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Transport-Limited Growth Rates in a Mutant of *Escherichia coli*

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Mutants of *Escherichia coli* B/r have been selected which require increased nutrient concentrations for half-maximal growth rate. This half-saturation constant (K_m) for growth on glucose is 10^{-6} and 7×10^{-4} M for the wild type (CP 366) and the mutant (CP 367), respectively. Similarly, the K_m is increased for growth on many other carbohydrates (20- to 500-fold), for the anions PO_4^{3-} and SO_4^{2-} (ca. 100-fold), and for the uptake of several amino acids (20- to 50-fold). At sufficiently high concentrations of the nutrients, mutant and wild type grow equally fast. The yield in terms of cell mass per milligram of substrate is unaffected by the mutation. The phenotype of the parent is reestablished in what appears to be the reversion of a single mutation (*kmt*) which maps between *strA* and *metB*. The pleiotropic decrease of the affinities for transport of the various nutrients seems to be the result of a modification of the cell envelope which weakens the attachment of the various specific binding proteins to the periplasmic membrane. Since the mutant K_m values are increased considerably, high cell densities can be reached in batch cultures at growth-rate-limiting substrate concentrations (10^7 to 10^8 cells/ml). This allows chemical analysis of the cell composition; the application of the mutant to studies of bacterial physiology as function of growth rate is discussed.

Studies of bacterial populations growing at different rates have provided considerable information about the in vivo states and potentials of these cells (21). It is against this background that we must try to assess the role and significance of the various biochemical mechanisms currently being studied in vitro.

At a given temperature, the growth rate can be varied by choosing media with different carbon or nitrogen sources, or both. In this way, a number of simple relationships between cell composition and growth rate have been observed, which permit the construction of fairly detailed models for the control of bacterial growth (20). However, many questions remain open. Thus, we do not know to what extent the state of a growing population is determined by the evident need for particular enzyme systems for the utilization of different carbon sources (e.g., glucose, succinate, acetate, or proline).

Alternatively, the growth rate can be varied by using chemostatic continuous cultures (25, 30). In this case, the growth rate (μ = doublings per hour) is determined by the effective concentration, S, of a given substrate, which is maintained as a balance between supply from outside and utilization by the cells [essentially according to Monod's equation (24): $\mu = \mu_{max} S/(K_m + S)$]. This technique has not been used as widely as batch cultures to study the general physiology of microbial growth (6, 41); however, certain complex metabolic patterns in yeast have been studied (2, 43) which could not have been analyzed thoroughly in batch cultures.

The obvious advantage of the chemostat is that the growth rate can be varied *without* qualitatively changing the substrate. However, the ideal chemostat requires continuous flow of substrate and perfect mixing as well as adequate control of aeration, foaming, and contamination. The usefulness of the chemostat is limited by these technical prerequisites and by the fact that large samples cannot be repeatedly withdrawn for analysis.

The approach presented here eliminates most of these difficulties by offering a practical solution to the problem of establishing *batch cultures in which different growth rates are defined by different concentrations of one particular substrate.* In principle, it has always been possible to do so at extremely low cell densities (24, 30). To obtain half-maximal growth rate of the common laboratory strains of *Escherichia coli* in a glucose minimal medium, the glucose concentration must be reduced to 10^{-6} M, i.e., the half-saturation constant K_m for growth on glucose. This implies

that the cell densities required for biochemical studies (at least 10^7 to 10^8 cells/ml) cannot be reached in batch cultures in which the growth rate is limited by the glucose concentration.

A mutant of *E. coli* B/r is described below, whose K_m for growth on various sugars is greatly increased. With this strain, cell densities can be obtained, which allow the chemical composition of the cells to be determined as function of μ in the range from 0.25 to 1.5 doublings/hour, at 37 C. The nature of this pleiotropic mutation is discussed in light of the additional finding that the K_m is increased not only for several sugars, but for some amino acids and anions as well.

MATERIALS AND METHODS

Organism. *E. coli* B/r *thy*$^-$, collection number CP 366, was used.

Media. Phosphate-buffered AB minimal medium according to Clark and Maaløe (5) was used; after sterilization, it was supplemented with various carbon sources at variable concentrations, and with 10 μg of thymine/ml. Tris(hydroxymethyl)aminomethane (Tris) minimal medium (18) with 0.2% glucose and 10 μg of thymine/ml was used to determine the K_m values for uptake of SO_4^{2-}, PO_4^{3-}, K^+, NH_4^+, or Mg^{2+} by varying the concentration of the respective ion. All growth experiments were performed at 37 C, except where noted.

Minimal medium for substrate gradient plates was as follows: 0.1 g of $MgSO_4 \cdot 7H_2O$, 5 g of $Na_2HPO_4 \cdot 2H_2O$, 1.7 g of $Na(NH_4)HPO_4 \cdot 4H_2O$, 0.75 g of KCl; *p*H adjusted with HCl to 7.0; 1% agar; 10 μg of thymine/ml; in 1,000 ml.

MacConkey-plates were prepared as follows: in 1,000 ml of distilled water, 40 g of MacConkey agar base (Difco) was added; after autoclaving, sterile glucose solution was added.

Measurement of growth and half saturation constant for growth on different substrates. The optical density (OD) for cultures was determined with a Zeiss PMQ II spectrophotometer at 450 nm in 1-cm cuvettes. An OD_{450} of 0.1 corresponds to 3.3×10^7 cells/ml (9 μg of protein/ml) of *E. coli* B/r *thy*$^-$ growing in AB medium with 0.2% glucose (doubling time, 42 min). At low cell densities (e.g., in experiments with low glucose concentrations), 2-cm cuvettes were used, but OD values are always given as absorbancy per 1 cm. Cell number and cell size distribution were determined with a modified Coulter counter (38). The growth rate μ is expressed in doublings per hour, and the doubling times g in minutes (specific growth rate $K = \mu \ln 2$). From the growth rates at different substrate concentrations, S, the K_m for growth was estimated graphically by plotting μ^{-1} against S^{-1} (*see* Fig. 2B).

Determination of the half saturation constant for amino acid uptake. Different radioactive amino acids (^{14}C- or ^3H-labeled; 2 to 5 mCi/mmole) were added at different concentrations to cultures growing in AB medium with 0.2% glucose. The rate of incorporation of radioactivity into protein was determined by sampling (0.2 ml) at intervals into 5% trichloroacetic acid, collecting the precipitate on membrane filters, and counting the radioactivity in a Nuclear-Chicago scintillation spectrometer (18). From the rates of incorporation (V, assumed to reflect the rate of amino acid uptake), the K_m for amino acid uptake was estimated graphically by plotting V^{-1} against (amino acid concentration)$^{-1}$; see Fig. 4C.

Determination of the cell composition. Protein and ribonucleic acid (RNA) were determined by a modification of the procedure described by Koch and Deppe (17). Portions (9 ml) of cultures with OD_{450} between 0.3 and 0.6 are sampled into 2 ml of ice-cold 1.2 N perchloric acid (PCA) and centrifuged after 20 min; the pellet is resuspended in 0.2 N PCA, and after a second centrifugation the supernatant fluid is completely removed. Two samples are used to determine protein (19); dried *E. coli* protein is used as a standard. Two other pellets are hydrolyzed in 2 ml of 0.3 N NaOH for 1 hr at 37 C, after which 1 ml of cold 1.2 N PCA is added. After centrifugation, the ultraviolet absorbancy at 260 nm of the supernatant fluid is measured (7, 8); the RNA content of the cells is calculated from this value, using an extinction coefficient of 10.5 mmole^{-1} for a mixture of the four 3'-ribonucleoside monophosphates corresponding to the composition of ribosomal RNA (23). After RNA extraction, the pellet which contains deoxyribonucleic acid (DNA) and other cell components is washed once with 2 ml of ice-cold 0.2 N PCA and dissolved in 0.1 ml of 0.06 N NaOH; 0.9 ml of TSM buffer, *p*H 7.3 (9), and 50 μg of DNase I (Worthington, 1 × crystallized) are then added, and (Worthington, 1× crystallized) are then added, and tation of the proteins with 0.5 ml of 0.2 N PCA (30 min, 0 C) and centrifugation, the concentration of oligodeoxynucleotides is determined spectrophotometrically at 260 nm and the DNA content of the cells is calculated by using an extinction coefficient of 10 mmole^{-1}. Hypochromicity is disregarded since it is less than 5% at the acid *p*H used (40).

Qualitative estimation of the K_m values for various carbon sources: the substrate gradient test. A few thousand cells are uniformly spread on petri dishes (diameter 8 cm), containing 25 ml of minimal agar with thymine but without a carbon source. A droplet of a 20% solution or some crystals of the substrate to be tested are then deposited on the surface; during subsequent incubation, a concentration gradient is formed. Cells with a low K_m value for the substrate tested rapidly produce colonies at the low substrate concentrations far from the "substrate spot," whereas cells with an increased K_m grow more slowly. The distance from the spot to the furthest visible colonies ("the colony front") is measured at half-day intervals (Fig. 3). A 20-fold difference in the apparent K_m is easily registered.

Selection of mutants with an increased K_m for the uptake of glucose. Cells (10^9) of a clone of *E. coli* B/r *thy*$^-$ growing exponentially in AB medium with 0.2% glucose were mutagenized with nitrosoguanidine (4). After washing with minimal medium, the cells were grown in AB medium with 0.5% glucose. Exponential growth was maintained for 20 generations by appropriate dilutions with fresh medium to reduce the frequency of slowly growing cells and to eliminate auxotrophs. The desired mutant was expected to grow as rapidly as the wild-type cells at a glucose concentration of 0.5%. Finally, 10^9 washed cells were resuspended in

250 ml of AB medium with 20 mg of glucose per liter. This concentration is about 100 times the K_m value of the wild type for growth on glucose; mutants with increased K_m values should grow slowly, and, therefore, penicillin selection (13) against the wild type should be possible. A glucose concentration of 10 to 15 mg was maintained by appropriate addition of sterile glucose solution to prevent cessation of growth of the wild-type cells. After 90 min of growth at low glucose concentration (doubling time of entire population, 47 min), penicillin [10,000 International Units (IU)/ml] was added. After 1 hr, the viable count was reduced 7,000-fold, and about 10^3 surviving cells were plated on MacConkey agar with 0.07% glucose. The development of the color of the colonies was checked repeatedly between 10 and 15 hr of incubation at 37 C. Wild-type colonies develop a red center after 11 to 12 hr; colonies which turned red later or developed weaker color, or both, were picked, restreaked, and tested in various ways for growth on glucose.

RESULTS

Growth characteristics of mutants with decreased affinity for the uptake of glucose. Among 24 colonies showing delayed coloring on MacConkey plates with 0.07% glucose, four were found to have a considerably increased apparent K_m value for growth on glucose. In AB medium with 0.2% glucose, all four grew with the same doubling time as the wild type (42 min); however, after a 40-fold dilution into AB medium to reduce the glucose concentration to 50 mg/liter, doubling times of 100, 120, 150, and 180 min, respectively, were recorded. The mutant (CP 367) with the highest apparent K_m for glucose growth is slightly larger and has a broader size distribution than the wild type when growing at 1.4 to 1.5 doublings/hour in AB medium with 0.2% glucose. The three other mutants showed about a twofold increased cell size. With respect to the uptake of sugars and amino acids, these four mutants exhibit the same pleiotropic increase of K_m as is shown below for CP 367.

Figure 1A shows the growth of CP 367 at various glucose concentrations. When these cultures approach the stationary phase, their growth rates decrease gradually even at the relatively high initial glucose concentration of 0.1%. Moreover, when a culture growing at 0.1% glucose (doubling time, 43 min) is diluted to give 100, 50, or 20 mg of glucose/liter, much lower growth rates are immediately established. At the same low glucose concentrations, the parent strain CP 366 (Fig. 1B) continues growth at its maximum rate ($\mu = 1.5$) until the glucose is practically used up. In the mutant CP 367, the same high growth rate can only be maintained at glucose concentrations higher than 0.1%.

In the wild type, μ begins to decrease at about 2 mg of glucose/liter (Fig. 2A). At such low concentrations, a defined growth rate can only be measured in very dilute cultures by following the increase in cell numbers, e.g., with a Coulter counter. The K_m for growth of CP 366 on glucose is about 10^{-6} M; for another *E. coli* B/r, which is *lac*$^-$, it was estimated at 2×10^{-6} M. Strains *E. coli* B and *E. coli* 15 TAU-bar (9) have K_m values below 5×10^{-6}, as judged from OD_{450} measurements. For strain CP 367, a value of 0.7×10^{-3} M can be read from the plot of μ versus glucose concentration S from the reciprocal plot (Fig. 2B). The linearity between μ^{-1} and S^{-1} in the latter agrees with Monod's conclusion (24) that the relationship between μ and S can be formulated in analogy with simple enzyme kinetics.

There is no gross temperature dependence of the K_m value for glucose; the same values are found at 30 and 37 C (Fig. 2B). Also, the yield, in terms of OD, is the same for mutant and wild type growing with maximal growth rate: 0.22 to 0.25 OD_{450} units per 100 mg of glucose per liter.

Pleiotropy of the mutation in CP 367. The growth of CP 367 has been studied with several sugars and carbon sources. The progression of the "colony front" on minimal plates with different substrates is illustrated in Fig. 3A, and actual measurements are shown in Fig. 3B for three different carbon sources. Delayed appearance of mutant colonies (compared to wild type) at a certain distance from the substrate spot is thought to reflect their slower growth at low concentrations of the substrate and therefore an increased K_m for growth on that substrate.

The following sugars and other simple carbon sources were tested: D-glucose, lactose, D-fructose, D-galactose, D-mannose, D-mannitol, maltose, D-raffinose, L-rhamnose, L-arabinose, D-ribose, glycerol, pyruvate, acetate, and succinate. All of them behaved qualitatively like glucose, and so did the amino acids which can support growth as carbon sources: i.e., aspartic acid, asparagine, glutamine, glutamic acid, tryptophan, serine, proline, alanine, lysine, leucine, tyrosine, and cysteine. Thus, the K_m values for the uptake of many carbon sources, including amino acids, appear to be increased in strain CP 367.

Quantitative evidence for a decreased affinity for the *uptake of sugars* was obtained by determining the growth rates at different concentrations and analyzing the data, as illustrated in Fig. 2 and 6 for glucose and lactose, respectively. Table 1 shows that the estimated K_m values for all the sugars tested are from 40 to 1,000 times higher in CP 367 than in the parent strain. For most of these substrates, the K_m values of the parental strain are too low to be determined accurately by

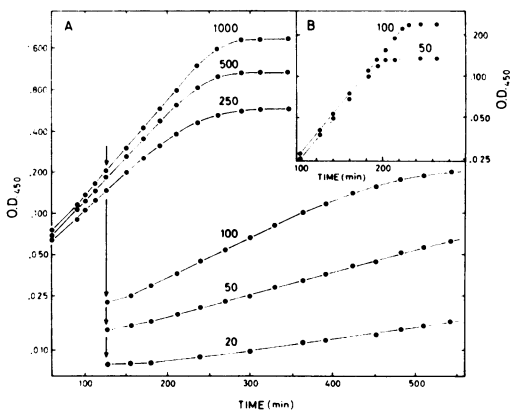

FIG. 1. *Growth of CP 366 and CP 367 at different glucose concentrations. (A) Cultures of the mutant CP 367 in AB medium with 1,000, 500, and 250 mg of glucose/liter, respectively, inoculated from a culture pregrown with 1,000 mg/liter. The 100, 50, and 20 mg of glucose/liter cultures were obtained by appropriate dilution of portions of the 1,000 mg/liter culture (arrows) into prewarmed medium without carbon source. (B) Growth of the wild type CP 366 with 50 and 100 mg of glucose/liter, respectively.*

OD measurements, and several of the values in Table 1 are therefore upper limits.

The characteristics of the *amino acid uptake* were obtained from the rates of incorporation into protein of radioactive amino acids added at different concentrations to exponentially growing glucose cultures (Fig. 4 and 5). The half-saturation constants for the uptake of several amino acids were estimated from such labeling kinetics (Table 2), and 20- to 50-fold increases were registered in agreement with the qualitative results obtained with the substrate gradient test. From the reciprocal plots, it appears that the maximal rate of incorporation of leucine or histidine in glucose cultures with a doubling time of 43 min is only about 50% of the theoretical maximal uptake rate (V_{max}) found by extrapolation (Fig. 4C and 5). The K_m for leucine uptake was determined independently by growth rate measurements on a leucine-requiring derivative of CP 367 at different leucine concentrations. Identical K_m estimates were obtained with the two methods (Fig. 5).

Finally, the dependence of growth rate on the external concentration of different ions was studied. For SO_4^{2-} and PO_4^{3-} a definite increase

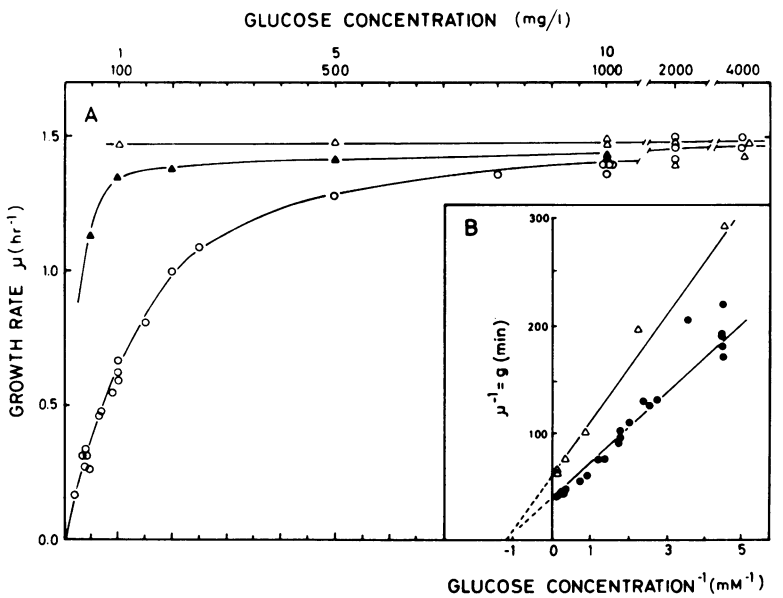

FIG. 2. *Relationship between glucose concentration and growth rate for CP 366 and CP 367. (A) Growth rates in doublings per hour, at 37 C, plotted as a function of the glucose concentrations. The upper scale (0 to 10 mg of glucose/liter) refers to cultures of the wild-type CP 366 (▲) in which the growth rates were determined with a Coulter counter. The lower scale (0 to 4,000 mg of glucose/liter) refers to CP 367 (○) and CP 366 (△) growing at glucose concentrations at which growth was measured in terms of OD_{450}. (B) The data from A for growth of CP 367 at 37 C (●), and additional data for growth at 30 C (△), plotted as μ^{-1} (doubling time, g, in minutes) versus (glucose concentration)$^{-1}$. At both temperatures, the K_m for growth of CP 367 on glucose is estimated at 7 $\times$ 10^{-4} M.*

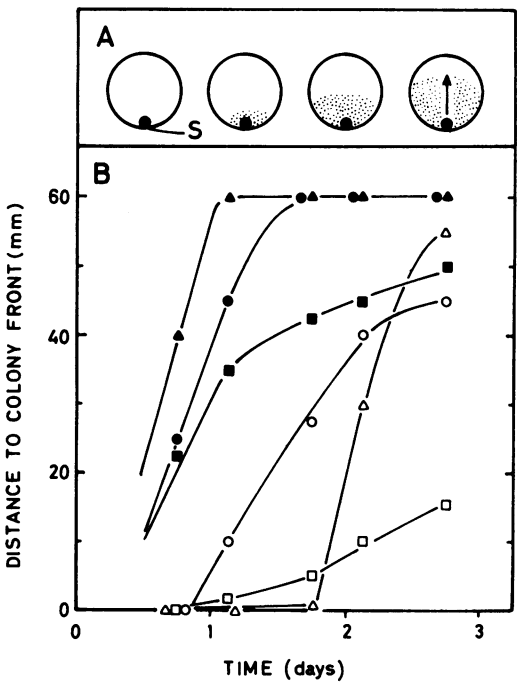

FIG. 3. *Substrate gradient test of the growth of CP 366 and CP 367 with different substrates. (A) Schematic presentation of the substrate gradient test. S, substrate spot. The small dots indicate colonies which, as incubation is prolonged, become visible at increasing distance (arrow) from S. (B) Progression of the colony front (as distance from the substrate spot) with time of wild-type CP 366 (filled symbols) and CP 367 (open symbols) with glucose (○), lactose (□), or proline (△) as substrate.*

of the apparent K_m for growth (or uptake) was observed, whereas for the cations K^+, NH_4^+, and Mg^{2+} there was no difference between wild type and mutant (Table 3).

A case of special interest is the *growth on lactose*. As in the wild type, the K_m of the mutant for growth on lactose is considerably higher than for any other sugar (Table 1). With strain CP 367, a lactose concentration of 1% is required to reach the growth rate of the wild type; at lower concentrations, the growth rate is reduced (Fig. 6A), and, as in the case of glucose, the μ^{-1} versus S^{-1} relationship is approximately linear (Fig. 6B).

The high K_m value for lactose is not due to impaired function of the *lac* operon, resulting in low levels of β-galactosidase or M-protein (12). Addition of isopropyl-β-D-thiogalactoside (5×10^{-4} M) to a culture growing in 0.15% lactose with a growth rate of 0.5 doublings/hour increases the β-galactosidase level about fivefold but does not affect the growth rate. Analogous results were obtained by comparing the growth on L-arabinose of CP 367 (*araC*$^+$) with that of a derivative of CP 367 (*araC*^{c}) constitutive for the arabinose enzymes, the specific permease, and the binding protein (39).

The lactose concentrations at which the growth rates are greatly reduced are still comparatively high (e.g., 0.15% for a doubling time of 120 min). At this low growth rate, a cell density of 3×10^8 cells/ml can therefore be reached without μ changing significantly as a result of substrate consumption.

Revertants. Cultures of strain CP 367 can be maintained in media with rate-limiting substrate concentrations only as long as they remain genetically stable. Under these conditions, wild-type revertants would have an obvious selective advantage and would soon outgrow the mutant.

To estimate the reversion frequency, cultures grown up from a single colony in 1% lactose were used to inoculate 30-ml volumes of minimal medium with 0.08% lactose. The initial density was approximately 10^7 cells/ml, and the doubling time was about 240 min. Usually, μ began to increase after about 20 hr, indicating that fast growing revertants were taking over. The reversion frequency was calculated to be no higher than 10^{-8}/generation. Thus, cultures with lim-

TABLE 1. *K_m for growth on carbohydrates of wild-type CP 366 and mutant CP 367 and uptake of nonmetabolizable sugars*[a]

Substrate	CP 366	CP 367
D-Glucose	10^{-6}	7×10^{-4}
Glucose-6-phosphate	$\leq 2 \times 10^{-6}$	2×10^{-3}
D-Galactose[b]	10^{-4}	3×10^{-3}
D-Fructose	$\leq 5 \times 10^{-6}$	10^{-3}
Lactose	5×10^{-5}	7×10^{-3}
L-Arabinose	$\leq 5 \times 10^{-6}$	3×10^{-4}
Glycerol	5×10^{-6}	5×10^{-5}
α-Methylglucoside (α-MG)[c]	0.5×10^{-4}	2×10^{-3}
Thiomethylgalactoside (TMG)[c]	2×10^{-4}	7×10^{-3}

[a] Values expressed as moles per liter.

[b] The μ versus S relationship for galactose is complex, probably due to the multiple galactose transport systems in *E. coli* (36).

[c] Cells were grown in AB minimal medium supplemented with thymine (10 μg/ml) and 0.2% glycerol at 25 C, for the determination of TMG uptake in presence of 10^{-3} M IPTG. At $OD_{450} = 1.0$, ^{14}C-labeled (0.2 mCi/mmole) α-MG and TMG, respectively, were added at various concentrations to portions of the cultures. At intervals of 10 sec, the cells from 0.5-ml portions were collected on membrane filters and washed with cold growth medium. The initial rates of uptake of radioactivity as a function of the concentration of the respective compound were analyzed as described for the amino acid uptake.

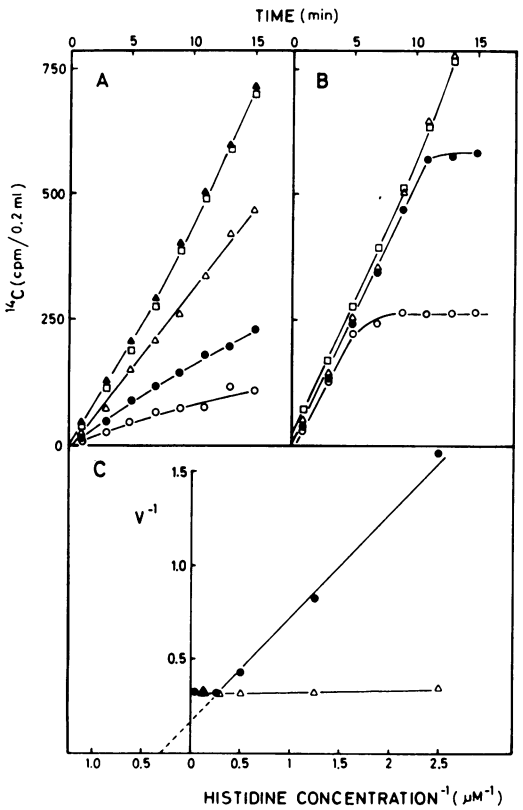

FIG. 4. *Histidine uptake by CP 366 and CP 367. Incorporation of ^{14}C-L-histidine (4.5 mCi/mmole) by the mutant CP 367 (A) and the wild type CP 366 (B), respectively, at 5 (▲), 1 (□), 0.25 (△), 0.1 (●), and 0.05 (○) μg of histidine/ml. At the time of histidine addition, the OD_{450} values of the cultures were 0.175 (CP 367) and 0.205 (CP 366), respectively. (C) Graphical estimation of the K_m for histidine uptake. The rates of histidine incorporation (V) are calculated as micromoles per (gram of protein × minute) from the incorporation data in A and B and plotted as V^{-1} versus the reciprocal of the histidine concentration. K_m for histidine uptake: CP 366 (△) ≤ 1.5 × 10^{-7} M; CP 367 (●) = 3 × 10^{-6} M. The extrapolated V_{max} for histidine uptake by CP 367 is 6.5 μmoles per (gram of protein × minute).*

iting lactose concentrations and with μ values as low as 0.2 to 0.25 remain stable for several doubling times. For most purposes, a culture may be considered to have reached a steady state of growth after three to four mass doublings at constant μ.

To test whether the increased K_m value for the uptake of the sugars, amino acids, and anions is due to a single mutation, 25 clones of CP 367 were grown in 10-ml volumes of AB medium with 0.05% lactose (doubling time, about 400 min). After 40 hr, these cultures were diluted 1/50 into the same medium; 30 hr later, all except two of the cultures were strongly enriched for revertants, as judged from their growth rate in the low lactose medium. Each "reverted" culture was streaked on broth plates, and isolated colonies were tested on lactose gradient plates. Some of the isolated clones appeared to have K_m values for growth on lactose as low as the wild type;

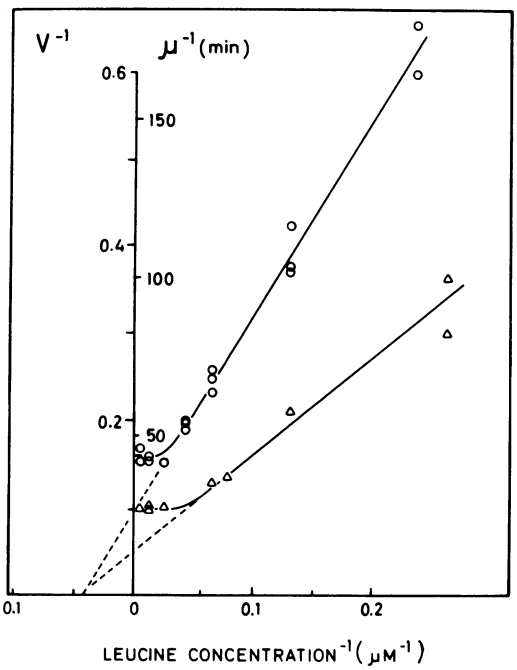

FIG. 5. *Uptake of leucine by CP 367 (△) and growth of CP 367 leu⁻ on leucine (○), plotted as in Fig. 4C and 2B, respectively. The K_m for leucine uptake as well as for growth on this amino acid is estimated at 2.5 × 10^{-5} M. Extrapolated V_{max} for leucine uptake = 18 μmoles per (gram of protein × minute).*

TABLE 2. *K_m for the uptake of L-amino acids, uracil, and uridine by wild-type CP 366 and mutant CP 367[a]*

Substrate	CP 366	CP 367
Arginine	10^{-7}	4×10^{-6}
Histidine	$\leq 1.5 \times 10^{-7}$	3×10^{-6}
Leucine[b]	$\leq 4 \times 10^{-7}$	2.5×10^{-5}[b]
Phenylalanine	10^{-5}	
Proline	2×10^{-7}	4×10^{-6}
Threonine	$\leq 2 \times 10^{-7}$	8×10^{-6}
Uracil	2.2×10^{-7}	9×10^{-7}
Uridine	1.6×10^{-6}	1.6×10^{-6}

[a] Values expressed as moles per liter.

[b] K_m for leucine uptake of the mutant determined from the rates of ^{14}C-leucine incorporation at different concentrations is the same as has been estimated from the dependency of the growth rate of a *leu⁻* derivative (*see* Fig. 5) on the leucine concentration.

TABLE 3. *K_m for the uptake (respective growth on) of ions by the wild type CP 366 and the mutant CP 367*[a]

Substrate	CP 366	CP 367
PO_4^{3-}.....	$\leq 4 \times 10^{-7}$	4×10^{-5}
SO_4^{2-}	$\leq 10^{-7}$	4×10^{-6}
NH_4^+	$\leq 5 \times 10^{-6}$	$\leq 5 \times 10^{-6}$
K^+	$\leq 2 \times 10^{-6}$	$\leq 2 \times 10^{-6}$
Mg^{2+}	10^{-6} to 2×10^{-6}	10^{-6} to 2×10^{-6}

[a] Values expressed as moles per liter. Cultures of CP 366 and CP 367 were grown in Tris-buffered minimal medium with 0.2% glucose at different concentrations of the respective ions. The K_m for growth on the different ions was estimated graphically (Fig. 2B).

others were intermediate between wild type and the original mutant (CP 367). This reversion of the K_m for lactose was invariably paralleled by reversion of the K_m for growth on glucose and on proline; furthermore, the degree of reversion seemed to be the same for all three carbon sources. Reversion seems, therefore, to be as pleiotropic as the original mutation, and phenotypically it can be partial as well as complete.

Genetic crosses have been made by using the three Hfr strains of *E. coli* B/r isolated by Boyer (3) to map the mutation in CP 367. Direct screening of the mutant phenotype was not practicable, and time-of-entry experiments could therefore not be performed. However, linkage analysis indicates that the mutation maps between *str* A (64 min) and *met* B (76.5 min) on the *E. coli* map (42).

DISCUSSION

Nature of the mutation. The characteristics of mutant CP 367 can be summarized as follows. The half-saturation constant for growth on or uptake of glucose, for which the selection was made, all other sugars tested, many amino acids, and some ions is increased 20- to 1,000-fold. This multiple effect is presumably the result of a single mutation. The mutant grows at wild-type rate if the substrate concentration is 10 or more times the K_m value, and the yield in terms of mass per unit of substrate is unaffected.

A mutation affecting an early step of glycolysis is excluded: first, because the K_m for growth on carbon sources metabolized quite differently from glucose (glycerol, acetate, succinate) is changed, and, second, because the mutation affects the apparent K_m for the uptake of various amino acids and ions. Therefore, the transport of the substrates through the cell membrane, not their subsequent metabolism, seems to be affected.

The incorporation measurements probably yield good estimates of the true rates of uptake and half-saturation constants for amino acid transport; the K_m and V_{max} values (18 μmoles per g of protein per 1 min) for leucine uptake agree with data obtained with a different method for *E. coli* K-12 (33). The exact meaning of the K_m for sugars, etc., is less clear, because the data were obtained by growing cells at different substrate concentrations, and hence at different rates and in different physiological states. However, the K_m values for the uptake of the nonmetabolizable sugars, α-methylglucoside and thiomethylgalactoside, are also increased 20- to 50-fold in the mutant (Table 1). I therefore consider the K_m values for growth on sugars as useful estimates of the true K_m value for transport, i.e., the first step of the metabolism of any substrate.

Alternatively, the general requirement for high substrate concentrations could be due to leakiness in strain CP 367. A leaky mutant would suffer a constant loss of amino acids and other metabolites, and its maximum growth rate would be expected to be less than that of the wild type. However, strain CP 367 grows as rapidly as the parent strain when the substrate concentration is high enough, and the medium in which it has grown contains no more material absorbing at 280 nm than is present in the supernatant fluid of a culture of the parent strain. Also, the fact that identical estimates of the K_m are obtained (i) from the incorporation rates of ^{14}C-leucine at different concentrations at maximal growth rate and (ii) from the dependence of μ on the external leucine concentration in a requiring strain points against leakiness, since the internal concentration of leucine on which the counterflow would depend obviously is different in the two experiments. In the former it is adequate to sup-

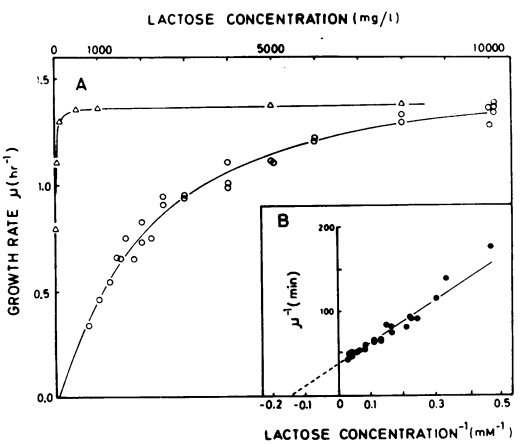

FIG. 6. *Growth of CP 366 and CP 367 with lactose as carbon source. (A) Growth rates (doublings per hour) of CP 366 (Δ) and 367 (O) as function of the lactose concentration. (B) Reciprocal plot of the data for CP 367 from which the K_m for growth on lactose is estimated at 7×10^{-3} M.*

port maximal growth rate, whereas in the latter it is the leucine concentration in the cells which limits the growth rate. Finally, an increased counterflow apparently does not yield the observed μ versus S relationship, as indicated by the results obtained with a mutant of *E. coli* ML 308 which appeared to be leaky for galactosides (47).

No permease, transport protein, or other factor has yet been described in which a mutational change could be expected to exert the pleiotropic effect on transport seen in strain CP 367. The proteins of the phosphotransferase system have been shown to be involved in the active transport of carbohydrates [see review by Roseman (34)]. They are specific either (i) for a single sugar and under substrate-specific control (enzyme II complex, factor III), or (ii) for a more or less defined group of carbohydrates (enzyme I, HPr), which does not include glucose-6-phosphate and pentoses (44) for which the K_m is markedly increased in our mutant (Table 1). Also, enzyme I and HPr lesions have been mapped at 46 min (45), whereas the mutation in CP 367 maps between *strA* and *metB*. Various proteins, which bind different substrates with high specificity, have been isolated from the supernatant fluid of osmotically shocked cells (16) and appear to be located in the periplasmic space between the cytoplasmic membrane and the cell wall (15, 27, 32). Dissociation constants of 10^{-7} to 10^{-6} M have been reported for binding proteins specific for leucine (1, 33), arginine (46), galactose and glucose (1), arabinose (39), SO_4^{2-} (31), and PO_4^{3-} (22). No common element seems to govern the synthesis of this class of proteins since substrate-specific control of the synthesis of the leucine- and arabinose-binding proteins has been reported (33, 39). With arabinose constitutive (*araC*^{c}) derivatives of strains CP 366 and 367, we could show that they produce arabinose-binding protein in similar amounts and with comparable K_m values; in contrast, the K_m value for growth on L-arabinose is 100-fold increased in the mutant (Table 1).

By definition, transport proteins are situated on or in the cytoplasmic membrane, i.e., they are structurally more or less fixed. All transport thus takes place in a highly complex protein-lipid membrane structure, and any mutation affecting the cooperativity in the system may have pleiotropic effects. A mutant has been isolated with a requirement for fatty acid for lactose transport (11), suggesting that the lactose permease, the M-protein of Fox and Kennedy (12), has to combine with fatty acids to associate with the membrane in the proper active configuration. Similarly, the function of a whole class of specific binding proteins might be impaired by a change in a structural membrane protein, or by a mutation affecting membrane assembly or the interaction between cell wall and membrane. In fact, it might be impaired by any change in the environment in which the binding proteins function.

The structure of the cell membrane or wall, or both, may actually be changed in strain CP 367 since these cells are slightly bigger and more heterogenous in size than wild-type cells. Moreover, ethylenediaminetetraacetic acid (EDTA, 10 mM) causes lysis of the mutant, but not of the wild-type cells. (The other three mutants isolated together with CP 367 show similar pleiotropic increases of their K_m values; however, they are not EDTA sensitive and the cells are unusually big.) This EDTA sensitivity reverts together with the other characteristics. We first envisaged a change of the cell envelope (membrane or wall; 29) which would result in loss of the binding proteins, but no arabinose-binding activity could be demonstrated in the supernatant fluid of CP 367 *araC*^{c} cultures. Alternatively, a mutational change of the cytoplasmic membrane might interfere with the passage of the binding proteins from the cytoplasm where they presumably are produced into the periplasmic space. However, arabinose-binding activity could be released by osmotic shock (28) from the *araC*^{c} derivatives of both CP 366 and 367 without concomitant loss of β-galactosidase, indicating that in both strains the binding proteins are located in the periplasmic space. [The *araC*^{c} derivatives were tested by Robert Schleif who also carried out some of the arabinose-binding protein determinations with the equilibrium dialysis assay (39).]

By varying the degree of osmotic shock, it was found that the arabinose-binding protein is easier to release from the mutant than from the wild type (Fig. 7). This could mean that the binding proteins are relatively loosely attached to the periplasmic membrane in the mutant. This in turn could impair their function which presumably is to trap substrate molecules and turn them over to the transport chain.

The mutation in strain CP 367 seems not to affect the outer surface of the cell, since the mutant can be efficiently synchronized by the Helmstetter-Cummings membrane elution technique (14), which requires that the cells stick to certain membrane filters; *E. coli* B/r, from which CP 367 is derived, sticks particularly well.

Since the functional analysis shows that the K_m of the mutant for the transport of a wide spectrum of nutrients is increased without revealing the exact nature of the mutational change, I designate its genetic locus *kmt*.

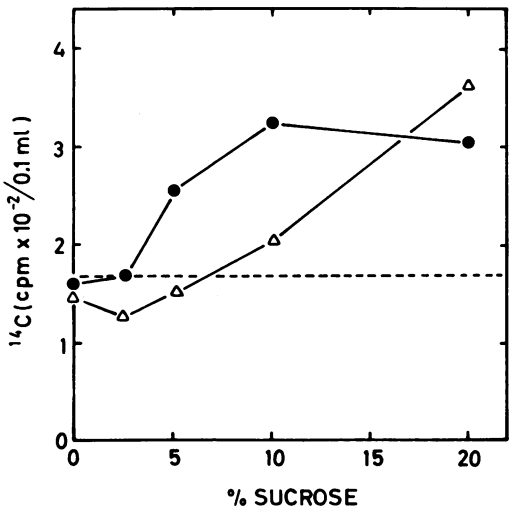

FIG. 7. *Release of L-arabinose-binding activity from wild-type CP 366 and mutant CP 367 by different degrees of osmotic shock. Arabinose constitutive (araC^c) derivatives of CP 366 and CP 367 were grown in AB minimal medium with 0.4% glycerol to an OD_{450} of 2.8, at which 100 ml of each culture was centrifuged. The cells were washed, and five portions of each strain were osmotically shocked as described by Schleif (39), except that the sucrose concentration was 0, 2.5, 5, 10, and 20%, respectively, during the incubation at 22 C in 1 ml of 0.03 M Tris-hydrochloride (pH 7.3)-10^{-4} M EDTA; the cells were then centrifuged and resuspended in 0.6 ml of ice-cold 10^{-4} M $MgCl_2$. We vary the degree of osmotic shock in proportion to the sucrose concentration (abscissa). Volumes (0.3 ml) of the shock fluids of CP 366 araC^c (△) and CP 367 araC^c (●) were dialyzed for 90 min at 4 C against 3 × 10^{-7} M ^{14}C-l-arabinose (3 mCi/mmole); ^{14}C counts per minute were determined in 0.1-ml volumes of the dialyzed samples and dialysis buffer (dashed line). Counts in the shock fluids exceeding the counts in the buffer reflect the amount of arabinose-binding protein released by osmotic shock.*

Application of the mutant to growth studies. The increase of the K_m for the uptake of many substrates makes strain CP 367 extremely useful for the study of several problems related to bacterial growth. Steady-state growth up to relatively high cell densities can be obtained at growth rates as low as 0.2 doublings/hour by limiting the concentration of a single substrate. It is obvious that, under such conditions, the substrate concentration and, thus, the growth rate, must decrease continuously as the culture grows. From the relationship between substrate concentration and μ (determined at low cell densities) and the yield in cell mass per milligram of substrate, we can calculate the cell density below which μ will remain within, e.g., 10% of the value characteristic of the initial substrate concentration. At low cell densities, a lactose concentration of 0.13% generates a μ value of 0.6, and, below an OD_{450} value of 0.550, $\mu \geq 0.5$. The corresponding figures for a lactose concentration of 0.07% are: $\mu = 0.3$ at low cell densities, and $\mu \geq 0.24$ below an OD_{450} value of 0.220. As indicated by the K_m values in Table 2, the corresponding substrate concentration and upper limits are 10 times lower when glucose is used as carbon source. Higher cell densities may be reached, but this requires carefully calculated and timed addition of substrate to keep μ within the desired interval. Furthermore, experiments involving prolonged growth at low rates are only possible if the level of revertants is kept low by using as inoculum a freshly isolated clone.

The RNA/DNA and RNA/protein ratios for strain CP 367 are shown in Fig. 8; they represent batch cultures in which the growth rate was defined by the lactose concentration. The results agree quite well with the data of Rosset et al. (35) and Forchhammer and Lindahl (10) on the composition of *E. coli* as a function of the growth rate when the latter depends on the actual substrate used, and not on its concentration. The overall composition of the cell thus varies similarly with the growth rate, whether this is determined by the rate at which a given substrate

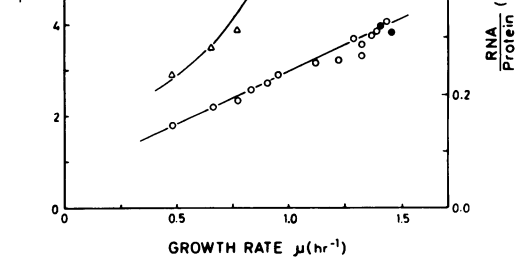

FIG. 8. *Cell composition of the mutant CP 367 as function of the growth rate. RNA/DNA (△) and RNA/protein (○) ratios were determined on cell samples harvested from batch cultures of CP 367 growing at different lactose concentrations. Wild-type CP 366 (▲, ●) growing on 0.2% lactose has the same cell composition as CP 367 with 1% lactose.*

is taken up or by the efficiency with which a given substrate can support catabolism. This suggests that the growth rate is controlled at a level at which it does not matter whether one or another set of enzymes serves to maintain the necessary flow of carbon and energy; a model which satisfies this condition has recently been proposed (20).

The general correspondence between growth rate and cell composition is particularly relevant for experiments involving rate changes. With strain CP 367, a simple change of the substrate concentration (by dilution or addition) will produce shifts which do not involve derepression or repression of the synthesis of new substrate specific enzyme systems. A shift-down of this kind is illustrated in Fig. 1. The dilution with minimal medium, without substrate, replaces the otherwise necessary filtration, washing, and resuspension of the culture. This simple procedure also has the advantage that the new growth condition (i.e., the new substrate concentration) is immediately established. In chemostat cultures, a shift performed by reducing or increasing the medium flow rate introduces a prolonged transient state (26), because in this case the substrate concentration is dependent on the growth response of the cell population.

Strain CP 367 also lends itself to studies of phosphate- and sulfate-limited growth rates; with suitable auxotrophs, amino acid-limited growth rates can be maintained in batch culture at useful cell densities. In this laboratory, the mutant has been successfully applied to studies of cell size as a function of lactose- or leucine-limited growth rates, yielding results similar to those obtained by Schaechter et al. (37) for *Salmonella typhimurium*. Finally, the timing of DNA synthesis in the division cycle at doubling times up to 250 min has been investigated by Jesper Zeuther (*unpublished data*) with the membrane elution technique of Helmstetter and Cummings (14).

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