Evolution of *Escherichia coli* During Growth in a Constant Environment

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ABSTRACT

Populations of *Escherichia coli*, initiated with a single clone and maintained for long periods in glucose-limited continuous culture, developed extensive polymorphisms. In one population, examined after 765 generations, two majority and two minority types were identified. Stable mixed populations were reestablished from the isolated strains. Factors involved in the development of this polymorphism included differences in the maximum specific growth rate and in the transport of glucose, and excretion of metabolites by some clones which were utilized by minority clones.

THE classical model of the evolution of asexual populations such as microorganisms, was proposed by MULLER (1932) and has remained essentially unchanged for more than 50 yr (CROW and KIMURA 1970). The model postulates that asexual populations consist of a single clone, except for those periods when a population experiences an adaptive shift and one clone is replaced by a second with a higher fitness. This model, coupled with the pervasive view that the (often) haploid nature of asexual microorganisms, and their virtual absence of recombination should preclude the development of any "interesting" features, such as the development of the large amounts of genetic variation found in sexually reproducing diploid organisms (HARRIS, HOPKINSON and EDWARDS 1977; BROWN 1979; COYNE 1982), may account for the lack of attention afforded such populations by population geneticists and evolutionary biologists.

Recent studies of the population genetics of *Escherichia coli* and yeast have suggested that these conceptions may not be correct. In natural populations, a number of studies have shown that the levels of genetic diversity among strains of *E. coli* are at least as great as the levels of genetic variation found in eukaryotes (HARTL and DYKHUIZEN 1984 and references therein). In laboratory populations growing in simple defined media, clonal replacements may occur so frequently that periods of stasis when no adaptive change is occurring may be small (ADAMS and HELLING 1983; PAQUIN and ADAMS 1983a; ADAMS *et al.* 1985, ADAMS and OELLER 1986). In addition, adaptive clones isolated from evolving populations exhibit a high degree of epistatic interaction (PAQUIN and ADAMS 1983b).

It is already well-known (NOVICK and SZILARD 1950; ATWOOD, SCHNEIDER and RYAN 1951; DYKHUIZEN and HARTL 1981; ADAMS *et al.* 1985) that adaptive mutants appearing during continuous growth under limiting substrate conditions, exhibit increased maximum specific growth rates (μ_{max}) and/or increased ability to grow under conditions of limited substrate availability [lower K_s; see *e.g.*, KUBITSCHEK (1970)]. These results are consistent with the theory of the dynamics of growth and reproduction in continuous culture under nutrient-limiting conditions (chemostat culture) (KUBITSCHEK 1970) which predicts selection of a single dominant phenotype optimized for the uptake and utilization of the limiting substrate. However, we are aware of only one study (NOVICK and SZILARD 1950) of a population which was maintained for more than 300 generations.

In this communication we analyze the evolution of populations of *E. coli* initiated with a single clone, and grown in glucose-limited chemostat culture for up to almost 1900 generations. Although organisms with improved growth characteristics were indeed selected, no one optimum clone appeared to be predominant in any population. In one population analyzed in detail, a polymorphism had developed after 800 generations, consisting of two mutant types in high frequency and at least two minority classes. Factors involved in establishing this polymorphism included differences in the maximum specific growth rate and in the transport of glucose, and the excretion of incompletely respired metabolites by some clones, which could be utilized by other, minority clones.

MATERIALS AND METHODS

Media and culture conditions: Tryptone broth (TB) contained 5 g NaCl and 10 g tryptone (Difco) per liter. Minimal salts medium (HELLING, KINNEY and ADAMS 1981) contained salts and thiamine HCl (1 μg/ml). When present, D-glucose was added to 0.0125% w/v for chemostats, 0.025% for batch cultures. Growth was followed by measuring optical density at 550 nm (A_{550}) in a Bausch and Lomb Spectronic 100 spectrophotometer. Batch cultures used for determining growth constants were initiated by dilution to a starting A_{550} of about 0.01 from cultures grown overnight in the same medium. Determination of growth rates was as

previously described (ADAMS *et al.* 1979; HELLING, KINNEY and ADAMS 1981). Batch cultures of strain CV103 were frequently overgrown by faster growing mutant derivatives. Therefore in all experiments involving batch culture, multiple replicates of that strain were initiated from different colonies. At the end of growth, samples were streaked from each culture to tryptone agar (TA) to test for the possible growth of contaminants or revertants.

Long-term chemostats were inoculated with a single clone of one of six different strains (see Tables 1 and 2) and maintained at 30° in minimal medium (HELLING, KINNEY and ADAMS 1981) in aerated culture vessels 145–190 ml in volume, at a dilution rate $D \simeq 0.2$ hr^{-1}, equivalent to a cell generation time of approximately 3.5 hr (KUBITSCHEK 1970). Under these conditions growth of the cells is limited by the concentration of glucose, and population densities were $\simeq 1.2 \times 10^8$ cells/ml, resulting in total population sizes of $1.7–2.4 \times 10^{10}$. The populations were maintained in this constant environment for up to 1867 generations, though occasionally the entire population was transferred to a new culture vessel to reduce any effects due to wall growth. Populations were sampled every 7–20 generations throughout the course of the experiment, plating directly onto TA to obtain estimates of population density. The resulting colonies were replicated onto TA plates containing ampicillin (40 μg/ml) to estimate the frequency of plasmid-containing organisms. Previous experiments had shown that the organisms in such ampicillin-resistant colonies invariably contained a plasmid (HELLING, KINNEY and ADAMS 1981). Periodically, an aliquot of the cell suspension was frozen in 40% glycerol and stored at −70° for later analysis.

The frequency of T5-resistant mutants was determined by mixing samples from the chemostat population with a suspension of bacteriophage T5 at a multiplicity of 50, and $CaCl_2$ present at 5 mM. The suspensions were maintained on ice for 15 min to allow adsorption, spread onto TA containing 5 mM $CaCl_2$ and at least 5×10^8 T5 per ml, and incubated at 37° overnight.

The frequency of small colony variants was estimated by scoring the plates after 24 hr of growth. In order to obtain precise estimates of their frequency it was necessary to restreak individual colonies, as the colonies of some of the S mutants eventually achieved normal size if given sufficient time, and because of overgrowth by faster growing variants originating from S mutants during growth on the plate. Nevertheless, these frequencies may be underestimates as the efficiency of "S" colony formation may have been less than one (0.75 in the case of CV103 when plated from batch culture, but approximately one for CV101, CV115 and CV116).

Detailed procedures for the competition experiments have been described (HELLING, KINNEY and ADAMS 1981). These chemostat cultures were aerated and maintained at 30° with culture volumes fixed between 150 and 190 ml and a constant dilution rate of 0.2 hr^{-1}. The frequency of plasmid-containing organisms was estimated by measuring the frequency of ampicillin-resistant cells as described above. Arabinose-resistant derivatives were obtained by plating on EMB (eosin-methylene blue) arabinose medium. L-Arabinose is toxic to strains containing the *araD139* mutation. These arabinose nonutilizing but resistant mutants have second mutations in the *araA*, *araB* or *araC* genes (BOYER, ENGLESBERG and WEINBERG 1962). In experiments involving mixtures of arabinose-resistant and arabinose-sensitive strains, the frequency of arabinose-resistant organisms was estimated by replicating colonies appearing on TA to TA + arabinose (0.1% w/v), and scoring growth after 5–8 hr at 37°. Control experiments showed that none of the mutations to arabinose-resistance exerted a selective effect in glucose-limited chemostat culture.

Growth in spent culture media: Organisms were grown into stationary phase in minimal salts + glucose (0.2% w/v or 0.025% w/v) medium at either 30° or 37°. The cells were removed by filtration through 0.45 μm nitrocellulose membrane filters, and resuspended in the spent medium (filtrate). The cultures were shaken at 30° or 37°. Growth was followed by measuring the optical density at 420 nm rather than 550 nm in order to increase sensitivity at low cell densities (see *e.g.*, STANIER *et al.* 1986, p. 187). Neither temperature nor glucose concentration affected the general pattern of results obtained.

Active transport studies: Organisms were grown in shaking flasks overnight at 30° in minimal salts + glucose (0.2%) medium. The cultures were diluted 10-fold in the same medium and grown for another 2 hr. The cells were harvested by filtration, washed with minimal salts medium (lacking glucose), resuspended in minimal salts medium to an absorbance of 0.5 at 550 nm (0.45 mg dry weight ml^{-1}), and maintained on ice. Chloramphenicol succinate was added to a final concentration of 170 μg ml^{-1}, temperature was equilibrated at 30°, and uniformly labeled ^{14}C-α-methylglucoside (αMG) (specific activity approximately 18.6 becquerels nmol^{-1}) was added to the desired concentration. One-milliliter samples were filtered on 0.45 μm nitrocellulose filters, and washed twice with 1 ml of minimal salts medium at 30°. Velocities were determined from the regression of the amount of αMG incorporated against time of sampling (at 0.15, 0.3 and 0.45 min after addition of label), passing through the origin. K_m values are least-squares estimates, determined from the linear Lineweaver-Burk form of the Michaelis-Menten equation. When transport was measured in the presence of D-fructose, the overnight cultures were diluted in minimal salts + fructose (0.1%). After 1–1.5 hr growth on fructose, the cells were harvested and resuspended in minimal salts + fructose (0.01%), and αMG uptake was measured as above. Fructose was chosen as the energy source in these experiments because CV103 utilized it better than other possible substrates.

In experiments involving the addition of azide, the cultures were diluted and grown in fructose-containing medium as described above. The cell suspensions were in minimal salts plus fructose (0.01%) at an A_{550} of 0.25. At time 0, ^{14}C-αMG was added to a final concentration of 10 μM. After 3 min sodium azide was added to a final concentration of 20 mM.

RESULTS AND DISCUSSION

Adaptation of a population to a glucose-limited environment: Figure 1 shows the frequency of T5-resistant mutants over the first 800 generations in a population initiated from a single clone of strain JA209 (Table 1; Table 2, experiment 4), and grown in minimal medium in a continuous culture apparatus operating as a chemostat at a generation time of approximately 3.5 hr with growth limited by the concentration of glucose. Each fluctuation in frequency marks the occurrence and fixation of an adaptive clone in the population ("adaptive shift"). Under our experimental conditions, T5-resistant mutants are approximately selectively neutral (KUBITSCHEK 1970; DYKHUIZEN and HARTL 1980). Thus, the frequency of T5-resistant mutants increases during growth solely

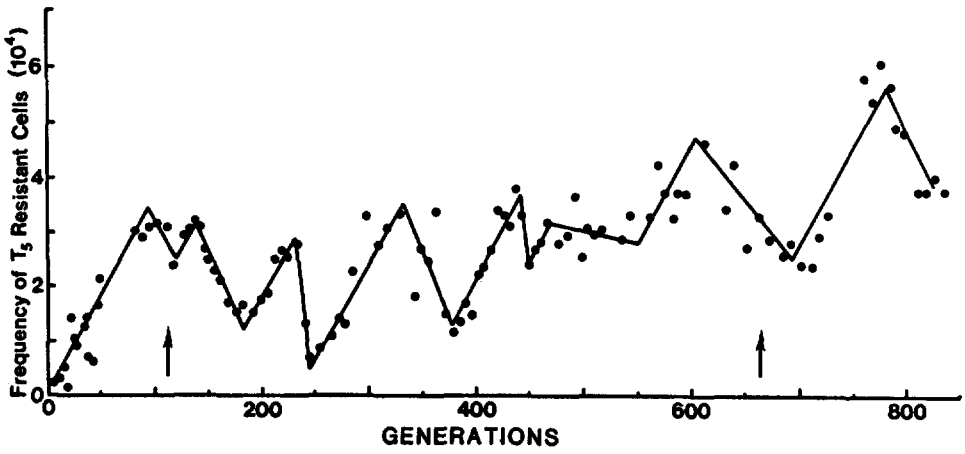

FIGURE 1. Fluctuations in the frequency of T5-resistant mutants during growth of the strain JA209 (experiment 4; Table 2). The line is drawn so as to correspond to the fixation of eight adaptive mutations. At the times indicated by *arrows*, a sample was transferred to a different chemostat and growth was continued.

as the result of recurrent mutation to T5 resistance. Periodically, adaptive mutations conferring a growth advantage occur and since the frequency of T5 resistant mutants is still extremely low, these adaptive mutations will almost invariably occur in cells which are T5-sensitive. As those adaptive mutants increase in the population, all other cells, including those which are T5-resistant, decrease in frequency. As the new adaptive clone becomes predominant, recurrent mutation to T5 resistance in this sector of the population will again result in an increase in the frequency of T5 resistant cells. Therefore a plot of the frequency of T5-resistant mutants against the number of generations of growth should show a series of increases and decreases (Figure 1), corresponding to the replacement of one clone by another. This phenomenon was originally termed "periodic selection" by ATWOOD, SCHNEIDER and RYAN (1951). More extensive discussion of the rationale and justification for this procedure are presented elsewhere (NOVICK and SZILARD 1950; PAQUIN and ADAMS 1983a). Eight adaptive mutants were estimated to have become dominant over the course of the first 800 generations (see Figure 1). There was no apparent decrease in the rate at which new adaptive mutants were selected in the population during this period. Furthermore, although changes in the frequency of T5 resistance were more difficult to follow beyond this point, the population continued to experience adaptive shifts until the termination of the experiment at generation 1867 (data not shown).

After 329 generations a polymorphism became apparent in the population. Variants that formed smaller colonies on tryptone agar than JA209 were evident and continued to be present throughout the remainder of the experiment. When organisms from individual small colonies were streaked to new TA plates, they again formed small colonies, although such colonies were frequently overgrown by mutants capable of forming colonies of normal size. The small colony variants (which we term S mutants) were found to be sensitive to λ, T5 and arabinose, indicating that they were indeed derived from the clone used to inoculate the culture at generation 0.

The S variants invariably appeared if a population was carried for a sufficiently long period. The results of 15 experiments, listed in Table 2 show that small colony variants were observed in all cultures maintained for longer than 100 generations. In each of the populations in which they were observed, the S mutants persisted until the end of the experiment, usually fluctuating in the range of 5–80% of the colony-formers, though occasionally their frequency dropped below 1% for periods of time.

In order to understand the bases for the development of the population polymorphism and the small colony phenotype, we focused on a set of clones isolated from one of the long-term cultures (Table 2; experiment 2) when the frequency of S variants was especially high. In this population the S variants attained a frequency of about 85% of the colony-forming organisms by generation 383, and continued to constitute a majority of the population until the isolation of the clones at generation 765. At that time only about 1% of the organisms formed colonies of normal size, and the rest were S mutants. Although the original experiment was terminated by a laboratory fire, shortly thereafter a new continuous culture was initiated from a 1-ml frozen sample taken at generation 765. The new population was maintained for a further 340 generations, with the S variants fluctuating in frequency between 99 and 50% during this period.

Two of the clones isolated were small colony variants (CV103 and CV116) and two were large colony variants (CV101 and CV115) with a colony morphology indistinguishable from JA122, the strain used to inoculate the population. In each group, one of the clones contained a plasmid (CV103 and CV101) and one was plasmid-free (CV115, CV116). Although the original population was initiated with a plasmid-bearing strain, plasmid-free derivatives soon appeared as

TABLE 1

Bacterial strains

Strain	Relevant characteristics	Origin
RH201	F⁻ *thr1 leu6 thi1 lacY1 tonA21 supE44 hss1*	ADAMS *et al.* (1979)
JA100	As RH201 but *leu*⁺ *ace*	Transduction to *leu*⁺, score for *ace*
JA103	As JA100 but *ace*⁺ *tonA*⁺	Transduction to *ace*⁺, score for *tonA*⁺
JA104	As JA103 but *thr*⁺ *araD139*, lysogenic for λ	Transduction to *thr*⁺, score for *araD139*
JA122	As JA104 plus plasmid pBR322Δ5	Transformation with plasmid of HEFFRON *et al.* (1981)
JA209	As JA104, but not lysogenic for λ	As for JA104
JA210	As JA122, but not lysogenic for λ	As for JA122
CV101	Derivative of JA122, isolated from chemostat, contains plasmid	Isolated after 773 generations of glucose-limited culture
CV103	As CV101 but independent isolate which forms small colonies on TA	As CV101
CV105	Arabinose-resistant, Ara⁻ derivative of CV101	Selection for growth on EMB arabinose
CV115	Derivative of JA122 isolated from chemostat, lacks plasmid	As CV101
CV116	Derivative of JA122 isolated from chemostat, lacks plasmid, forms small colonies on TA	As CV101
CV117	Arabinose-resistant derivative of CV115	As CV105
RH434	As JA209 plus plasmid RSF2124	Transformation with plasmid RSF2124 of SO, GILL and FALKOW (1975)

All strains are derivatives of *E. coli* K12. The presence of the nonconjugative plasmids pBR322Δ5 and RSF2124 confer ampicillin resistance. RSF2124 is a derivative of the nonconjugative plasmid ColE1 containing transposon Tn3. JA104 and derivatives are lysogenic for λ, originating from a P1bt transducing lysate grown on a donor strain lysogenic for λ.

TABLE 2

Summary of the long-term continuous culture experiments

Experiment No.	Strain	Duration (generations)	Small variants first seen
1	JA104	773	359
2	JA122	765	340
3	JA209	148	136
4	JA209	1867	329
5	JA209	66	Not seen
6	JA209	58	Not seen
7	JA209	244	92
8	JA209	468	198
9	JA210	90	Not seen
10	JA210	232	187
11	JA210	688	381
12	JA210	537	222
13	JA210	372	88
14	JA220	49	Not seen
15	RH434	304	189

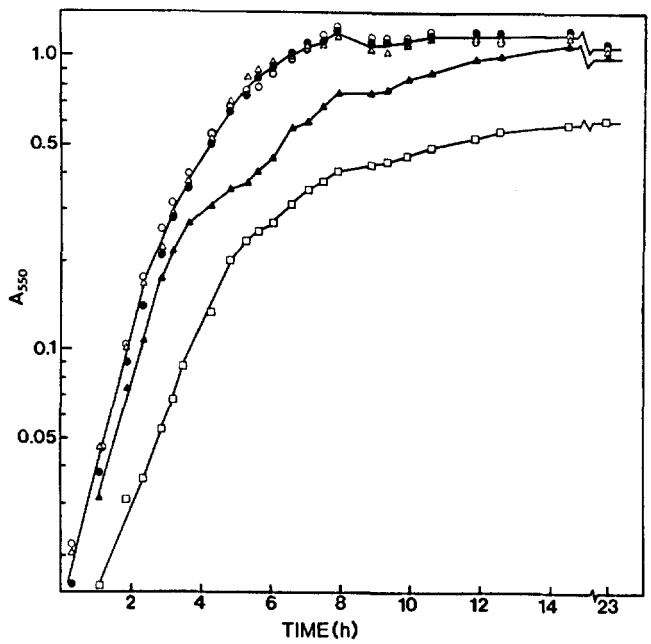

FIGURE 2.—Growth of input strain JA122 and evolved strains on tryptone broth at 30°. Symbols designate JA122 (○); CV101 (△); CV103 (□); CV115 (●); CV116 (▲).

expected (HELLING, KINNEY and ADAMS 1981), and at generation 765 only a minority of the cells contained a plasmid. The presence of a plasmid was not responsible for the difference in colony size as (i) plasmid-containing strains exhibited both colony morphology phenotypes, and (ii) plasmid-free derivatives of the small colony variant CV103 retained the small colony phenotype.

The difference in the growth phenotypes of the small and large variants was confirmed by growth in tryptone broth. Figure 2 shows the growth kinetics of JA122, and of the four clones isolated from the chemostat at generation 765. The large colony variants, CV101 and CV115 (termed L strains), exhibited growth kinetics indistinguishable from each other and from that of the strain used to initiate the population (JA122). However, the two S variants, CV103 and CV116, differed from each other as well as from the other strains. The small variant with a plasmid (CV103) not only grew significantly slower, but also exhibited a growth yield (final cell density) only about 55% of that of the other strains. The small variant without a plasmid (CV116) exhibited a complex growth pattern, showing transitions similar to those sometimes seen when changing substrates (diauxic

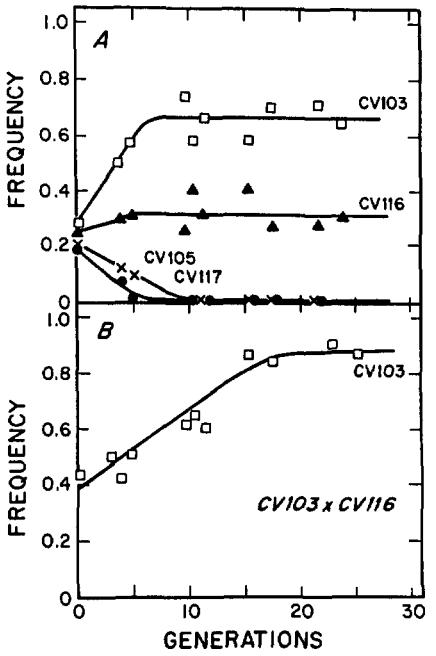

FIGURE 3.—Competition among chemostat-derived strains during growth in glucose-limited continuous culture. (A) Competition among four variants isolated after 773 generations of growth in continuous culture. The small variants are CV103 (□, plasmid-containing) and CV116 (▲, plasmid-free), and the large variants are CV105 (●, plasmid-containing, arabinose-resistant derivative of CV101) and CV117 (×, plasmid-free, arabinose-resistant derivative of CV115). Strains were identified by plating on TA and replicating the colonies to test for ampicillin resistance and for arabinose resistance. Arabinose resistance was shown to have no selective effect in control experiments. (B) Competition between small variants CV103 and CV116.

growth), but eventually achieved the same final cell density as JA122 and the L strains.

Since the two S variants differed in their growth patterns, 54 random clones of small-colony-forming organisms plated from a frozen sample taken at generation 759 were examined in order to determine if CV103 and CV116 were representative of the dominant organisms in the chemostat. Forty-one percent (22 of 54) of the clones resembled CV116 in that they formed colonies of normal size if incubated for a sufficiently long time (2–3 days). The growth of the remainder (59%) resembled that of CV103. We conclude that both CV103- and CV116-like organisms were present at high frequency in the chemostat.

If CV103 and CV116 are indeed representative of organisms predominant in the chemostat at the time sampled, it should be possible to reconstruct the composition of the population at generation 753 by inoculating a new culture with the two S strains and the two L strains. Figure 3A shows the results from such an experiment initiated with the four strains in equal frequency. During growth in competition the S variants (CV103 and CV116) were selected over L variants, consistent with the low frequency of L variants in the original chemostat. Furthermore, as predicted both S strains were maintained during continued growth, an equilibrium being reached after 10 generations with CV103 at a frequency (approximately 65%) not greatly different from the frequency (59%) of CV103-like cells in the original chemostat at generation 753. The initial presence of the L variants had no obvious effect on the creation of this polymorphism, as a similar result was obtained when the S variants were placed in direct competition with each other, without the L variants (Figure 3B).

TABLE 3

Growth rates and relative yields in glucose-containing minimal medium at 30°

Strain	Specific growth rate (hr^{-1}) ± SEM	Relative growth yield ± SEM
JA122	0.44 ± 0.01	1.00
CV101	0.50 ± 0.02	1.14 ± 0.02
CV115	0.55 ± 0.02	1.11 ± 0.02
CV116	0.60 ± 0.01	1.20 ± 0.03
CV103	0.40 ± 0.01	0.81 ± 0.04

Growth rates are least square estimates and are reported together with the standard error of the mean (SEM) for a representative experiment. The growth rate of JA122 is significantly greater than the growth rate of CV103 ($\alpha < 0.01$) as determined using the Wilcoxon Mann-Whitney nonparametric test (*e.g.*, CONOVER 1971) on results from replicate experiments. Similarly the growth rates of CV101, CV115 and CV116 are significantly higher ($\alpha < 0.001$) than that of JA122. Growth rates of CV101, CV115 and CV116 are significantly different ($\alpha < 0.05$) as determined by the nonparametric Friedman test (*e.g.*, CONOVER 1971). Growth yields (±SEM calculated from results for replicate experiments) are expressed as the ratio of A_{550} of the strain relative to that of JA122 at the completion of batch-style growth in medium containing glucose at concentrations up to 0.05%. JA122 attained an A_{550} of 0.16 ± 0.008 on 0.025% glucose (1.39 mM).

Growth kinetics of the evolved strains: The growth rate in chemostat culture is generally considered to be dependent on two parameters, the maximum specific growth rate, μ_{max}, and the saturation coefficient, K_s, the substrate concentration at half the maximum growth rate (KUBITSCHEK 1970; ADAMS *et al.* 1985). The simplest form of this relationship is due to MONOD (1942) who suggested that the kinetics of growth in chemostat culture was analogous to first order enzyme kinetics, namely,

$$\mu = \mu_{max} s/(K_s + s)$$

where μ is the growth rate of the cells within the chemostat, and s is the equilibrium concentration of the limiting substrate in the chemostat.

In order to determine if the strains which had evolved in the chemostat had an increased μ_{max}, the growth kinetics of the four evolved strains and of JA122 were determined during unlimited (batch-type) growth in medium identical to that used in the chemostat experiments, except that glucose was present at double the concentration. Surprisingly, the relative growth patterns (Table 3) were quite different from those in TB. In glucose-minimal medium, three (CV101, CV115, CV116) of the four strains isolated

from the chemostat possessed an increased maximum specific growth rate relative to JA122. Both the growth rate and the yield of the fourth strain, CV103, were lower than those of all the other strains including the input strain. The addition of more glucose led to further growth of each strain, showing that the growth yield in each case was limited by substrate-availability and not by accumulation of toxic products. Our results contrast with those of DYKHUIZEN and HARTL (1981) who found no significant changes in maximum growth rate among strains isolated from glucose-limited continuous cultures. However, their experiments were of comparatively short duration, extending for less than 200 generations.

Improved glucose transport in adaptive mutants: The second parameter involved in determining reproductive rate in continuous culture environments, the saturation coefficient, is a measure of the ability of the organism to sequester the limiting substrate, in this case glucose. Thus, mutations which increase the ability of the organism to scavenge the extremely low quantities of glucose present during chemostat culture would be expected to have a selective advantage. Glucose enters *E. coli* by means of two transport systems (POSTMA, NEIJSSEL and VAN REE 1982). The two systems (IIB^{Glc}/III^{Glc} and II^{Man}) each carry glucose into the organism concomitant with phosphorylation to form glucose-6-phosphate (POSTMA and LENGELER 1985). The first phosphotransferase system is of considerable interest because, in addition to catalyzing the transport of its substrate, glucose, it plays a major role in regulating the activities of other transport systems and in regulating transcription of specific genes (POSTMA and LENGELER 1985; SAIER 1985). In the absence of glucose, the protein III^{Glc} is phosphorylated and stimulates adenylate cyclase to form cyclic AMP, a cofactor required for transcription of genes involved in catabolism of many compounds other than glucose. During unlimited growth on glucose, the protein is largely unphosphorylated, fails to stimulate cyclase, and inhibits transport systems for other compounds such as glycerol and lactose.

In *E. coli* K12 the two transport systems may be assayed separately by using isotopically labeled glucose derivatives not metabolized beyond transport-coupled phosphorylation (POSTMA and LENGELER 1985). Assays of the kinetics of 2-deoxyglucose transport, a substrate specific for II^{Man}, showed no significant differences among the strains (results not shown). In contrast, for each chemostat isolate, the kinetics of transport of αMG, generally used as a specific indicator of IIB^{Glc}/III^{Glc} activity (POSTMA and LENGELER 1985), differed from the kinetics of the starting strain (Figure 4, see dashed lines). The input strain JA122 shows kinetics of transport reflecting a transport system with a K_m for αMG of approximately 0.08 mM, a

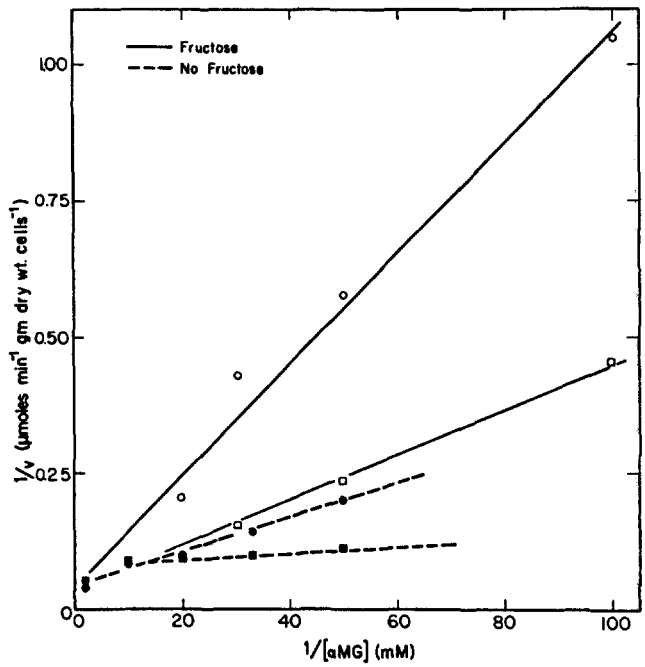

FIGURE 4.—Double-reciprocal plots showing the kinetics of transport of αMG by original and chemostat-derived strains. Circles designate JA122; squares, combined average of CV101, CV103, CV115 and CV116. (Results for the latter four strains did not differ significantly.) *Dashed lines* indicate transport in the absence of an added energy source; *solid lines*, in the presence of fructose.

result similar to that obtained by others (GACHELIN 1972; HERNANDEZ-ASENSIO, RAMIREZ and DEL CAMPO, 1975). The evolved strains have a higher affinity transport system for the αMG ($K_m \approx 0.01$ mM), as shown by the decreased slope relative to that of JA122 (Figure 4). It is reasonable to conclude that the evolved organisms have a transport system with a higher affinity not only for αMG, but also for glucose itself, as these strains were selected during long-term competition for a low concentration of glucose.

These glucose uptake results show that the strains adapted to the chemostat environment are superior to the input strain in their ability to scavenge a low concentration of glucose, but they do not reveal any differences among the chemostat strains. However, when αMG uptake is followed over a longer time, differences are evident (Figure 5). The final pool size in JA122, (before the addition of azide) is less than that of all the chemostat strains tested. The difference between the uptake kinetics of JA122 and those seen for CV101, CV115 and CV116 is not surprising and may be ascribed to the higher affinity transport already demonstrated (Figure 4) for the latter strains. However, the pool size of CV103 is larger than that of any other strain, a result not predictable from the uptake kinetics described earlier.

Since strains grown in aerated glucose-limited chemostat culture actively respire (HARVEY 1970), and since respiration inhibits glucose uptake, the evolved strains were assayed for differences in the regulation

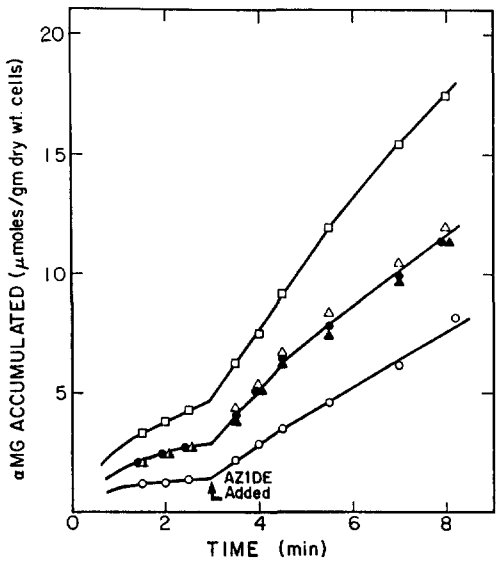

FIGURE 5.—Concentration of αMG in the presence and absence of azide by JA122 and by strains selected in glucose-limited continuous culture. Symbols designate JA122 (○); CV101 (△); CV103 (□); CV115 (●); CV116 (▲). The averaged results from duplicate experiments are shown.

of αMG transport by respiration. Respiration has been shown to inhibit the uptake of glucose (or αMG) via IIB^{Glc}/III^{Glc} by raising the K_m of glucose-uptake (GACHELIN 1972; HERNANDEZ-ASENSIO, RAMIREZ and DEL CAMPO 1975) as the consequence of a high proton-motive force (DEL CAMPO, HERNANDEZ-ASENSIO and RAMIREZ 1975; SINGH and BRAGG 1976; REIDER, WAGNER and SCHWEIGER 1979; ROBILLARD and BEECHEY 1986), and also to stimulate an exit reaction in which internal glucose-6-phosphate is converted to glucose and excreted (POSTMA and LENGELER 1985; HOFFEE, ENGLESBERG and LAMY 1964; GACHELIN 1970; HAGUENAUER and KEPES 1971; HERNANDEZ-ASENSIO and DEL CAMPO 1980). As a result both the rate of uptake of αMG and the pool size of the phosphorylated derivative are increased by the addition of a respiratory uncoupler or an agent which inhibits respiration (such as azide) to cells which are respiring.

Two series of experiments were carried out to test for differences in the regulation of αMG transport by respiration. In the first series, αMG transport was compared in the presence and absence of fructose. Fructose is not a substrate for the IIB^{Glc}/III^{Glc} transport system but it is respired. The feedback effects exerted by respiration should be minimal in its absence. The results (Figure 4, solid lines) show that the presence of fructose reduced the rate of αMG uptake in the input strain JA122 by increasing the K_m for transport to about 0.22 mM without changing the V_{max} significantly, consistent with results reported by others (HERNANDEZ-ASENSIO, RAMIREZ and DEL CAMPO 1975; GACHELIN 1970). The respiration of fructose also increased the K_m for αMG uptake by the evolved strains (Figure 4) suggesting that the higher affinity glucose-uptake system of the evolved strains is subject to inhibition by respiration. However, no differences among the evolved strains were observed.

In the second series, the effect of a respiratory inhibitor (azide) on αMG uptake was measured in cells respiring fructose. The results in Figure 5 show that all strains exhibit increased uptake of αMG coincident with the blocking of respiration by azide. These results and those shown in Figure 4 are consistent; there is no evidence for differences among the strains in the respiratory inhibition of glucose uptake. All strains showed the same degree of inhibition by respiration. Thus, the evolved strains do not differ in initial rates even during respiration, and the pool size differences among these strains do not appear to result from differences in inhibition of transport by respiration.

Excretion of metabolites by CV103: A poorer growth yield on glucose might result from incomplete metabolism of the glucose, from "slip-reactions" in which energy is dissipated instead of being used productively in metabolism (NEIJSSEL and TEMPEST 1976), or from less efficient use of the energy derived from respiration. NEIJSSEL and TEMPEST (1976) have argued that the glucose-limitation imposed on the starting strain during continuous culture is carbon-limitation rather than energy-limitation. If glucose were incompletely metabolized, the CV103-type organisms would be expected to excrete incompletely oxidized products and such products might serve as carbon and energy sources for other organisms.

In order to test this hypothesis, CV101 and CV103 (L and S strains, respectively) were grown to completion on glucose and then the cells of each strain were removed from the medium by filtration. The cells were resuspended (separately) in a portion of each filtrate. Growth of the suspensions was followed (Figure 6). CV101 grew on added glucose at the same rate in spent medium from either CV101 or CV103, indicating that neither strain released growth inhibitory compound(s) into the medium. Similarly CV103 grew normally on its own spent medium when glucose was provided but not in its absence. However, CV101 was able to continue growth on the spent medium from CV103 without added glucose, indicating that CV103 excretes a compound or compounds that can be used by CV101 as a carbon and/or energy source. CV103 did not grow in the filtrate from CV101. The growth yield of CV101 on CV103-spent medium quantitatively accounts for the difference between the growth yields of the two strains on fresh medium, thus indicating that the reduced yield of CV103 results from incomplete respiration of the glucose.

In an extensive series of experiments of the same type, we were unable to detect any growth of either

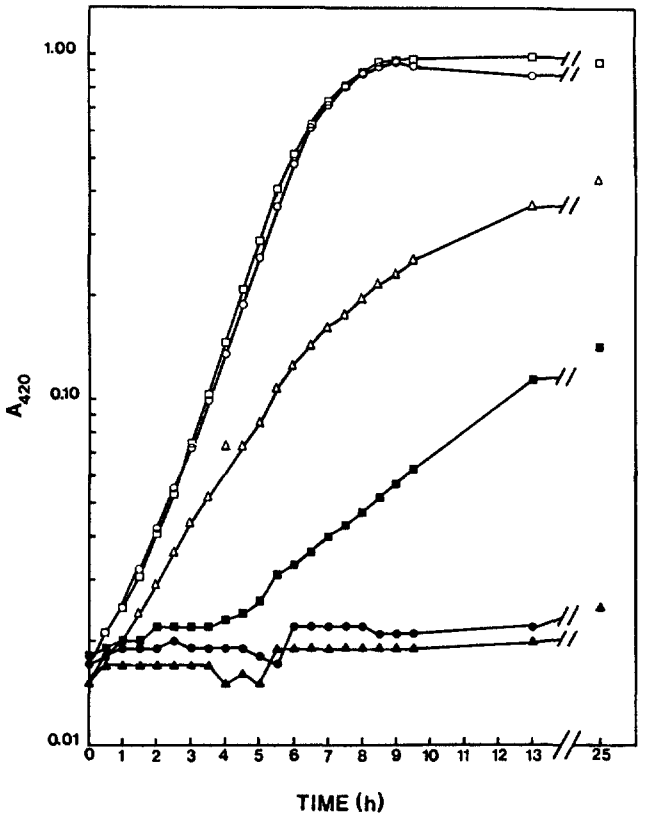

FIGURE 6.—Growth of large and small variants in spent culture media at 37°. (□), CV101 in CV103 spent medium plus glucose (0.2% w/v); (○), CV101 in CV101 spent medium plus glucose; (△), CV103 in CV103 spent medium plus glucose; (■), CV101 in CV103 spent medium; (●), CV101 in CV101 spent medium; (▲), CV103 in CV103 spent medium.

JA122 or CV116 on the filtrate from CV103 or vice versa.

CONCLUSIONS

Implications of the selection of S variants: We have observed the appearance and selection of S variants in long-term glucose-limited chemostat cultures initiated with strains of *E. coli* containing and lacking a plasmid, and with and without a λ prophage (Table 2). In every case where the cultures were maintained for several hundred generations, small-colony-formers appeared. These mutants were maintained at different frequencies ranging from approximately 0.99 to less than 0.01. The frequency of the small-colony-formers occasionally dropped below the level of easy detection but they typically appeared again many generations later. Fluctuations in their frequency are expected as the result of the serial selection of adaptive mutants (PAQUIN and ADAMS 1983a; HELLING, KINNEY and ADAMS 1981; NOVICK and SZILARD 1950; ATWOOD, SCHNEIDER and RYAN 1951). Similar mutant phenotypes were selected in populations of *Saccharomyces cerevisiae* maintained in long-term glucose-limited continuous culture (ADAMS *et al.* 1985). In the population from which we analyzed individual clones, small-colony-formers constituted the majority class for more than 400 generations. The appearance of such variants thus appears to be a predictable feature of adaptation to a glucose-limited environment. A substantial proportion of the variants not only grow poorly on broth, but even on glucose, when in batch culture.

These overall results suggest that the correlation between a growth advantage in the glucose-limited chemostat environment for an organism like CV103 and poor growth on glucose in batch culture is not coincidental. It would not be surprising to find that unrestricted uptake of glucose by CV103-like organisms during growth in excess glucose leads to bottlenecks in central metabolism, and thus to poor growth in batch culture. However, the diversity of mutant types seen in long-term chemostat culture suggests an intriguing possibility, namely that some organisms are more successful *because* they utilize the substrate less well. CV103 has a superior rate of glucose uptake, but also the same strain metabolizes glucose incompletely as evidenced by its excretion of incompletely oxidized products. In glucose-limited chemostat culture, the reduced oxidation is expected to result in a lower respiratory rate resulting in less inhibition of glucose uptake and an improved rate of glucose transport compared to the other evolved strains. If growth on limiting glucose selects for mutants with reduced respiration, there should be a large repertoire of genes in which such mutations occur. This would explain the variety of S variants observed in this and other populations. Feedback inhibition of glucose uptake by a product of glucose or by respiration is presumably an important component of the Pasteur effect (the greater utilization of glucose under fermentative conditions than during respiration). The unrestricted uptake of any compound would lead to metabolic imbalance. Selection for a reduction or elimination of feedback is a response to an unusual environment in which the compound is always at low concentration.

Maintenance of a stable polymorphism: CV103-like organisms are successful at sequestering low levels of glucose. In comparison, the other strains from the chemostat have a poorer secondary rate of glucose uptake (or greater rate of efflux). How then can those strains be maintained during growth in a glucose-limited chemostat culture? We believe that the L-type organisms such as CV101 maintain themselves not by competing for glucose, but rather by growing on the incompletely oxidized metabolites excreted by organisms similar to CV103. This ability must have evolved during growth in the chemostat as JA122 is unable to grow on the products excreted by CV103. It is not unreasonable to imagine that more than one compound is excreted during growth on glucose by CV103 and similar strains. It is plausible, therefore,

that during long-term chemostat culture different genotypes each optimized for utilization of a different overflow product evolve and fill niches in which each is superior.

We do not know if the mutation(s) resulting in poor growth on TB is(are) advantageous to CV116 during growth in the chemostat, but we have found mutants in other populations which appear to be similar. The maintenance in continuous culture of CV116-like organisms, together with CV103-like and CV101-like organisms, suggests interaction beyond a simple competition for a limiting substrate. However, in batch culture experiments we have been unable to demonstrate growth of either CV103 or CV116 on the spent growth medium from the other.

Glucose transport: Under glucose limitation in chemostat culture, all the evolved strains examined were superior to the input strain because they possessed improved glucose transport, and transport in CV103 may be better than that of the other chemostat strains. The higher affinity glucose-transporting system found (as predicted from measurement of αMG uptake) may result from addition of a distinct transport system or from alteration of the IIB^{Glc}/III^{Glc} system. The evolved system has a relatively poor affinity for L-arabinose, D-galactose, D-fructose and mannose (R. B. HELLING and J. ADAMS, unpublished results), suggesting that it is unlikely to have evolved from a transport system for one of those compounds.

The significance of the greater αMG pool in CV103 than in the other chemostat strains is unclear. The pool size reflects the balance between αMG uptake and efflux. An increased pool might result from (i) αMG transport in CV103 that is better than in the other chemostat strains, but not sufficiently so to see a difference over the brief period of standard assay, (ii) a change in the transport rate after the initial period so as to increase uptake in CV103 (positive feedback) or to decrease uptake in the other strains (negative feedback), and/or (iii) less efflux of accumulated αMG from CV103. Although the initial rates would seem the best indicators of relative transport activity during growth, we suspect that the greater pool in CV103 may be a manifestation of superior glucose uptake.

Similarities to tumor progression in eukaryotes: Our results show that populations in a seemingly simple environment with a single limiting nutrient differentiate to become complex and polymorphic. This evolution is strikingly similar to the process of tumor progression in higher animals. It has long been known that that process often is accompanied by a shift from respiratory metabolism to fermentation, and the excretion of lactic acid (WARBURG 1930; RUDDON 1981). Furthermore, tumors develop subpopulations of cells with different properties (MARX 1982; KLEIN and KLEIN 1985; NOWELL 1976). Transformation by tumor viruses or chemicals leads to increased uptake of glucose and certain amino acids, and a shift to glycolysis. Tumor promotors and growth factors such as transforming growth factor β, which effect many of the changes associated with transformation, also stimulate the uptake of glucose and amino acids and the shift from respiratory to fermentative metabolism (INMAN and COLOWICK 1985; BOERNER, RESNICK and RACKER 1985). Transforming growth factor β is thought to act through a cell-membrane-associated protein kinase.

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