mm i.d. GLT (glass-lined stainless steel), packed with 100/120 Chromasorb 101 coated with 0.5% free fatty acid phthalate (FFAP) (SGE, Austin, TX). The column temperature was 170°C and the carrier gas was N_2 at 80 ml/min. Ethanol eluted at 0.5 min and acetate at 1.1 min under these conditions. The limit of detection of both analyses was 0.05 g/l. The acetate, ethanol, and succinate GC assays were adapted from information provided by Supelco (Bellefonte, PA).

Succinate was assayed on the same GC. Samples were prepared by acidifying 2 ml culture supernatant with 33 μl 50% (v/v) H_2SO_4 in septum vials. 4 ml methanol and 0.8 ml 50% (v/v) H_2SO_4 were added. The vials were capped and heated at 55°C for 30 min in a water bath to methyl esterify the organic acids. The vials were cooled on ice, and 1.0 ml $CHCl_3$ was added to each. The vials were inverted 20 times to mix. 1.0 μl of the $CHCl_3$ layer was injected onto a 6 ft × 1.4 in o.d. × 2 mm i.d. glass column, packed with 10% SP-1000/1% H_3PO_4 on 100/120 Chromasorb WAW (Supelco). Column temperature was 140°C and carrier gas was N_2 at 30 ml/min. Succinate eluted at 5.6 min under these conditions; the sensitivity of the assay was 0.1 g/l.

Pyruvate and lactate were determined on duplicate samples using spectrophotometric assay kits (Sigma Diagnostics) which employ lactate dehydrogenase.

Phenylalanine was assayed on duplicate samples using the PaT Stat Test Kit (Pharmacia P-L Biochemicals), which employs phenylalanine ammonia lyase in a kinetic spectrophotometric assay.

Product, byproduct, and cell yield from each fermentation were computed as averages of the two to four data points after the cells had ceased to grow exponentially but before the culture turbidity had started to fall.

RESULTS

Growth properties

ppc$^-$ strains grew two-fold more slowly than *ppc*$^+$ strains (t_d = 4.0 vs. 1.8 h as shown in Fig. 2). The *ppc*$^+$ strains used glucose about three times as efficiently as the *ppc*$^-$ strains (Table 1). The presence of the plasmid in either strain did not alter the growth or cell yield on glucose significantly (Table 1, and data not shown). *Enterobacter* is reported to produce 0.42 g cell/g glucose [9], which is very close to the value we obtained for the *ppc*$^+$ *E. coli* strains. The low yield of the *ppc*$^-$ strains on glucose is consistent with the slow growth rate, and suggests that glucose carbon has fates other than cell mass. Although the *ppc*$^-$ strains absolutely require succinate for growth in this medium, they consumed less than 10% as much succinate as they did glucose. *ppc*$^+$ strains, although not requiring succinate for growth, consumed succinate throughout the fermentations, irrespective of glucose concentration (data not shown).

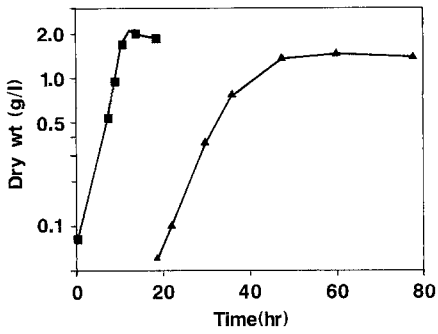

Fig. 2. Growth of *ppc*$^-$ and *ppc*$^+$ strains, under fermentation conditions described in Materials and Methods. ■, *ppc*$^+$/Phe^o; ▲, *ppc*$^-$/Phe^o. Specific growth rate (μ), h^{-1}: *ppc*$^+$/Phe^o, 0.39; *ppc*$^-$/Phe^o, 0.17. Judging by Klett readings (data not shown), growth of the *ppc*$^-$/Phe^o strain was exponential from the time of inoculation. Due to a smaller inoculum size, dry weight of the *ppc*$^-$/Phe^o strain was not detectable until 18.5 h.

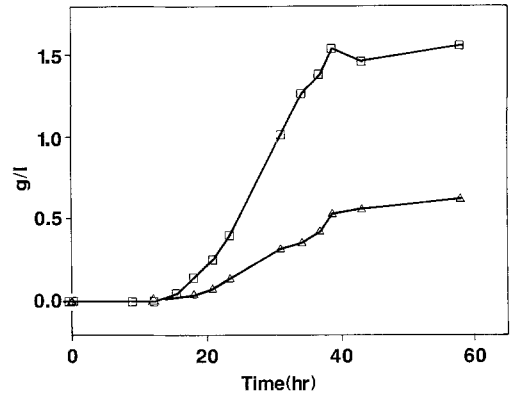

Fig. 3. Kinetics of phe production by the *ppc*$^-$/Phe^c strain. □, dry weight; △, phe.

Product formation

Phenylalanine production was growth-related in the plasmid-containing strains (Fig. 3). Kinetics of phe production were similar for the *ppc*$^+$/Phe^c strain (data not shown).

The *ppc*$^-$/Phe^c strain produced considerably more phe than *ppc*$^+$/Phe^c (data not shown), about ten times as much on a dry weight basis (0.32 g phe/g cells for *ppc*$^-$/Phe^c versus 0.03 g phe/g cells for *ppc*$^+$/Phe^c) and about four times as much on a glucose yield basis (0.04 g phe/g glucose consumed for *ppc*$^-$/Phe^c, versus 0.01 for *ppc*$^+$/Phe^c). These estimates are based on the assumption that the plasmid was 100% stable in both strains. In fact, only 60% of the clones obtained by streaking at the end of the *ppc*$^+$/Phe^c fermentation were amp-resistant, but 100% of the *ppc*$^-$/Phe^c clones were amp-resistant. The most conservative estimate (see Materials and Methods) of the difference between the two strains would assume that only 60% of the *ppc*$^+$/Phe^c cells contained the plasmid at the time that the samples were taken, in which case the *ppc*$^-$/Phe^c strain produced six times as much phe on a dry weight basis and 2.5 times as much on a glucose yield basis as the *ppc*$^+$/Phe^c strain.

Table 1

Average[a] cell yield on glucose and succinate

Strain	g dry wt/g substrate consumed	
	glucose	succinate
ppc$^+$/Phe^o	0.41	–[b]
ppc$^+$/Phe^c	0.38	–[b]
ppc$^-$/Phe^o	0.12	1.77
ppc$^-$/Phe^c	0.13	2.10

[a] Average of the time points after logarithmic growth had slowed and before Klett reading started to fall.

[b] Cell yield on succinate for the *ppc*$^+$ strains has no meaning, since the *ppc*$^+$ strains do not require succinate for growth.

Neither the *ppc*$^-$/Phe^o nor the *ppc*$^+$/Phe^o strains produced any detectable extracellular phe. This suggests that an accumulation of precursors to aromatic biosynthesis is not sufficient, by itself, to affect phe biosynthesis. Specific genetic enablement of phe biosynthesis is necessary in order to detect the effects of *ppc* mutations on that synthesis.

Organic acid formation

The amount of carbon consumed by overproduction of phenylalanine by the *ppc*$^-$/Phe^c strain is insufficient to explain its decreased cell yield on glucose. The low cell yield of the *ppc*$^-$/Phe^o strain also suggested that other by-products should be present. Therefore, we analyzed culture supernatants for some of the products of mixed acid fermentation.

Acetate was the by-product present in the most

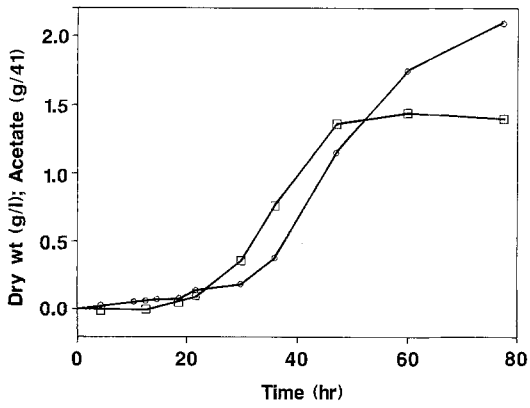

Fig. 4. Kinetics of acetate production by the ppc^-/Phe^o strain. □, dry weight; ○, acetate.

significant quantity. The ppc^-/Phe^o strain produced 7.0 g acetate/l; this is equivalent to 0.42 g acetate per g glucose consumed, or to 1.41 mol of acetate produced per mol of glucose consumed. The ppc^-/Phe^c strain produced somewhat less (data not shown), 1.08 mol acetate per mol glucose consumed. The ppc^-/Phe^c strain produced about ten times as much acetate as phenylalanine, as measured in grams per liter. Both ppc^+ strains produced much less acetate (0.12 and 0.03 mol acetate per mol glucose consumed for the Phe^o and the Phe^c strains, respectively) (data not shown) than did the ppc^- strains. Although the 7 g/l of acetate produced by the ppc^- strains is considerably lower than the 40 g/l which is generally taken to represent a toxic level, the cell densities we worked with here are low (about 1.6 g cell mass/l). Acetate continued to be produced by all strains after growth and phenylalanine production had ceased (Fig. 4, and data not shown).

Pyruvate was produced by the ppc^- cells, in much lower quantities (1 g/l, corresponding to 0.18 mol pyruvate per mol of glucose consumed) than was acetate (data not shown). No pyruvate was detectable in the ppc^+ broths. Neither lactate nor ethanol (data not shown) were produced in detectable quantities by any of the strains.

Carbon balancing was done for each fermentation, by adding up the total carbon that was accounted for by the production of cell mass, phenylalanine, acetate, and pyruvate, and by comparing it with the amount of glucose and succinate consumed. For both ppc^- strains, virtually all of the consumed carbon was accounted for by these products (Table 2). In the case of the ppc^+ strains, there appears to be an undetermined product or products that we have not accounted for.

Table 2

Fraction of consumed carbon (glucose and succinate) accounted for[a] at the end of the experiment[b]

	ppc^+	ppc^-
Phe^o	0.91	0.97
Phe^c	0.81	1.01

[a] Cell mass accumulation was assumed to account for 1 g glucose for each 0.42 g dry weight produced. Acetate, pyruvate, and lactate were assumed to account for 1 mol glucose for each 2 mol by-product produced. The synthesis of 1 mol phe was taken to require 2 mol glucose. The consumption of 2 mol succinate was taken to be equivalent to the consumption of 1 mol glucose.

[b] Fermentation runs described in Materials and Methods.

DISCUSSION

The ppc^- mutation uncouples the EMP and TCA pathways, and resulted in the overproduction of pyruvate-related intermediates. The overproduction of PEP was anticipated (see Introduction), and most likely contributed to enhanced phe production. The overproduction of acetate and pyruvate probably reflects accumulation of PEP which exceeds the ability of the cells to utilize it for phe biosynthesis. The fact that acetate production and glucose consumption continued after growth and phe synthesis had ceased suggests that glycolysis in these cells persists after other metabolic activities have largely ceased.

The theoretical yield of phenylalanine on glucose is 0.46 g phe/g glucose consumed; however, this theoretical yield does not account for the glucose necessary to make the cell mass which synthesizes phenylalanine. With the ppc^-/Phe^c strain,

we achieved roughly 10% of theoretical yield (0.04 g phe/g glucose consumed). The *ppc*$^-$ mutation, in combination with genetic changes which enable constitutive phenylalanine biosynthesis, is nevertheless effective in directing carbon into increased phenylalanine biosynthesis, as shown by the fact that the *ppc*$^-$ mutation increases phenylalanine production six- to ten-fold over the *ppc*$^+$ control.

The *ppc*$^-$ mutation also significantly enhances acetate and pyruvate by-product formation. It is noteworthy that by-products which are NADH sinks do not appear to be produced. This suggests that, under our growth conditions, no excess reducing power accumulated. This suggestion is corroborated by the dissolved oxygen profiles during the fermentation runs, which showed that all four strains consumed oxygen at equivalent rates.

Although we have couched our discussion in terms of the effect of *ppc* mutations on phe production, the data can also be cast in terms of the effect of enabling phe production in a *ppc*$^-$ background. In these terms, we see that enabling phe production diverts carbon that is otherwise forced to appear as acetate and pyruvate. In no case, however, did enablement of phe production significantly affect growth rate or cell yield, probably because the amount of carbon which went into phe production was small compared with total glucose consumption. We are continuing to investigate routes by which our observations can be utilized in production processes.

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REFERENCES

1 Backman, K., Y.-M. Chen and B. Magasanik. 1981. Physical and genetic characterization of the *glu*A-*glu*G region of the *Escherichia coli* genome. Proc. Natl. Acad. Sci. USA 78: 3743–3747.

2 Bolivar, F., R.L. Rodriguez, P.J. Greene, M.C. Betlach, H.L. Heyneker, H.W. Boyer, J.H. Croso and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2: 95–113.

3 Casadaban, M.J. and S.N. Cohen. 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: *in vivo* proof for transcriptional control sequences. Proc. Natl. Acad. Sci. USA 76: 4530–4533.

4 Cheldelin, V.H. 1961. Evaluation of metabolic pathways. In: E.R. Squibb Lectures on Chemistry of Microbial Products, pp. 61–74, Wiley, New York.

5 Goochee, C.F., R.T. Hatch and T.W. Cadman. 1985. The response of a nitrogen-limited chemostat culture of *Escherichia coli* B/r to pH and dilution rate steps. Biotechnol. Bioeng. 27: 439–446.

6 Gottschalk, G. 1986. Bacterial Metabolism, Springer-Verlag, New York.

7 Miller, J.H. 1972. Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

8 Roberts, R.B., P.H. Abelson, D.B. Cowie, E.T. Bolton and R.J. Britten. 1963. Extracellular products of metabolism. In: Studies of Biosynthesis in *Escherichia coli,* p. 193, Carnegie Institute of Washington Publication 607, Washington, DC.

9 Shiloach, J. and S. Bauer. 1975. High-yield growth of *E. coli* at different temperatures in a bench-scale fermentor. Biotechnol. Bioeng. 17: 227–239.