Microbial Evolution in a Simple Unstructured Environment: Genetic Differentiation in *Escherichia coli*

R. Frank Rosenzweig,*[,1] R. R. Sharp,[†] David S. Treves* and Julian Adams*[,2]

*Departments of *Biology and [†]Chemistry, University of Michigan, Ann Arbor, Michigan 48109*

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ABSTRACT

Populations of *Escherichia coli* initiated with a single clone and maintained for long periods in glucose-limited continuous culture, become polymorphic. In one population, three clones were isolated and by means of reconstruction experiments were shown to be maintained in stable polymorphism, although they exhibited substantial differences in maximum specific growth rates and in glucose uptake kinetics. Analysis of these three clones revealed that their stable coexistence could be explained by differential patterns of the secretion and uptake of two alternative metabolites acetate and glycerol. Regulatory (constitutive and null) mutations in acetyl-coenzyme A synthetase accounted for different patterns of acetate secretion and uptake seen. Altered patterns in glycerol uptake are most likely explained by mutations which result in quantitative differences in the induction of the glycerol regulon and/or structural changes in glycerol kinase that reduce allosteric inhibition by effector molecules associated with glycolysis. The evolution of resource partitioning, and consequent polymorphisms which arise may illustrate incipient processes of speciation in asexual organisms.

NATURAL populations of most species appear to be highly polymorphic at the level of individual proteins. It has been argued that such variation provides raw material for the evolutionary process allowing populations to respond to the demands of an ever-changing environment (LEWONTIN 1974; MCDONALD 1983; *cf.* KIMURA 1983). The fuel for this argument has largely consisted in assessing the relative importance of the various mechanisms which can explain maintenance of the polymorphisms. Lost in the debate has been the more fundamental issue of how the more complex polymorphic state evolves from the simpler condition of monomorphism.

Evidence is accumulating that monocultures of asexual microorganisms reproducing in simple unstructured nutrient-limited environments rapidly become polymorphic (ADAMS *et al.* 1992; CLAASEN *et al.* 1986; HELLING *et al.* 1987; JOHNSTON and MCKAY 1977). The component clones of the polymorphisms have been distinguished on the basis of growth parameters (CLAASEN *et al.* 1986; HELLING *et al.* 1987) on patterns of gene expression (KURLANDZKA *et al.* 1991), and on gross chromosomal changes (ADAMS *et al.* 1992). Both direct and indirect evidence indicates that these evolved polymorphisms can persist over hundreds of generations (HELLING *et al.* 1987). Such findings are of particular significance as they appear to contradict two related tenets of evolutionary biology: the classical model of the evolution of asexual organisms (MULLER 1932; ATWOOD *et al.* 1951), and the so-called competitive exclusion principle (GAUSE 1934; HARDIN 1960).

The classical model (MULLER 1932) postulates that in the absence of recombination a population will evolve through a succession of clonal replacements. Except for those periods when the population undergoes adaptive shifts, its composition will remain monomorphic. In its simplest form, the classical model operates under the assumption of the competitive exclusion principle (GAUSE 1934), namely that clonal succession involves a series of "complete competitors" (HARDIN 1960) whose niches perfectly overlap; the strain possessing the highest intrinsic rate of increase will be expected to displace all others.

Spatial (CHAO and LEVIN 1981; COUTTS *et al.* 1987) or temporal (DAVISON and STEPHANOPOULOS 1986; PAVLOU *et al.* 1990; STEWART and LEVIN 1973) variation may allow the maintenance of otherwise unstable polymorphisms. Pairs of microbial species whose niches overlap may also coexist in continuous culture under certain conditions (TILMAN 1976; TILMAN *et al.* 1982). However, stable coexistence of more than one type on a single limiting resource in a constant homogeneous environment does not appear possible, unless there are interactions between the component members of the population. SLATER and BULL (1978) have described a number of interactions which can favor stable coexistence of species within a microbial community. These include metabolic interactions that result in mutualism (YEOH *et al.* 1968) or commensalism (MEGEE *et al.* 1972). Typically, such interactions are positive in nature and involve net

[1] Present address: Department of Biological Science, University of Idaho, Moscow, Idaho 83843.
[2] To whom reprint requests should be sent.

TABLE 1

Bacterial strains

Strain	Relevant characteristics	Source
JA122	F⁻ *thi*1 *lacY*1 *ara*D139 *gdh sup*E44 *hss*1 lysogenic for λ contains plasmid pBR322Δ5. Ampicillin and tetracycline resistant	ADAMS *et al.* (1979), HELLING (1990), HELLING *et al.* (1987)
CV101	Derivative of JA122–evolved strain isolated after approximately 773 generations of glucose limited continuous culture. Ampicillin and tetracycline resistant	HELLING *et al.* (1987)
CV103	As CV101 but independent isolate which forms small colonies on TA; contains plasmid pBR322Δ5. Ampicillin and tetracycline-resistant	HELLING *et al.* (1987)
CV116	As CV101, but independent isolate which forms small colonies on TA plates; lacks plasmid. Ampicillin sensitive	HELLING *et al.* (1987)

excretion of usable metabolite(s) by the species best adapted to growth on the primary resource. This compound in turn serves as a secondary resource to another species which either specializes in that resource or gains a transient advantage in growth rate. Such metabolic interactions can be more complex and involve negative, density-dependent effects (*e.g.*, LENSKI and HATTINGH 1986; YEOH *et al.* 1968).

Here we present evidence that similar interactions underlie genetic diversification within a population, initiated with a single clone of *Escherichia coli* grown in long term glucose-limited continuous culture. We show that three distinct adaptive clones isolated at generation 773, coexist in stable equilibrium, mediated by cross-feeding between strains of normal products of aerobic fermentation. Genetic and physiological differentiation of these strains with respect to the metabolism of glucose, acetate and glycerol has transformed a simple, constant glucose-limited environment into one whose chemical complexity has resulted in the evolution of a balanced polymorphism.

MATERIALS AND METHODS

Bacterial strains: Bacterial strains used in this study are listed in Table 1. CV103, CV101 and CV116 were isolated after 773 generations of growth from a glucose-limited chemostat initiated with JA122 (HELLING *et al.* 1987). Arabinose resistant, otherwise isogenic derivatives of CV101 and CV103 were used in some competition experiments to distinguish strains (see below).

Media and culture conditions: Tryptone agar (TA) contained 10 g Tryptone (Difco), 5 g NaCl and 14 g Bacto-agar per liter. For both batch and continuous (chemostat) cultures the minimal medium was that of DAVIS and MINGIOLI (1950), lacking citrate and containing 1 μg ml^{-1} thiamine·HCl. Unless otherwise indicated, carbon source in the form of D-glucose (Sigma Chemical Co.) was present at a concentration of 0.05–0.1% (w/v) in batch culture and was varied between 0.00625% (w/v) and 0.025% (w/v) in chemostats. Sodium acetate (0.1%, w/v) and glycerol (0.1%, v/v) were added as carbon sources where specified. D-Glucose (Sigma) and thiamine·HCl were sterilized by filtration though a 0.22-μM pore size cellulose nitrate filter prior to use in the chemostat medium. Where indicated, ampicillin (50 μg ml^{-1}), and L-arabinose (1 mg ml^{-1}) were added after autoclaving.

In all cases cultures were maintained at 30°. To ensure adequate aeration, batch cultures were grown in Erlenmeyer flasks at no more than a 1:5 media volume:flask volume ratio. Constant temperature and agitation (150 rpm) were maintained using a New Brunswick G67 water bath shaker. Changes in population density were monitored by measuring absorbance at either 420 or 550 nm using a Gilford model 250 spectrophotometer. The former value was chosen for experiments where increased sensitivity was required to detect changes at low cell densities (STANIER *et al.* 1986).

Both single-strain and mixed continuous cultures were initiated with bacteria previously grown 14–18 hr in 0.025% glucose batch culture, supplemented as necessary with ampicillin. Prior to inoculation, cells were harvested by centrifugation in a Sorvall GSA (DuPont, Wilmington, Delaware) rotor at 6,000 rpm for 5 min, at 25°, and washed once with minimal medium lacking metabolizable carbon. Chemostats were then inoculated with a total cell number, equivalent to the equilibrium cell density for a given glucose concentration. For mixed cultures, the inoculum was composed of approximately equal number of cells from each strain. Just before the addition of cells the outflow tube of the chemostat was clamped, the inoculum was allowed to mix thoroughly, and the clamp was removed. Cells were grown in aerated culture vessels 145–210 ml in volume at a dilution rate of $D \approx 0.2$ hr^{-1}, equivalent to a generation time of approximately 3.5 hr (KUBITSCHEK 1970). Culture absorbance was monitored at 550 nm using a Bausch and Lomb Spectronic 100 spectrophotometer.

In the absence of an antibiotic resistance marker, an arabinose resistance marker was used to distinguish strains. Although previous experiments have established that mutations to arabinose resistance exert a small selective effect in glucose-limited continuous culture (MODI and ADAMS 1991), this was not sufficient to significantly change the stable equilibria attained. Changes in the frequency of two strains was estimated by plating samples of appropriate dilutions onto TA plates and then replica-plating colonies onto either TA plus ampicillin (TAamp) or TA plus arabinose (TAara), depending on the strains involved. TAamp and TAara plates were incubated at 37° and scored within 8–12 hr. An average of 150 colonies was replicated per sample. Continuous culture experiments were terminated within 30–35 generations of growth in order to avoid complications due to faster growing adaptive mutants (HELLING *et al.* 1987).

Identification and analysis of metabolites: The following procedures were used.

Nuclear magnetic resonance studies: For each strain at least two replicate 50-ml samples of spent batch culture media or continuous culture effluent were filter sterilized by passage through a 0.2-μM cellulose nitrate filter. The cell-free filtrate was then lyophilized and resuspended in 2.5 ml sterile deuterium oxide. Standards (50 mM) of 33 different metabolites were prepared in Davis minimal medium, concentrated 20-fold by lyophilization, and resuspended in deuterium oxide.

Acetone (50 mM) was added to all samples as an internal standard for chemical shift measurements. Samples and standards were analyzed by proton NMR spectrometry using a Bruker 360 nuclear magnetic resonance spectrometer. Congruence of spectra was established by visual inspection. Metabolite standards were as follows. Glycolytic intermediates were 2-phosphoglycerate, 3-phosphoglycerate, acetaldehyde, dihydroxyacetone phosphate, fructose 1,6-diphosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, phosphoenolpyruvate and pyruvate. Tricarboxylic acid (TCA) cycle intermediates were 2-ketoglutarate, α-ketoglutarate, citrate, fumarate, isocitrate, malate, oxaloacetate and succinate. Fermentation products were 2,3-butanediol, acetate, acetoin, ethanol, formate, glycerol and lactate. Others were 6-phosphogluconate, α-glycerophosphate, arabinose, fructose, fucose, glucosamine, glucose and ribulose 5-phosphate.

Enzymatic analyses: Analysis of the fermentation products acetate, ethanol, formate, glycerol, lactate, pyruvate and succinate was performed using modifications of near-UV spectrophometric assays (BERGMEYER, 1983). (i) Acetate was determined by its conversion to acetyl-CoA in the presence of acetyl-CoA synthetase (ACS), CoA, adenosine triphosphate, malic acid, citrate synthase, malate + dehydrogenase and nicotinamide-adenine dinucleotide (NAD^+). Fresh filtered samples of either batch or chemostat media were assayed at 25° in a 3-ml reaction mixture consisting of 100 mM triethanolamine buffer, pH 8.4, and containing 4 mg L-malic acid, 2 mg magnesium chloride, 5 mg ATP, 500 μg CoA, 2.4 mg NAD^+, 27 units malate dehydrogenase (malate:NAD^+ oxidoreductase; EC 1.1.1.37), 6 units citrate synthase [citrate oxaloacetate-lyase (CoA acetylating); E.C. 4.1.3.7] and 0.4 units ACS. This and all subsequent colorimetric assays were monitored by following absorbance change at 340 nm using a Gilson model 250 spectrophometer and model 4019 recorder. (ii) Ethanol was determined by following the alcohol dehydrogenase reaction at 25° in 100 mM potassium diphosphate buffer, pH 9.0. Appropriate volumes of fresh filtered chemostat effluent were assayed in a 3 ml reaction mix that contained 4 mg NAD^+, 0.8 units aldehyde dehydrogenase (EC 1.2.1.5), and 219 units of alcohol dehydrogenase (EC 1.1.1.1). (iii) Fresh chemostat filtrates were assayed for formate by following the formate dehydrogenase reaction at 25° in 100 mM potassium phosphate buffer, pH 7.5. The 3-ml reaction mixture contained 19 mg NAD^+ and 3.3 units of formate dehydrogenase (EC 1.2.1.2). (iv) Glycerol concentrations were estimated by following at 25° the ATP-dependent glycerol kinase reaction coupled to the pyruvate kinase/lactic dehydrogenase reactions. An appropriate volume of sample was assayed in a 3-ml reaction mixture consisting of 250 mM glycylglycine, 6 mM magnesium sulfate, pH 7.4, with 636 μg reduced nicotinamide adenine dinucleotide (β-NADH), 2 mg ATP, 1 mg phosphoenolpyruvate, 6 units pyruvate kinase (EC 2.7.1.40), 5.5 units lactate dehydrogenase (EC 1.1.1.27), and 0.9 units glycerokinase (EC 2.7.1.30). (v) Lactic acid was assayed by following the lactate dehydrogenase reaction at 25° while trapping product using the glutamate-pyruvate transaminase reaction. Samples were analyzed in a 3 ml reaction mixture that consisted of 200 mM glycylglycine buffer, pH 10, and contained 14.6 mg L-glutamic acid, 7 mg NAD^+, 31 units glutamate-pyruvate transaminase (EC 2.6.1.2), and 108 units of L-lactate dehydrogenase. (vi) Succinate was assayed by coupling the succinate thiokinase (EC 6.2.1.4) reaction to pyruvate kinase/lactate dehydrogenase. Fresh filtered samples of chemostat effluent were analyzed in a 3-ml reaction mixture consisting of 200 mM glycylglycine buffer, pH 8.4, 460 μg β-NADH, 750 μg CoA, 700 μg inosine-5′-triphosphate, 300 μg phosphoenolpyruvate, 25 units pyruvate kinase, 23 units lactate dehydrogenase, and 1 unit succinate thiokinase. (vii) Pyruvate was assayed by following the lactate dehydrogenase-mediated conversion of pyruvate to lactate. Fresh filtered samples of chemostat effluent were assayed in a 1.0-ml reaction mixture consisting of 160 mM triethanolamine, 1.18 mM EDTA, pH 7.6, containing 100 μg β-NADH and 10 units of lactate dehydrogenase. (vii) Glucose was estimated by following the hexokinase (EC 2.7.1.1)/glucose-6-phosphate dehydrogenase (EC 1.1.1.49) reactions at 25° in a 3-ml reaction mixture that consisted of 250 mM triethanolamine buffer, pH 7.6, and contained 2.4 mg nicotinamide dinucleotide phosphate ($NADP^+$), 5.8 mg ATP, 2.8 mg magnesium sulfate, 5.8 units hexokinase, and 2.9 units glucose-6-phosphate dehydrogenase. Samples were concentrated 20-fold prior to analysis by lyophilizing fresh 50-ml chemostat filtrates, resuspended in 2.5 ml sterile glass distilled water, and clarified by passage through a 5-μM Acrodisc filter (Gelman Products, Ann Arbor, Michigan).

Enzyme assays: The following procedures were used.

Acetate kinase and ACS: Batch cultures were grown in the media specified and harvested at an OD ≈ 1.0 (A_{550}) by centrifugation at 8000 rpm, at 4° for 5 min in a Sorvall GSA rotor. The cell pellets were resuspended and washed twice in ice-cold 100 mM sodium phosphate, 10 mM $MgCl_2$, 1 mM EDTA, 50 mM phenylmethylsulfonyl fluoride (PMSF), 50 μM 2 dithiothreitol, pH 7.5, then passed twice through a French press at 15,000 psi. These extracts were then centifuged at 13,000 rpm, 4° for 30 min in a Sorvall SS-34 rotor and the undiluted crude supernatant used for the assays. Chemostat cultures were grown in the media specified, and harvested after a minimum of ten generations of growth by centrifugation at 8000 rpm, 4° for 5 min in a Sorvall GSA rotor. The cell pellets were resuspended and washed twice in ice-cold 100 mM sodium phosphate, 10 mM $MgCl_2$, 1 mM 2 EDTA, 50 μM PMSF, 50 μM dithiothreitol, pH 7.5 and stored at −80°. Since equilibrium cell densites attained in continuous culture under glucose limitation are low, it was necessary to pool from each chemostat monoculture three 100-ml samples harvested at 24-hr intervals. These pooled samples were resuspended in extract buffer, disrupted by French press, centrifuged at 13,000 rpm in an SS-34 rotor for 30 min at 4°, and the undiluted crude supernatant used for the assays. ACS was assayed by a modification of the procedure used to determine acetate as described in BERGMEYER (1983). Specific activity was measured at 25° as the reduction of NAD^+ in the presence of L-malate, ATP, CoA, malic dehydrogenase, citrate synthase and potassium acetate. The reaction mixture (2.85 ml) contained: 142 mM triethanolamine hydrochloride, pH 8.4, 8 mM $MgCl_2$, 11 mM L-malate, 3 mM ATP, 0.45 mM CoA, 1.3 mM NAD^+, 31 units malic dehydrogenase, 16.7 units citrate synthase and cell-free extract. After reaching equilibrium the reaction was initiated by the addition of 30 μM potassium acetate and the change in absorbance followed at 340 nm using a Gilford model 250 spectrophotometer and model 4019 recorder. Total soluble protein was estimated in cell free extracts by the method of BRADFORD (1976) using the Bio-Rad (Richmond, California) protein assay kit. Bovine serum albumin was used as standard. Acetate kinase was assayed in the acetyl phosphate-forming direction essentially as described by BERGMEYER (1983). Activity was measured at 25° by coupling ADP formation to the oxidation of NADH. The reaction mixture consisted of 67 mM triethanolamine-HCl, 1.33 mM $MgCl_2$, 5.4 mM ATP, 1.1 mM phosphoenolpyruvate, 0.35 mM NADH, 8.1 units pyruvate kinase, and 25.2 units of lactic dehydrogenase, pH 7.0. For determination of K_m values, reactions were initiated by the addition of sodium acetate in concentrations ranging from 333 mM to 1 mM, and the change in absorbance at 340 nm monitored as above.

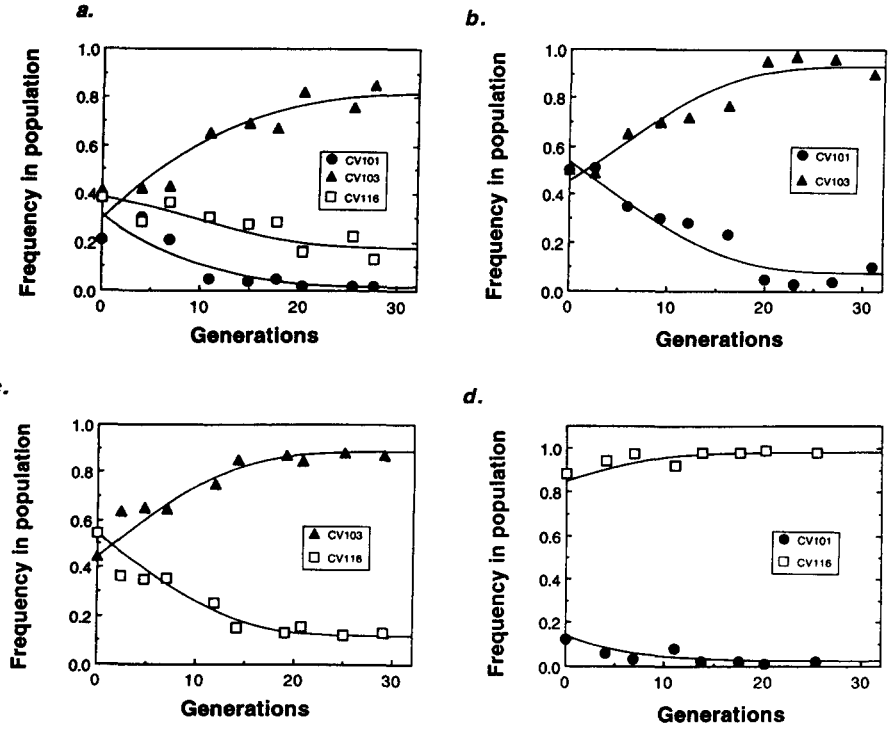

FIGURE 1.—Reconstruction experiments demonstrating that the three strains, CV101, CV103 and CV116 are maintained in stable polymorphism. (a) Competition between strains CV101, CV103 and CV116; ▲, CV103; ●, CV101; □, CV116. (b) Competition between CV101 and and CV103; ▲, CV103; ●, CV101. (c) Competition between CV103 and CV116; ▲, CV103; □, CV116. (d) Competition between CV101 and CV116; ●, CV101; □, CV116. Glucose concentration in the medium was 0.00625% for all experiments. In each case results from a representative experiment are shown; replicate experiments gave concordant results in all cases.

Glycerol kinase and glycerol-3-phosphate dehydrogenase: Chemostat grown cells were harvested by centrifugation at 8,000 rpm, 4°, for 5 min in an SS-34 rotor. The cell pellet was washed twice in 50 mM Tris-HCl, 1 mM dithiothreitol, 100 μM PMSF, pH 7.5 (TDP buffer), and then resuspended in TDP to a density of 2–5 × 10^9 cells ml^{-1}. The suspension was placed in an ethanol/ice water bath at −10° and cells disrupted by three 30-sec bursts of sonication at 100 W using a Braun 1510 sonicator. The preparation was then centrifuged in an SS-34 rotor at 13,000 rpm, 4°, for 15 min to remove cellular debris. Glycerol kinase activity was estimated in fresh cell extracts according to the procedure described by FREEDBURG and LIN (1973) and modified by Boehringer-Mannhein Biochemicals (7th Edition Quality Control Manual Assay No. 5181). Appropriate dilutions of crude cell extract were assayed at 25° in a 3.13-ml reaction mixture consisting of 0.2 M glycine/hydrazine buffer, 2 mM $MgCl_2$, pH 9.5, containing 2.5 mg ATP, 1 mg NAD^+, 1 mg glycerol, and 51 units glycerol-3-phosphate dehydrogenase (EC 1.1.1.8). The reaction was followed spectrophotometrically by monitoring change in absorbance at 340 nm due to the reduction of NAD^+. Aerobic glycerol-3-phosphate dehydrogenase activity was estimated in fresh cell extracts by using a modification of the procedure described by KISTLER and LIN (1971). Then 10–50 μl of crude extract were assayed at 25° in a 1.0-ml reaction mix consisting of 67 mM potassium phosphate, pH 7.5, 10 mM potassium cyanide, pH 7.5, containing 33 μg of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, 200 μg phenazine methosulfate, 0.2% (v/v) Triton X-100 and 33 μmol D,L-α-glycerophosphate. The reaction was followed spectrophotometrically by monitoring change in absorbance at 570 nm. Specific activity was expressed as micromoles of substrate utilized per min per unit protein using an extinction coefficient of 17 mM^{-1} cm^{-1}. Total soluble protein content of crude extracts was estimated by the Bio-Rad, microassay based upon the method of BRADFORD(1976) using bovine serum albumin as a standard.

Glycerol transport studies: Cells were removed from batch culture or chemostats as indicated, harvested by centrifugation in a Sorvall GSA rotor at 6,000 rpm for 5 min at room temperature then resuspended in carbon-free Davis minimal media to a density of 5 × 10^8 cells ml^{-1}. Glycerol uptake was estimated at 30° according to a modification of the procedure described by SWEET *et al.* (1990). Experiments were initiated by adding 500 μl of cell suspension to 4.5 ml of minimal media containing 1.5 μmol [u-^{14}C]glycerol (153.5 mCi/mmol; Amersham) that had been preincubated for 5 min at 30°. At indicated time points over a period of 60 sec, 1 ml of the mixture was withdrawn, vacuum-filtered over a Millipore 0.45-μM filter using a Millipore 700 sampling manifold, and washed twice with 1 ml of carbon-free minimal media. Each filter was then placed into 10 ml of Ecoscint A (National Diagnostics, Manville, New Jersey) scintillation cocktail and counted in a Beckman LS7000 scintillation counter. The data were plotted as counts/min substrate incorporated per 10^8 cells *vs.* time, then converted to picomoles glycerol/min/10^8 cells.

RESULTS

A population of *E. coli*, initiated with a single clone, and maintained in long term glucose-limited chemostat culture, developed extensive polymorphisms. Two majority and two minority types, defined by colony size on TA and presence/absence of a plasmid carrying ampicillin resistance, were identified in a sample taken after 773 generations (HELLING *et al.* 1987). In addition, these strains are highly differentiated with respect to global patterns of gene expression (KURLANDZKA *et al.* 1991). Reconstruction experiments, in which a mixed population was initiated with these four strains in equal frequency, demonstrated that three (CV101, CV103 and CV116) of the four were stably maintained in the population (Figure 1a), while the fourth was rapidly eliminated (HELLING *et al.* 1987). In addition, a population, established from a sample taken at generation 765, remained polymorphic for an additional ~450 genera-

TABLE 2

Physiological parameters

Strain	Specific growth rate (hr^{-1}) [a]	Rate of glucose uptake (μmol α-MG/min/gm) [b]	Equilibrium glucose concentration (nmol/ml)	Equilibrium acetate concentration (nmol/ml/10^8 cells)	Rate of glycerol uptake (pmol/min/10^8 cells)
JA122	0.44 ± 0.01	1.19 ± 0.09	1.84 ± 0.48	194 ± 20	99 ± 8
CV101	0.50 ± 0.02	1.66 ± 0.06	0.88 ± 0.31	0 ± 0	93 ± 7
CV103	0.40 ± 0.01	2.46 ± 0.16	0.07 ± 0.03	252 ± 70	104 ± 7
CV116	0.60 ± 0.01	1.61 ± 0.11	0.19 ± 0.05	40 ± 25	146 ± 14

Values are presented ± standard error of the mean.
[a] Data from Table 3 of HELLING *et al.* (1987).
[b] Data from Figure 5 of HELLING *et al.* (1987).

tions, with at least three components having the same phenotypic characteristics as strains CV101, CV103 and CV116. Replicate populations maintained for similar periods of time under glucose-limited conditions also developed similar polymorphisms (HELLING *et al.* 1987).

Estimation of maximum growth rates under non-limiting conditions, of glucose uptake kinetics, and of the equilibrium glucose concentrations revealed significant differences among all three strains (Table 2). Thus, CV103 possessed superior glucose uptake kinetics, reflected in the lower equilibrium concentrations of glucose in the chemostat, whereas CV116 showed a higher maximum growth rate.

The kinetics of growth in continuous culture, where fitness is determined by competition for a single limiting substrate, predicts that no stable equilibrium is possible (POWELL 1958). In mixed culture, cells with the phenotypic characteristics of CV103 should be selectively favored, whereas those with the characteristics of CV101 and CV116 should disappear, contrary to the results shown in Figure 1a. To determine the factors responsible for the maintenance of this stable polymorphism, we analyzed the system as a set of three two component polymorphisms.

Analysis of the CV101, CV103 polymorphism: Growth of strains CV101 and CV103 in mixed culture showed that these two strains were maintained in stable equilibrium (Figure 1b) and thus were not maintained simply due to the presence of cells with the phenotypic characteristics of the third strain CV116.

Previous results had indicated that CV101 was able to grow in the culture filtrate of CV103 grown to stationary phase, but not the reverse, suggesting that CV103 secretes a metabolite upon which CV101 can grow. This indication that stationary phase cultures of CV103 contain unmetabolized carbon in the surrounding media, is consistent with the finding that CV103 possesses a significantly lower cell yield in batch culture compared to the parent strain, JA122, and the other evolved strains (HELLING *et al.* 1987). We therefore initiated analyses to determine the chemical nature of this compound.

Proton nuclear magnetic resonance spectra can provide detailed information about the structure of organic molecules. For a given buffer and pH, each compound presents a distinct signature as a consequence of the number and arrangement of its constituent atoms (SILVERSTEIN 1991). NMR spectra were obtained for the culture filtrates of the three strains grown to stationary phase, as well as for 33 common metabolites including glycolytic and TCA cycle intermediates and compounds known to be common substrates and products of bacterial fermentation (see MATERIALS AND METHODS). Examination of the spectra for CV103 revealed the presence of a strong signal at 0.8 ppm in the spent media of CV103, absent in CV101 and CV116 as well as in JA122, the strain used to initiate the population (data not shown). Comparison with the standards putatively identified the filtrate of CV103 as containing acetate. The presence of acetate was then confirmed by conventional enzymatic analysis. This finding suggests that a positive interaction between the CV103 and CV101 involving cross-feeding of acetate may be responsible for their maintenance in stable polymorphism.

Acetate is known to be the predominant by-product of *E. coli* fermentation under aerobic conditions (LANDWALL and HOLME 1977; DOELLE *et al.* 1982). However, during extended glucose batch culture the acetate which accumulates extracellularly is normally metabolized after glucose is exhausted from the media (BROWN *et al.* 1977). To determine whether strain CV103 was oversecreting and/or failing to resorb this metabolite, we monitored bacterial growth and acetate production in 0.1% glucose batch cultures. The results (Figure 2) show that by early stationary phase CV103 has excreted approximately three times more acetate into the surrounding media than strain CV101, and that acetate levels for CV103 remain elevated throughout stationary phase. In addition, CV101 secretes less than half the amount of acetate produced by JA122 by late log-phase, and that acetate levels for CV101 diminish rapidly thereafter. These data indicate that with respect to the parent strain both CV101 and CV103 have undergone genetic changes resulting in altered patterns of acetate metabolism, and that CV103 may be altered with respect to both acetate efflux and uptake.

To determine the ability of the strains to grow on low levels of acetate, we assayed the ability of cells, grown on

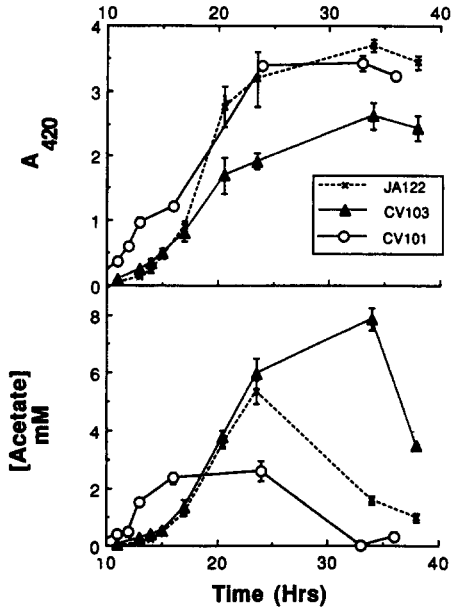

FIGURE 2.—Strains CV103 and CV101 have differentiated from the parent strain JA122 with respect to acetate metabolism. Population growth and extracellular acetate concentration in 0.1% glucose batch cultures of JA122 (×-----×), CV101 (○——○) and CV103 (▲——▲). Standard errors for some time points were smaller than the symbols on the graphs and are therefore not shown.

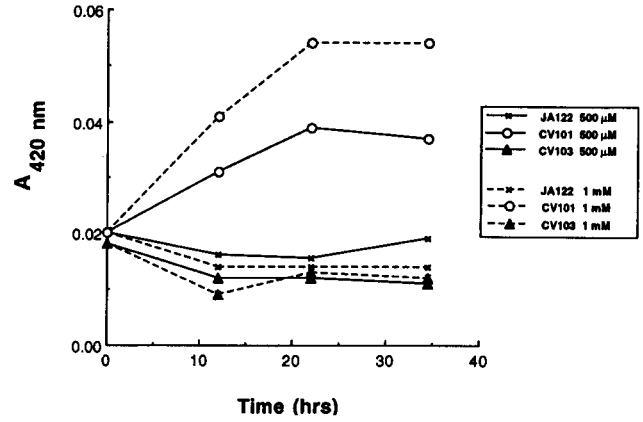

FIGURE 3.—Strains CV103 and CV101 have differentiated from the parent strain JA122 with respect to acetate metabolism. Growth in batch cultures containing either 500 µM or 1 mM acetate as sole carbon source. (×——×), JA122 in 500 µM acetate; (○——○), CV101 in 500 µM acetate; ▲——▲, CV103 in 500 µM acetate; ×-----×, JA122 in 1 mM acetate; ○----○, CV101 in 1 mM acetate; ▲-----▲, CV103 in 1 mM acetate.

glucose minimal media and washed in medium containing no carbon source, to divide in minimal media containing 500 µM, 1 mM (Figure 3) and 5 mM acetate (data not shown). The results show that population density increased only in CV101 cultures.

Physiological basis of the interaction between CV101 and CV103: During growth on a fermentable carbon source, a fraction of the carbon metabolized via pyruvate to acetyl-CoA is diverted and excreted by a two-step reaction sequence involving its phosphorylation by phosphoacetyl transferase and dephosphorylation by acetate kinase (LEVINE *et al.* 1980). These enzymes can catalyze the reverse reaction in the acetyl-CoA-forming direction. However, the Michaelis constant for acetate kinase, the first enzyme in the acetate dissimilation pathway is comparatively high $K_m^{acetate}$ = 7 mM; FOX and ROSEMAN 1986). During the course of batch culture as the titer of fermentable carbon drops, acetate which has accumulated extracellularly first reenters central metabolism via this high K_m (low affinity) pathway. ACS, a high affinity enzyme ($K_m^{acetate}$ = 200 µM), then becomes derepressed. This enzyme then acts to scavenge acetate as its concentration diminishes in the media (BROWN *et al.* 1977; NUNN 1987).

The structure of these pathways suggests that genetic changes in acetate kinase (ACK) and/or ACS would most likely result in altered patterns of acetate dissimilation. Accordingly, we estimated kinetic parameters for these enzymes *in vitro*. Both the parental strain, JA122, and the adaptive clones exhibit a pattern of acetate kinase expression consistent with that reported as wild-type (BROWN *et al.* 1977; KOPLOVE and COONEY 1978), with specific activities two to three times higher on a non-fermentable carbon source, than on glucose (Table 3). However, there are no significant differences in the specific activities of adaptive clones cells grown in glucose-limited chemostat culture. Although the K_m estimates for CV103 and CV116 are lower than the other two strains, they are at least one to two orders of magnitude greater than the residual acetate concentration measured in glucose-limited chemostats (Table 2). We therefore conclude that there are no changes in the structure or regulation of acetate kinase that can explain the differences in the secretion and uptake of acetate observed.

In contrast, the adaptive clones differ markedly with respect to their expression of the high affinity enzyme, ACS (Table 4). The parental strain, JA122, and one of the adaptive clones, CV116, exhibit a wild-type pattern of expression (BROWN *et al.* 1977): cells harvested from late log-phase glucose batch cultures are completely repressed for ACS, whereas cells harvested from batch cultures growing on non-fermentable carbon sources are derepressed and chemostat grown cells show low levels of enzyme activity. However, CV103 shows no ACS activity under any of the conditions tested, while CV101 constitutively overexpresses the enzyme. Consistent with these results is the observation that glucose-limited chemostat monocultures of CV101 contained no detectable acetate while cultures of CV103 grown under the same conditions (0.0625% glucose) contained 150 µM acetate (Table 2). Similarly, the data explain the growth of CV101 in low levels of acetate (Figure 3) and in the filtrate from CV103 grown to stationary phase, reported previously (HELLING *et al.* 1987).

TABLE 3

Acetate kinase

I. Specific activity

Strain	Growth conditions: 0.2% Glucose batch culture	0.025% Glucose chemostat	0.1% Glycerol + 0.1% acetate batch culture
JA122	1885 ± 102[a]	1566 ± 61[a]	4629 ± 73[a]
CV101	1367 ± 70	1191 ± 270	5350 ± 745
CV103	1424 ± 336	1185 ± 66	3266 ± 288
CV116	774 ± 63	1264 ± 116	NA

II. Apparent K_m for acetate

Strain	K_m
JA122	31 ± 5[b]
CV101	32 ± 5
CV103	14 ± 5
CV116	12 ± 2

[a] Values are presented as units/g soluble protein ± standard error of the mean. NA, not assayed.

[b] Values are presented as mM acetate ± standard error of the mean.

TABLE 4

Specific activity of acetyl-CoA synthetase

Strain	Growth conditions: 0.2% Glucose batch culture	0.025% Glucose chemostat	0.1% Glycerol + 0.1% acetate batch culture
JA122	0	18 ± 5	71 ± 14
CV101	150 ± 23	122 ± 25	179 ± 8
CV103	0	0	2 ± 2
CV116	0	3 ± 1	81 ± 6

Values are presented as units/g soluble protein ± standard error of the mean.

Since CV103 possesses a significantly improved glucose transport capacity (HELLING *et al.* 1987), and CV101 has gained exclusive access to the acetate secreted as a byproduct of fermentation, a stable equilibrium between these two strains is expected, which should be dependent on acetate levels in the culture. We tested this hypothesis directly by growing continuous mixed cultures of the two strains in medium with and without additional acetate. Figure 4a shows that the equilibrium frequencies of the two strains are strongly affected by the addition of acetate to the medium. In the absence of additional acetate, CV101 attains an equilibrium frequency of <20% of the population, whereas when additional acetate was added to a concentration of 1 mM, CV101 constitutes >80% of the population at equilibrium. Since acetate concentration in the medium will be determined by the population density, the equilibrium frequencies of CV101 and CV103 will also be expected to be density-dependent. At densities yielding acetate levels on the order of the K_m for ACS, the ability of CV101 to utilize glycerol as an alternative carbon source, will be significantly attenuated. Thus, below a critical population density the concentration of acetate will be insufficient to provide a growth advantage to CV101. We tested this prediction by growing mixed populations of CV101 and CV103 at different population densities, by varying the concentration of glucose in the incoming medium. As expected, the equilibrium frequencies were strongly dependent on population density (Figure 4b).

Analysis of the CV103, CV116 polymorphism: Figure 1c shows that CV103 and CV116 are maintained in stable polymorphism, independent of the presence of the third strain CV101 (see also HELLING *et al.* 1987). Since CV116 is not capable of growing on the filtrate of CV103 cultures grown to stationary phase, and *vice versa* (HELLING *et al.* 1987), we considered that equilibrium concentrations of other growth-active compounds would be present in culture filtrates at concentrations significantly lower than that of acetate. Proton-NMR analysis is relatively insensitive, requiring sample concentrations in the millimolar range. We therefore used standard spectrophotometric enzyme assays (BERGMEYER 1983) to analyze fresh unlyophilized chemostat samples for known products of *E. coli* fermentation. Pyruvate, lactate, ethanol, formate and succinate were not present in the culture filtrates at detectable levels. However, small quantities of glycerol (<10 μmol/liter) were detected in continuous monocultures, raising the possibility that differences in glycerol metabolism between CV103 and CV116 could explain their maintenance in stable equilibrium. To determine the effect of glycerol on the stable equilibrium between CV103 and CV116, we initiated a mixed culture of CV103 and CV116, allowed the two strains to approach stable equilibrium, and then added 1 mM glycerol to both the culture vessel and the incoming medium. The results shown in Figure 5a, show that the equilibrium frequencies of the two strains are dramatically affected by the concentration of glycerol in the medium. In contrast, addition of glycerol to mixed cultures of CV101 and CV103 had no effect on their final equilibrium (Figure 4c).

To determine if acetate also was instrumental in maintaining the CV103, CV116 polymorphism, we grew mixed cultures of these two strains in medium containing glucose as the sole carbon source, and in medium containing both glucose and acetate. The results, shown in Figure 5b show that the addition of acetate appears to have a small effect on the equilibrium frequencies attained. This result is expected given the difference in equilibrium acetate levels observed in monocultures of CV103 and CV116 (see Table 2), and is consistent with data indicating that CV116 expresses low levels of ACS when grown in under nutrient limitation (see Table 4).

Strain CV116 shows an enhanced ability to assimilate glycerol: Figure 6 shows population growth and extracellular glycerol concentration in 0.1% glucose batch cultures. Extracellular glycerol levels remained elevated through mid-log and early stationary phase

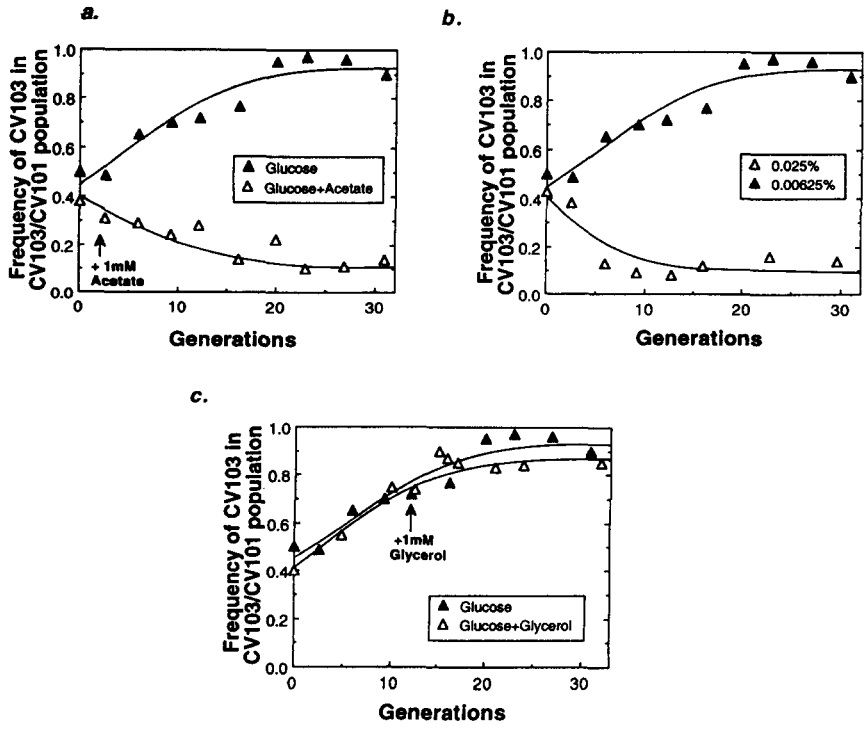

FIGURE 4.—Stable polymorphism between CV101 and CV103. (a) The frequencies of CV101 and CV103 at equilibrium are affected by the level of acetate in the medium. ▲, Frequency of CV101 in medium with 0.00625% glucose (w/v). △, Frequency of CV101 in medium with 0.00625% glucose; 1 mM acetate was added to the medium reservoir and culture vessel at the time indicated by the arrow. (b) The frequencies of CV101 and CV103 at equilibrium are affected by total population density. ▲, Frequency of CV101 in medium with 0.00625% glucose (w/v). Population density was ~5.9×10^7 ml^{-1}; △, frequency of CV101 in medium with 0.025% glucose (w/v). Population density was ~2.4×10^8 ml^{-1}. (c) The frequencies of CV101 and CV103 at equilibrium are not affected by the level of glycerol in the medium. ▲, Frequency of CV101 in medium with 0.00625% glucose (w/v). △, Frequency of CV101 in medium with 0.00625% glucose; 1 mM glycerol was added to the medium reservoir and the culture vessel at the time shown by the arrow. In each case results from one representative experiment are shown; replicate experiments gave concordant results in all cases.

among batch cultures of the parent strain, JA122, and the two adaptive clones, CV101 and CV103. In contrast, glycerol concentration in batch cultures of CV116 declined precipitously as soon as such populations ceased to grow exponentially. This difference is significant as the physiological state of cells undergoing the transition to stationary phase is similar to that of cells growing in nutrient-limited chemostat culture (KUBITSCHEK 1970). To determine whether this phenomenon was reproduced in continuous culture we estimated the glycerol uptake kinetics of cells grown under glucose limitation. As expected, CV116 possesses a significantly enhanced capacity to take up glycerol compared to the parent strain and the other two adaptive clones (see Table 2). In addition, we then estimated the time required for cells growing in glucose-limited monocultures to reach a new steady-state after glycerol was added to the medium. Consistent with the differences in glycerol uptake kinetics (see Table 2), the population density of monocultures of CV116 increased much more rapidly after the addition of glycerol than CV103 or CV101 (data not shown).

The stable polymorphism between CV103 and CV116, like that between CV101 and CV103 may be explained by growth rate differences between the two strains, coupled with a differential ability to utilize an alternative metabolite, in this case glycerol. CV103 possesses a superior glucose uptake system (see Table 2), which should endow it with a higher relative fitness than CV116, when glucose is the only carbon source available and is present in limiting concentrations. CV116, however, possesses a superior ability to assimilate glycerol compared to CV103. Since glycerol is secreted into the medium by both strains, the level of glycerol in the medium will be strictly determined by population density. The stable polymorphism between CV103 and CV116 may therefore also be expected to be density dependent. At population densities yielding glycerol levels on the order of the K_m for the glycerol assimilation machinery of the cell, the ability of CV116 to utilize glycerol as an alternative carbon source, will be significantly attenuated. Thus, below a critical population density the concentration of glycerol will be insufficient to provide a growth advantage to CV116. We tested this prediction by maintaining cultures of the two strains at different culture densities. The results (Figure 5c) show that, as expected, the equilibrium frequencies of the two strains are dependent on the density of the culture.

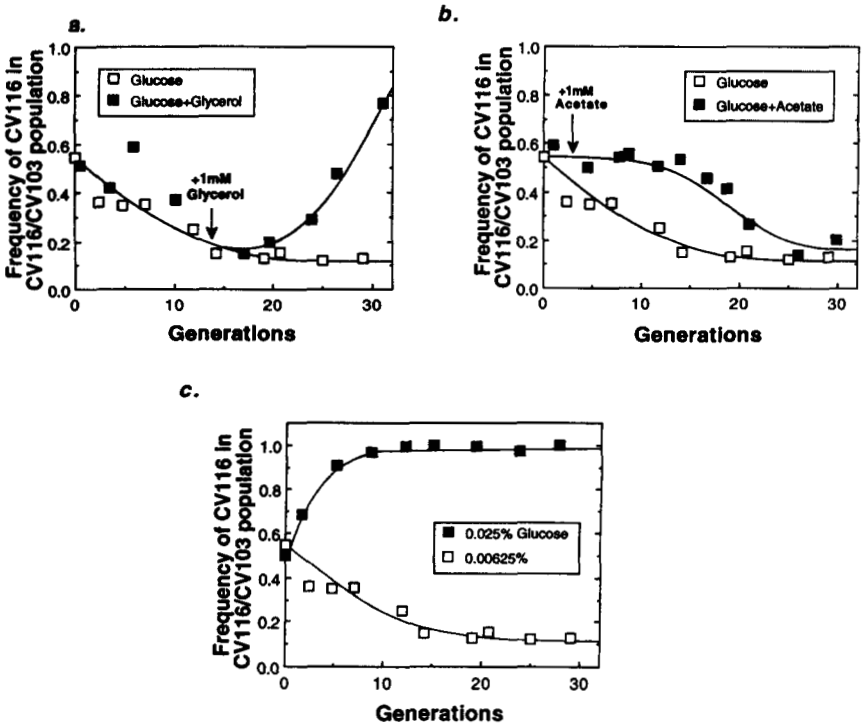

FIGURE 5.—Stable polymorphism between CV103 and CV116. (a) The frequencies of CV103 and CV116 at equilibrium are not affected by the level of acetate in the medium. □, Frequency of CV116 in medium with 0.00625% glucose (w/v). (■), Frequency of CV116 in medium with 0.00625% glucose + 1 mM acetate. (b) The frequencies of CV103 and CV116 at equilibrium are affected by the level of glycerol in the medium. □, Frequency of CV116 in medium with 0.00625% glucose. ■, Frequency of CV116 in medium with 0.00625% glucose; 1 mM glycerol was added to the medium reservoir and the culture vessel at the time shown by the arrow. (c) The frequencies of CV103 and CV116 at equilibrium are affected by total population density. □, Frequency of CV116 in medium with 0.00625% glucose (w/v). Population density was ~5.9×10^7 ml^{-1}; ■, frequency of CV116 in medium with 0.025% glucose (w/v). Population density was ~2.4×10^8 ml^{-1}. In each case results from a representative experiment are shown; replicate experiments gave concordant results in all cases.

Physiological basis for enhanced glycerol uptake in CV116: In *E. coli*, glycerol transport and dissimilation is governed by genes encoded by the glycerol regulon (FREEDBURG and LIN 1973). Glycerol is assimilated by a two-step pathway involving first, facilitated diffusion mediated by the glpF protein, and second, phosphorylation by the product of the *glpK* gene, glycerol kinase (LIN 1984). Glycerol 3-phosphate (G3P) may then enter either biosynthetic or energy-yielding pathways. Entry into the latter requires that G3P be converted to the glycolytic intermediate dihydroxyacetone phosphate, a reaction mediated either by the enzyme encoded by the *glpD* locus, aerobic glycerol-3-phosphate dehydrogenase, or by that encoded by the *glpA* locus, anaerobic glycerol-3-phosphate dehydrogenase. These four loci plus another, *glpT*, that encodes a protein which facilitates diffusion of extracellular G3P, are thought to be co-ordinately regulated through the *glpR* locus (LIN 1987; IUCHI *et al.* 1990).

We therefore estimated the specific activities of glycerol kinase, believed to catalyze the rate-limiting step in glycerol dissimilation (ZWAIG *et al.* 1970) and an enzyme metabolically downstream, aerobic glycerol-3-phosphate dehydrogenase. The results show (Table 5) that the activities of both enzymes are significantly reduced in strain CV103 compared to JA122 and the other adaptive clones. It would appear then that the equilibrium between CV103 and CV116 could be explained by differences in glycerol metabolism reflected both in their uptake capacity and regulation of enzymes involved in glycerol transport and dissimilation.

Analysis of the CV101, CV116 polymorphism: CV116, in comparison to CV101, possesses a superior growth rate, when all components of the medium are in excess, and scavenges a larger proportion of the glucose present in the medium (Table 2). Therefore if glucose were the sole carbon source available, CV116 should rapidly displace CV101 from the population. The stable equilibrium seen (see Figure 1d) with CV101 maintained at a low frequency may be explained by the utilization of acetate as an alternative carbon source by CV101. Monocultures of CV116 possess equilibrium acetate levels lower than CV103 but significantly higher than CV101. Addition of acetate to mixed cultures of CV101 and CV103 approaching equilibrium result in an increase in the frequency of CV101. Figure 7a shows that, as predicted, the frequency of CV101 increases after addition of acetate.

Compared to CV101, CV116 possesses an increased rate of uptake of glycerol, the second alternative metabolite identified. However, the equilibrium frequency of CV101 will be expected to be determined primarily by the acetate levels and should not be affected significantly by the addition of glycerol, unless this metabolite increases or decreases the pattern of secretion and/or uptake of acetate. Figure 7b shows that, as expected addition of glycerol does not affect the equilibrium frequency detectably.

DISCUSSION

We have presented evidence that the evolutionary process by which a clonal microbial population adapts to a simple, constant environment can favor the establishment of stable polymorphisms. In addition, a number of reports in the literature lead us to believe that this is not an isolated occurrence, and that polymorphisms

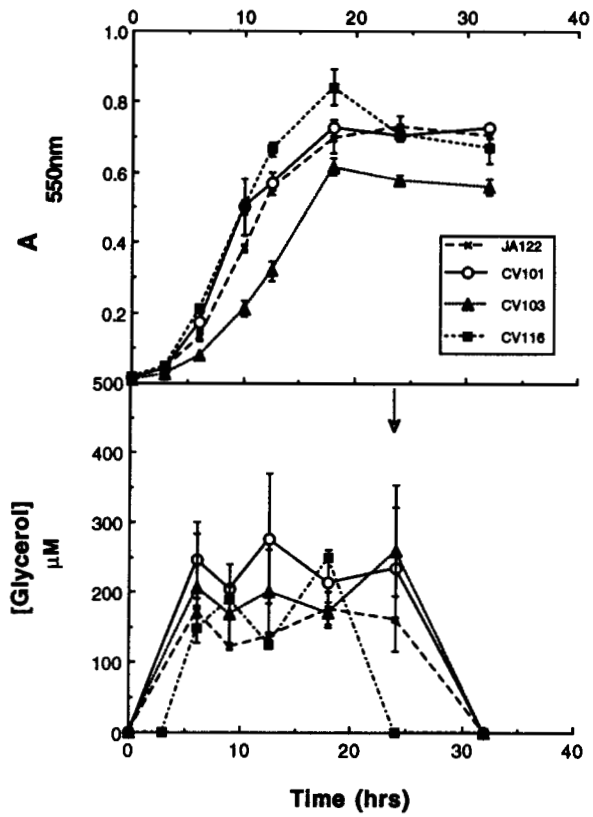

FIGURE 6.—Strain CV116 has differentiated from the parent strain JA122 and the other evolved strains with respect to glycerol metabolism. Population growth and extracellular glycerol concentration in 0.1% glucose batch cultures JA122 (×-----×), CV101 (○——○), CV103 (▲·····▲), and CV116 (■- - - -■). Glycerol is eliminated from the culture media of CV116 during early stationary phase (see arrow). Standard errors for some time points were smaller than the symbols on the graphs and are therefore not shown.

have been observed to develop in other organisms, growing in comparably simple environments (ADAMS *et al.* 1992; CLAASEN *et al.* 1986; HELLING *et al.* 1987; JOHNSON and MCKAY 1977). We may expect that in more complex environments and among more complex multicellular organisms, where the range of secreted metabolites may be greater, the opportunity for the development of polymorphisms maintained by interactions between the components, will be correspondingly greater.

Adaptive genetic changes that allow some members of the population to exploit a limiting resource more successfully appear to render them less efficient in their capacity to assimilate secondary metabolites. These residual metabolites comprise alternative resources and therefore offer niches which allow the evolution of strains with an enhanced capacity to exploit those resources. The evolution of resource partitioning which has occurred, compounded by the evolution of other differences among the three components may illustrate incipient speciation within the population. Our findings suggest that polymorphisms which evolve within an asexual microbial population can be maintained by the same type of density-dependent interactions sustaining microbial species diversity in nature. They also suggest that, in *E. coli*, metabolism of glucose, acetate and glycerol are interrelated and subject to interacting regulatory constraints.

TABLE 5

Specific activity of glycerol assimilation enzymes

Strain	Glycerol kinase	Glycerol-3-phosphate dehydrogenase
JA122	5763 ± 376	61 ± 6
CV103	2181 ± 411	29 ± 4
CV101	5608 ± 449	56 ± 7
CV116	5996 ± 340	61 ± 9

Values are presented as units/g soluble protein ± standard error of the mean.

Interactions within the evolved polymorphism resemble interspecific interactions within microbial communities: Many workers have shown that metabolic interactions are essential to the establishment and maintenance of microbial communities encountered in nature (WILKINSON *et al.* 1974; REANNEY *et al.* 1983; ZEIKUS 1983; COUTTS *et al.* 1987). In a classic study, SLATER and co-workers (SENIOR *et al.* 1976) isolated by enrichment a microbial community growing on the herbicide Dalapon (2,2-dichloropropionic acid) that included four pseudomonads, a fungus, *Trichoderma viride*, a flavobacterium, and an unidentified gram-negative bacterium. Maintenance of this seven-membered assemblage was explained by a putative suite of metabolic interactions.

The conditions which prevail in mixed cultures of CV103, CV116 and CV101 are analogous not only to conditions in natural communities, but also to those in artificial communities established for the purpose of investigating mixed substrate utilization by competing microbial species [reviewed by GOTTSCHAL (1986); also see DYKHUIZEN (1990)]. For example, a mixotrophic generalist Thiobacillus A2, capable of growth on either acetate or thiosulfate, and an autotrophic specialist *Thiobacillus neopolitanus*, capable of growth on thiosulfate only, can be stably maintained in continuous culture using thiosulfate as the sole limiting nutrient. This association is made possible by the specialist excreting a metabolite, glycollate, that can be used by the generalist as an alternative energy source (GOTTSCHAL *et al.* 1979). A third component, a heterotrophic Spirillum which specializes on acetate, can be added to this association provided that the growth media is supplemented with acetate (GOTTSCHAL *et al.* 1979; GOTTSCHAL and THINGSTAD 1982). However, this three-membered association appears to be driven by an additional factor: not only can Thiobacillus A2 metabolize glycollate, it also demonstrates higher yield on a combination of the primary substrates than on either acetate or thiosulfate alone (GOTTSCHAL and KUENEN 1980).

The present study is unique, however, in that the members of the assemblage we have characterized,

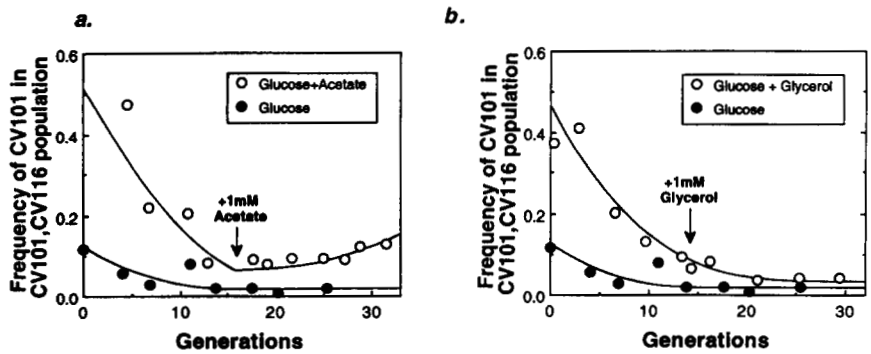

FIGURE 7.—Stable polymorphism between CV101 and CV116. (a) The frequencies of CV101 and CV116 at equilibrium are affected by the level of acetate in the medium. ●, frequency of CV101 in medium with 0.00625% glucose (w/v). ○, Frequency of CV101 in medium with 0.00625% glucose (w/v); 1 mM acetate was added to the culture and the medium reservoir at the time shown by the arrow. (b) The frequencies of CV101 and CV116 at equilibrium are not significantly affected by the level of glycerol in the medium. ●, Frequency of CV101 in medium with 0.00625% glucose (w/v). ○, Frequency of CV101 in medium with 0.00625% glucose; 1 mM glycerol was added to the medium reservoir and the culture vessel at the time shown by the arrow. In each case results from a representative experiment are shown; replicate experiments gave concordant results in all cases.

descended from a common ancestor. Thus the association is not ecologically fortuitous, but rather the result of evolution. Previous work has shown that the adaptive clones are strikingly different from the parent strain and one another with respect to global patterns of gene expression revealed by two-dimensional gel electrophoresis (KURLANDZKA *et al.* 1991). Phylogenies constructed with these data consistently root CV103 closest to the common ancestor, JA122 (A. KURLANDZKA and J. ADAMS, unpublished observations).

We can therefore imagine the following evolutionary scenario. In a constant, homogenous environment where bacterial growth is limited by the availability of glucose, selection initially favored genetic change(s) that increased the capacity to scavenge the primary resource. Thus, a CV103-like strain possessing superior glucose uptake kinetics (HELLING *et al.* 1987) arose first. However, pleiotropic effects of these adaptive changes resulted in a phenotype that both overproduced and was unable to resorb acetate, and that was compromised in its ability to assimilate glycerol. Selection thereafter favored genetic change(s) which either conferred restrictive access to these secondary resources or which resulted in higher yield on some combination of glucose, acetate and glycerol as a consequence of efficient cometabolism. It is possible that CV101 and CV116 arose independently from minority clones that persisted in the population even as the CV103 ancestor proliferated.

Physiological-genetic differentiation among adaptive clones may be constrained by co-ordinate regulation of catabolic pathways: It is surprising that 773 generations of growth under highly selective conditions failed to produce a strain that combines maximum glucose uptake with maximum ability to recover secondary metabolites. Furthermore, both the morphological and ampicillin resistance polymorphisms initially used to identify CV101, CV103 and CV116 strains were maintained in a long term reconstruction experiment extending over 400 further generations (HELLING *et al.* 1987). This finding suggests that the glucose, acetate and glycerol catabolic pathways are coupled in a manner that precludes the creation of a single optimal strain.

The production of overflow metabolites such as acetate, pyruvate, lactate and glycerol is a common feature of bacterial growth on a fermentable carbon source (TEMPEST and NEIJSSEL 1978; GOTTSCHALK 1979). Production of these metabolites is inevitably associated with anaerobic metabolism (TEMPEST and NEIJSSEL 1987). For example, most ATP generated under anaerobic conditions results from the acetate kinase-mediated conversion of acetyl phosphate to acetate (KOPLOVE and COONEY 1978). Under aerobic conditions excretion of overflow metabolites does not appear to be energetically costly, and in fact has been found to be associated with increased energy flux measured in terms of respiration rate (TEMPEST and NEIJSSEL 1987). In particular, acetate overflow appears to be associated with high rates of glucose catabolism, and has been related to the problem of maximizing ATP and GTP equivalents subject to the constraints of rate limitation both in the capacity of the electron transport system to turn over NADH and FADH as well as the capacity of TCA cycle enzymes such as α-ketoglutarate dehydrogenase to process triose generated by glycolysis (EL-MANSI and HOLMS 1989; MAJEWSKI and DOMACH 1990).

The extent to which such metabolites are secreted by *E. coli* growing in aerobic glucose-limited chemostats may depend on both the strain and operating parameters (HEMPFLING and MAINZER 1975; FIESCHKO and RITCH 1986; BAJPAI 1987; TEMPEST and NEIJSSEL 1987), though acetate is most commonly found. Furthermore, measurements of metabolite concentrations in cultures at physiological equilibrium will represent a balance between secretion and uptake. The low level of glycerol may therefore obscure a high flux through a secretion-uptake cycle. In addition, to our knowledge, no previous

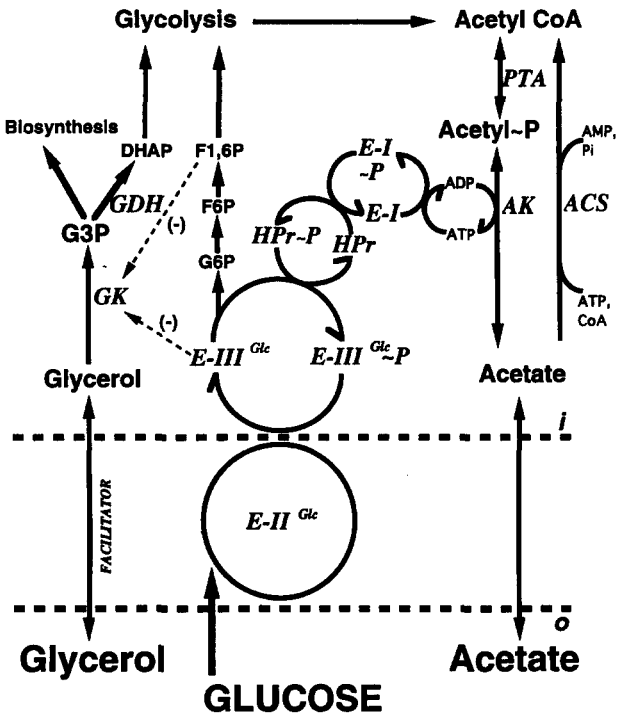

FIGURE 8.—Possible sites at which glycerol and acetate metabolism may be integrated with the sugar phosphotransferase system (PTS) in *E. coli*. The phosphoenzyme intermediate of acetate kinase (AK) can serve as a phosphoryl group donor to enzyme I (E-I) of the PTS. Glycerol kinase (GK) is subject to noncompetitive allosteric inhibition by the unphosphorylated form of glucose-specific GLC enzyme III (E-III^{Glc}). Other abbreviations: glycerol-3-phosphate dehydrogenase (GDH), phosphotransacetylase (PTA), acetyl-CoA synthetase (ACS), dihydroxyacetone phosphate (DHAP), glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), fructose 1,6-diphosphate (F1,6P), adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), coenzyme A (CoA).

studies have examined changes in steady-state levels of overflow metabolites as a consequence of adaptive changes taking place under long term nutrient limitation.

Two recent findings support the idea that evolution of an enhanced glucose uptake system could result in increased levels of residual acetate and glycerol. Figure 8 diagrams the essential features of the dissimilatory pathways of these three metabolites. Glucose is imported into the bacterial cell and converted to the glycolytic precursor glucose 6-phosphate by the four-enzyme phosphoenolpyruvate:glycose phosphotransferase (PTS) system [see POSTMA (1987) and references therein]. Glycerol and acetate metabolism both appear to be directly coupled to the PTS system. Glycerol kinase is subject to noncompetitive allosteric inhibition by both fructose 1,6-diphosphate and the unphosphorylated form of enzyme III^{Glc} (POSTMA *et al.* 1984; NOVOTNY *et al.* 1985; DE BOER *et al.* 1986; HURLEY *et al.* 1993; LIN 1987). By this means threshold concentrations of PTS sugars inhibit glycerol kinase activity and restrict the formation of its product, glycerol 3-phosphate (G3P). Since this intermediate is the true inducer of the glycerol regulon, expression of the facilitator protein and G3P dehydrogenases are also restricted. Genetic adaptation(s) resulting in improved glucose uptake would effectively lower the threshold value of PTS sugar that maintains this inhibition, therefore, any concomitant increase in extracellular glycerol due to enhanced carbon flow would remain unmetabolized. Phosphorylated acetate kinase effectively substitutes for phosphoenolpyruvate (PEP) by donating its phosphoryl group to enzyme I of the PTS system (FOX *et al.* 1986). In a glucose-limited environment restrictions on available PEP could make PTS enzyme I phosphorylation the rate-limiting step in glucose uptake. Since the conversion of acetyl phosphate to acetate produces the phosphoenzyme intermediate of acetate kinase (FOX and ROSEMAN 1986), overexcretion of acetate could provide an additional source of phosphoryl groups to drive sugar uptake.

Adaptive significance of genetic changes in CV103: During the course of evolution the majority clone CV103 has accumulated genetic changes that alter glucose uptake kinetics (HELLING *et al.* 1987), as well as the excretion and assimilation of acetate and glycerol. The selective advantage of improving glucose recovery in a glucose-limited environment is obvious, and consistent with other reports that prolonged exposure of a population to nutrient limitation results in adaptive mutants which better scavenge the limiting substrate (COLLINS *et al.* 1976; HARDER and DIJKHUIZEN 1983; HOFLE 1982, 1983). Overexcretion of acetate may be part of a suite of adaptations that enhance glucose recovery. Under carbon limitation increased acetate formation through the acetate kinase reaction could increase the total pool size of phosphoryl group donors for enzyme I of the PTS system. However, increased glucose recovery by this mechanism may be secondary to the imposition of tighter constraints on respiration either at the level of electron transport or within the TCA cycle.

The apparent null mutation at ACS may impose such a constraint. Acetate is a known to act as a potent growth rate inhibitor, even at micromolar concentrations among *E. coli* growing at neutral pH (LULI and STROHL 1990). This activity is thought to be related to its ability to uncouple metabolism by disrupting the proton motive force (REPASKE and ADLER 1981; SMIRNOVA and OKTYABR'SKII 1985, 1988). CV103 is incapable of metabolizing acetate at concentrations of 1 mM or less. We may expect therefore that even at acetate levels observed in chemostat cultures, respiration in CV103 will be restricted, and glucose transport capacity commensurately improved. Moreover, the acetate produced by CV103 would act to inhibit the growth of competing strains. In this regard, we have observed that CV116 grown in spent CV103 batch culture media to which 0.1% glucose was added, grows more slowly than when cultured in fresh

media containing glucose; neither strain exhibits growth rate depression when cultured in its own spent media plus glucose (data not presented). However, the presence of the acetate scavenging strain CV101 would mitigate the negative effect of acetate on the three member stable polymorphism.

The observation that CV103 shows reduced activity of both glycerol kinase and glycerol dehydrogenase may not have a direct genetic basis, but simply reflect improvements in the enzymatic machinery that recovers glucose. We have already noted that glucose restricts glycerol regulon expression through the interaction of unphosphorylated enzyme III with glycerol kinase. At any given glucose concentration, therefore, a superior uptake system should allow that substrate to act as a stronger repressor. Alternatively, decreased expression may be the consequence of pleiotropic mutations which result in an overall reduction of protein synthesis (KURLANDZKA *et al.* 1991). The observed changes are consistent with a diminished capacity to assimilate glycerol relative to CV116. Surprisingly, glycerol assimilation is comparably reduced in the parent strain JA122 without significant decreases in the activity of either glycerol kinase or glycerol dehydrogenase. It is difficult to reconcile these results without invoking the operation of additional, unknown metabolic constraints on glycerol assimilation in the parent.

Adaptive advantage of genetic changes in CV101: It is reasonable to assume the appearance of a CV103-like strain early in the evolutionary sequence that established this polymorphism. The proliferation of such a strain created a selective environment that favored mutation(s) giving cells access to low concentrations of acetate. The population response to this pressure was a CV101-like strain, wherein ACS was constitutively overexpressed. ACS is normally subject to glucose repression, although complete derepression seems to depend critically upon minimum threshold values of both glucose and acetate (BROWN *et al.* 1977; F. R. ROSENZWEIG and J. ADAMS, unpublished observations). The CV101 ACS mutant is derepressed in the absence of both signals and shows a twofold increase in activity relative to the parent strain growing under inducing conditions.

Adaptive advantage of genetic changes in CV116: CV116 presents a suite of traits that suggest it uses a generalist strategy to persist in the nutrient-limiting environment. All three adaptive clones show enhanced glucose recovery compared to the parent strain JA122 (HELLING *et al.* 1987). However, a comparison of residual substrate levels suggests that while CV116's capacity to recover glucose does not match CV103, it does exceed that of CV101 (Table 2). In addition, CV116 appears to retain the parent strain's ability to express low levels of ACS under nutrient-limiting conditions. This ability is consistent with observations that CV116 monocultures have significantly lower residual acetate levels than CV103, and that the addition of acetate to mixed CV101/CV116 populations does not promote takeover by CV101. The former observations are also consistent with evidence that adaptive changes have occurred in CV116 that increase yield presumably by restricting efflux of overflow metabolites such as acetate (HELLING *et al.* 1987).

Compared to the parent strain and the other adaptive clones, CV116 exhibits a significantly greater capacity to recover glycerol. However, in comparison with the parent strain this difference cannot be explained on the basis of relative activities of either glycerol kinase or aerobic glycerol-3-phosphate dehydrogenase. It may be that CV116 has achieved increased capacity by changes in the expression levels or substrate affinities of other enzymes in glycerol assimilation such as the glycerol facilitator protein or anaerobic glycerol-3-phosphate dehydrogenase. Alternatively, mutation(s) may have occurred within the CV116 *glpK* locus that remove allosteric inhibition by unphosphorylated glucose specific E-III and/or fructose 1,6-diphosphate thereby increasing *in vivo* activity. Such a *glpK* mutant has been isolated by DE BOER *et al.* (1986).

An evolved polymorphism is maintained by a combination of adaptive strategies. Diversification of a clonal population of *E. coli* growing in a simple, glucose-limited environment has given rise to a polymorphism consisting of strains which have adopted either a specialist or generalist strategy. Physiological data suggest that strain CV103 is a glucose specialist, however, the genetic changes which have allowed CV103 to adopt this strategy have resulted in the net efflux of metabolizable carbon. These same data suggest that CV101 can be considered acetate specialist. By contrast, CV116 appears to be a generalist, capable of cometabolizing glucose and overflow metabolites. The structure of the evolved polymorphism is therefore similar to the stable three-species association assembled by GOTTSCHAL *et al.* (1979) and GOTTSCHAL and THINGSTAD (1982).

It seems clear from our analyses that even starting with the simplest possible genetic and environmental conditions, complexity is generated from uniformity, allowing biodiversity to build upon itself. It is appropriate to point out that the polymorphisms analyzed here were detected because the component clones possessed easily identifiable phenotypes. It is possible that more extensive screens may uncover more subtle polymorphisms, as well as additional interactions.

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