Identification of Adaptive Changes in an Evolving Population of *Escherichia coli:* The Role of Changes with Regulatory and Highly Pleiotropic Effects[1]

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A population of *Escherichia coli* initiated with a single clone developed extensive morphological and physiological polymorphism after being maintained for 773 generations in glucose-limited continuous culture. To understand the mechanisms of adaptation to this environment, total protein patterns of four adaptive clones and of the parent strains were examined by two-dimensional gel electrophoresis. Approximately 20% of the proteins (~160 in absolute numbers) showed significantly different levels of expression in pairwise comparisons of parent and adapted clones. The extent of these changes points to the importance of mutations with regulatory and/or highly pleiotropic effects in the adaptive process. The four evolved clones all expressed fewer proteins than did the parent strain, supporting the hypothesis of energy conservation during evolutionary change. Forty-two proteins that could be assigned to known cellular functions were identified. The changes in some of them indicated that the evolved clones developed different adaptive mechanisms to glucose-limited environment. Changes were observed in the expression levels of proteins associated with translation, membrane composition, shock response, and active transport. A fraction of the changes could not be either explained or predicted from a consideration of the nature of the environment in which the clones evolved.

Introduction

A number of studies have shown that microbial populations adapt to a simple laboratory environment by repeated sequential replacement of one clone by another, each having an increased level of adaptation to the environment (e.g., see Dykhuizen and Hartl 1981; Paquin and Adams 1983*a*; Helling et al. 1987). The lack of recombination in such populations allows clonal replacements to be detected without a knowledge of the phenotype being selected, by monitoring the frequency dynamics of an unselected or neutral marker (e.g., see Paquin and Adams 1983*a*). In populations of *Escherichia coli,* clonal replacements were seen, with no apparent decrease in their rate of appearance, until the termination of the experiments after almost 2,000 generations (Helling et al. 1987; J. Adams and R. B. Helling, unpublished data). Similar results have been reported for evolving populations of *Saccharomyces cerevisiae,* maintained for shorter periods of time (Paquin and Adams 1983*a*). These results

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suggest either that the populations may be significantly removed from an "adaptive peak," representing the optimum genotype, or that a single optimally adapted genotype does not exist (Paquin and Adams 1983*b*; also see Gould and Lewontin 1979).

Although many studies have documented the extent and rate of adaptive change in simple laboratory environments, identification of the molecular and biochemical bases for these changes has proved more difficult. When growth and fitness are determined by limiting concentrations of a single substrate, it is reasonable to expect that mutations in the loci coding for proteins involved in the uptake and metabolism of that limiting substrate will be selected. Thus, Adams et al. (1985) and Helling et al. (1987) demonstrated significant increase in the rate of glucose transport in adaptive clones isolated from populations maintained for long periods of time under conditions of glucose limitation. Similarly, Francis and Hansche (1972, 1973) were able to detect, at the locus *pho5* coding for acid phosphatase, structural changes that resulted in increased activity, in clones isolated after long-term growth under conditions where growth was limited by the concentration of organic phosphate. It is equally clear that many changes occurring in response to growth under defined conditions cannot easily be explained by changes in the uptake systems for the relevant substrates (Francis and Hansche 1972, 1973); neither can they be easily predicted (e.g., see McDonald 1955).

Where the specific phenotype selected has been identified, it has sometimes also been possible to distinguish between changes at the structural loci responsible, changes in gene dosage, and regulatory changes (e.g., see Horiuchi et al. 1962; Rigby et al. 1974; Hall 1984). In many of these cases, changes have occurred at the level of gene dosage or gene regulation. However, the number of such cases remains small and only involve those phenotypes for which an increase in gene expression would be expected to confer a selective advantage, such as increases in transport rates. When the totality of all phenotypic changes are considered, it is not clear how important the regulatory changes are in the process of genetic adaptation of microbial populations. As Ayala and Kiger (1984) have suggested in a more general context, "the role of gene regulation in adaptive evolution remains one of the major unsolved issues in evolutionary genetics."

The analysis of total cellular proteins by two-dimensional electrophoresis has been extensively used in different types of experiments because of its ability to display a global picture of genome functioning during cell differentiation or during adaptation to different growth conditions (for reviews, see Celis and Bravo 1984; and Neidhardt and Van Bogelen 1987). The high sensitivity of this technique (up to 1,000 proteins resolved on one gel) allows the overall characterization of a bacterial strain. Therefore, it seems reasonable that, by comparing original and adaptive strains, this tool might be useful for illustrating the changes which occur when microorganisms evolve.

In the present study we analyzed four clones isolated from a polymorphic population of *E. coli* that had been grown for 773 generations in a glucose-limited chemostat (Helling et al. 1987). Our purpose was to understand the mechanisms of adaptation to this environment as well as to characterize the range of strain-to-strain protein changes. The number of changes found point to the importance that regulatory mutations with highly pleiotropic effects have in adaptation. A number of proteins exhibiting variation in their abundance among strains could be assigned to specific functional groups. These classifications include translation, shock and stringent response, and the lambda-phage life cycle and therefore span a wide range of cell functions. Although some of the changes were not unexpected from a consideration of the en-

vironment in which the population was evolving, the functional significance of others remains refractory.

Material and Methods

Strains, Media, and Culture Conditions

Escherichia coli strains are listed in table 1. Strains were grown in batch culture in Davis (Helling et al. 1981) or MOPS (Neidhardt et al. 1974) minimal media for pilot experiments and were grown in Davis minimal medium in chemostats. Glucose concentration was 0.025% w/v (14 mM) in batch cultures and was 0.0125% w/v (7 mM) in chemostats. The growth temperature was 30°C. In chemostats, cells were grown in aerated culture vessels 145–190 ml in volume, at a dilution rate of $D \cong 0.2$ h^{-1} following procedures described by Helling et al. (1987). Cell densities were 1–2 $\times 10^8$ cells/ml for chemostats and were 2–3 $\times 10^8$ cells/ml in batch cultures, at the moment when the isotope was added. In chemostats, cells were maintained for 19–20 generations prior to labeling; in batch culture, cells were grown overnight, and the next day a new culture was inoculated to $OD_{420} \cong 0.1$ ($\sim 3.8 \times 10^7$ cells/ml) and was maintained for about 3 generations.

Labeling Procedure

In batch cultures, ^{35}S-methionine (tran ^{35}S-label; ICN Radiochemicals) was added to 1 ml of culture to a final concentration of $\sim$30 μCi/ml. The culture was incubated with isotope for 30 min at 30°C with shaking, and then cells were treated as described in the following section. In chemostat cultures, samples (4–6 ml, depending on cell densities) were taken from a chemostat and were transferred to 15-ml tubes containing 50–75 μCi ^{35}S methionine (NEN Research Products) to give the isotope a final concentration of 12.5 μCi/ml. The specific activity of the isotope used was 1,140–1,198 Ci/mmol. Cells were incubated with the isotope for 30 min with vigorous shaking. Cells were transferred to test tubes and were incubated with isotope at 30°C to avoid any temperature change. After incubation, test tubes were put on ice, and a few ice crystals were added into each tube for rapid cooling. Cells were spun down in a microfuge for 5 min at 4°C. The pellet was washed four times with ice-cold growth medium and once with ice-cold water.

Table 1
Bacterial Strains

Strain	Relevant Characteristics
JA122	F⁻ *thr1 leu5 thi1 lacY1 tonA21 supE44 hss1* lysogenic for *lambda* contains plasmid pBR322Δ5
CV101	Derivative of JA122, isolated after 773 generations of growth in glucose-limited chemostat culture; contains plasmid
CV103	As CV101, but independent isolate which forms small colonies; contains plasmid
CV115	As CV101, but independent isolate; colony size as JA122; lacks plasmid
CV116	As CV101, but independent isolate; lacks plasmid; forms small colonies

SOURCE.—Helling et al. (1987).

Sample Preparation

In batch cultures, samples were prepared for resolution on two-dimensional electrophoresis according to the method of O'Farrel (1975), as modified by Phillips (1988), by lysis in SDS/β-mercaptoethanol/Tris-HCl buffer at 100°C. In chemostat cultures, samples were prepared according to Protein Databases, Inc. (PDI; Huntington Station, N.Y.) protocols, with PDI sample kit PDI-1001. Cells were solubilized by the addition of hot SDSBME buffer (0.3% sodium dodecyl sulfate, 5% β-mercaptoethanol, and Tris buffer-HCl pH 8.0) and were kept at 100°C for 4 min. The lysate was treated with DNARNASE solution (a mixture of DNase and RNase) and was snap-frozen in liquid nitrogen. Samples packed in dry ice were shipped to PDI and were electrophoresed there.

Electrophoresis

Two-dimensional electrophoresis was carried out on batch-culture samples in an O'Farrell (1975) system, with modifications described in detail by Phillips (1988). The isoelectric-focusing first-dimension gel contained 9.5 M urea, 2% Nonidet P-40 or Triton X-100, and a 0.5% blend of LKB (LKB Produkter AB, Bromma, Sweden) ampholites pH 5–7:pH 3.5–10 in an 8:2 proportion. An SDS-polyacrylamide gel in 11.5% acrylamide was run in the second dimension. First-dimension gels were 12 cm long, and second-dimension (hereafter referred to as "small") gels were 12.5 cm ×12.5 cm. Chemostat-culture samples were run by PDI with isoelectric focusing in the first dimension and with SDS in second dimension, according to the method of Garrels (1983). In the first dimension the pH was in a range maintained as described above. The acrylamide concentration in second dimension was 11.5%, as before. In addition, cellular proteins resolved by using nonequilibrium pH gradient electrophoresis in the first dimension were run according to the method of Garrels (1983). In first dimension, chemostat-culture samples were separated on 20-cm-long gels, and in the second dimension they were separated on 24-cm × 24-cm (hereafter referred to as "large") gels.

The amounts of radioactivity loaded for each sample on first-dimension (i.e., small) gels were $\sim 10^6$ cpm (as counted on a Beckman LS 7000 scintillation system; efficiency > 90%), and those loaded for each sample on second-dimension (i.e., large) gels were 357,711–401,840 cpm (as counted by PDI). The large gels, with their corresponding calibration strips containing known amounts of radioactive protein, were then processed for fluorography, and three exposures of each gel were made. The small gels were subjected to autoradiography without fluorography and without calibration strips. Single exposures of the small gels were selected for scanning and analysis.

Scanning and Image Processing

Autoradiograms and fluorograms were scanned with an Eikonix 78/99 camera system at a resolution of 200 μm (1,120 × 1,120 pixels). PDQUEST computer-analysis software release 2.7 (from PDI) was used to scan the gels and to aid in their comparison and analysis. The three exposures of each large gel were first merged into one image. This allowed accurate quantitation over a much greater range than would be possible with a single exposure. For both gel types, two critically important steps in the analysis of the two-dimensional-gel patterns of protein expression are (1) the resolution of the individual spots on the gels and (2) the comparison of two or more gels which exhibit subtle variations in mobility in both dimensions (pattern matching). As required by the software, it was first necessary to create a composite image "standard" which

consisted of all spots present in at least one gel being studied. The standard for batch culture (small gels) contained 317 spots, while that for chemostat cultures (large gels) had 743 spots. In the following discussion we refer to these two types of gels as "batch-culture gels" and "chemostat gels." The relative levels of protein expression are calibrated in disintegrations per minute (dpm). For batch-culture gels these levels were 0–5,119, and for chemostat gels they were 0–>10,000.

Statistical Analysis

To estimate the error associated with the density of the spots, replicate cultures (chemostats) were set up, and replicate protein preparations were made. The replicate chemostats were inoculated with single clones obtained from different streakings from the stock of the parent strain stored at −70°C. Replicate samples were prepared by using different batches of media, different protein-preparation stock solutions, and different radioisotopes, and the two-dimensional gels were run more than a month apart in different batches. However, the chemostat cultures of the five strains examined were initiated at approximately the same time, the protein extracts were prepared at the same time, and the two-dimensional gels were run in the same batch. This procedure therefore will tend to overestimate *within*-strain error relative to differences *between* strains and thus make our test conservative.

The variance between the replicates was then calculated for each spot. Since the error associated with spot density is dependent on the density of that spot, we then performed a variance-stabilizing transformation to obtain an expected-error term associated with a particular spot density. A third-degree polynomial regression was carried out on the individual variance estimates regressed against spot density, and the resulting estimates of the polynomial regression coefficients were used for the transformations. The fits of the third-degree polynomial to the data were very good, giving r^2 values of ~0.98. Strains were then compared pairwise, by calculating the differences (d) between the spot densities. Since the error of a difference is two times the error variance associated with the individual spots, the test statistic, T, is $t_{0.05}\sqrt{2}\hat{\sigma}_d$. Since the number of spots was on the order of 300 (for the small gels) to 700 (for the large gels), the number of degrees of freedom is extremely large. However, for both gel types, the number of degrees of freedom used was 60, the largest number available.

This test procedure assumes that the error in spot density is distributed normally, with the variance a function of spot density—i.e., $N(\mu, \sigma_x)$, where x is the spot density. Thus, the error associated with each spot is assumed to be dependent on its density and *not* on the particular spot. If the error associated with each spot were different, this would not necessarily affect the validity of the test, since the *average* variance would be estimated. However, it is possible that the majority of the spots have more or less the same error associated with them but that there are a few which are intrinsically much more variable than the rest. In this case, the distribution of error terms would be skewed to the right, and our test would be conservative. In addition, the test assumes that the error in spot density has the same distribution in all the strains.

Enzyme Assays

Cells were grown in chemostat culture, under the same conditions used for two-dimensional gel analyses. Aliquots (100 ml) of cell suspension were removed from the chemostat and were centrifuged for 10 min at 12 *K*, 4°C. The resulting pellets were resuspended in an extract buffer containing 50 mM potassium phosphate, 2 mM ethylenediaminetetraacetate, 2 mM β-mercaptoethanol, 100 μM phenylmethylsulfonyl

fluoride, 50 μM cocarboxylase (thiamin pyrophosphate), pH 7.1 and were centrifuged for 3 min at 13 *K*. Cells were then stored at −20°C prior to disruption and assay. Since the amount of cellular material obtained from any sampling was small, two consecutive samples from each chemostat were pooled. These were resuspended in extract buffer to a concentration of 200 mg/ml (wet wt/vol), were passed twice through a French press at 12,000 psi, and then were centrifuged for 10 min at 12 *K*, 4°C. Enzyme assays were performed on the supernatant containing the crude extract. All assays were conducted at 25°C in 1-ml volumes by using conventional spectrophotometry. Changes in absorbance were followed by using a Gilford model 250 spectrophotometer and a Gilford model 6051 recorder. Pyruvate dehydrogenase complex was assayed, by a modification of the method of Visser et al. (1982), in 60 mM potassium phosphate, 2 mM $MgCl_2$, 2.6 mM dithiothreitol, 0.13 mM coenzyme A, 0.75 mM β-NAD^+, 0.4 mM TPP, 5 mM sodium pyruvate, pH 7.0. After equilibration for 5 min at 25°C, the reaction was initiated by the addition of 50–100 μl of appropriately diluted extract. The change in absorbance was followed at 340 nm.

Malate dehydrogenase, isocitrate dehydrogenase, and isocitrate lyase were all assayed essentially according to the method of Holms and Bennett (1971). For malate dehydrogenase, 10 μl of appropriately diluted sample was added to 25 mM glycylglycine, 1 mM ethylenediaminetetraacetate, 0.15 mM NADH, pH 8.9 and was equilibrated for 3 min. The reaction was initiated by addition of oxaloacetate to a final concentration of 0.2 mM, and the disappearance of reduced dinucleotide was monitored as above. Isocitrate dehydrogenase was assayed in 50 mM Tris, 0.25 mM $MnCl_2$, 1 mM isocitrate, pH 7.5. The reaction was initiated by the addition of $NADP^+$ to a concentration of 1 mM. The increase in absorbance due to NADPH formation was measured at 340 nm. Isocitrate lyase was assayed in 67 mM KH_2PO_4, 5 mM $MgSO_4$, 2 mM L-cysteine-HCl, 3.3 mM phenylhydrazine, pH 6.8. After a 3-min equilibration with 20 μl of sample, the reaction was initiated by the addition of DL-isocitrate to a final concentration of 5 mM. The change in optical density due to the formation of osazone was measured at 324 nm. ADP-dependent phosphoenolpyruvate carboxykinase was assayed by coupling the formation of oxaloacetate to malate dehydrogenase. Favorable equilibria were maintained by removal of ATP by using hexokinase. A reaction mix consisting of 50 mM N-[2-hydroxyethyl]-piperazine-N′-[2-ethanesulfonic acid], 50 mM KCl, 2 mM $MnCl_2$, 50 mM $NaHCO_3$, 0.5 mM dithiothreitol, 0.1 mM NADH, 1 mM ADP, 50 mM glucose, 1 U malate dehydrogenase (Sigma), 1 U hexokinase (Boehringer-Mannheim), pH 7.1 was equilibrated with a 10-μl sample for 3 min. The reaction was initiated by addition of phosphoenolpyruvate to a concentration of 1 mM, and the change in absorbance due to conversion of NADH to NAD^+ was followed at 340 nm. One enzyme unit is defined as the amount of enzyme which either (*a*) reduces 1 μmol of NAD^+ or $NADP^+$ or (*b*) oxidizes 1 μmol of NADH or (*c*) forms 1 μmol glyoxylic acid/min at 25°C. The extinction coefficient of NADH or NADPH was assumed to be 6.3×10^6 (Bergmeyer 1981), and that of glyoxylic acid phenylhydrazone was assumed to be 1.7×10^4 (Dixon and Kornberg 1959). All activities are expressed as U/g soluble protein. Protein content in the crude extracts was determined by the method of Bradford (1976) by using bovine serum albumin as standard.

Results

The four strains of *Escherichia coli* analyzed here were isolated after ~773 generations of growth, from a long-term chemostat culture initiated with a single clone

of the strain JA122. All four strains could be distinguished on the basis of physiological characteristics, colony-size phenotype, and presence of a plasmid (Helling et al. 1987). The relevant characteristics of these strains, together with those for JA122, the strain used to initiate the population, are given in table 1. To analyze further the evolutionary changes occurring in these clones, we chose to determine the global patterns of gene expression by using two-dimensional electrophoresis.

Culture Conditions

The global patterns of protein expression were analyzed for the strains grown under two different environmental conditions. (1) In the first set of experiments, cells were grown in batch culture and were harvested during log phase when all components of the medium were expected to be present in nonlimiting concentrations. We elected to analyze first the patterns of gene expression under batch-culture conditions, as the physiological differences between the strains reported elsewhere (Helling et al. 1987) were manifested under batch-culture conditions. We expected that these physiological differences would be reflected in noticeable differences in the protein patterns under the same conditions. Proteins from such cells were resolved by using the small-gel system. Approximately 400 different proteins could be resolved on these gels. To facilitate direct comparison with this map, we also grew our strains in batch culture in MOPS minimal medium (Neidhardt et al. 1974). The protein expression patterns of MOPS-grown cells were visually indistinguishable from those of cells grown in Davis minimal medium. In contrast, the protein expression patterns of batch culture were strikingly different visually from those of chemostat culture–grown cells (e.g., see fig. 1). (2) Since the results from batch-culture experiments showed a large number of differences between the strains, we therefore analyzed the strains under the same chemostat conditions in which they evolved. For the strains grown under chemostat conditions, we chose to analyze the patterns of protein expression by using the large-gel system (for details, see Material and Methods), which gave greater resolution (up to 1,000 spots).

Analysis of the Two-dimensional Gels

Typical examples of small and large gels are shown in figure 1. Table 2 summarizes the results obtained from the comparison of the protein expression patterns of the parent strain JA122 with those of the adaptive clones CV101, CV103, CV115, and CV116, for both batch-culture and chemostat environments. It can be seen that the number of proteins with significantly different levels of expression was surprisingly high for all four adaptive clones, being 26%–39% of all proteins in the batch-culture environment and 21%–22% in the chemostat environment. We attach no significance to the differences between the values for the chemostat gels and those for the batch-culture gels, as the conditions under which they are run—and their powers of resolution—are markedly different. From the dynamics of the frequency change of a neutral genetic marker (T_5 resistance) in the population, Helling et al. (1987) had previously estimated that the number of clonal replacements between the initial strain (JA122) and the clones isolated at the end of the experiment was on the order of 10. Since each clone should differ from its predecessor at only one locus, we can conclude that the number of allelic differences between the initial strain and the adaptive clones described here is comparatively small. The difference between this estimate and the number of significant differences in protein expression levels shown in table 2 suggests an important role for mutations with regulatory and/or highly pleiotropic effects.

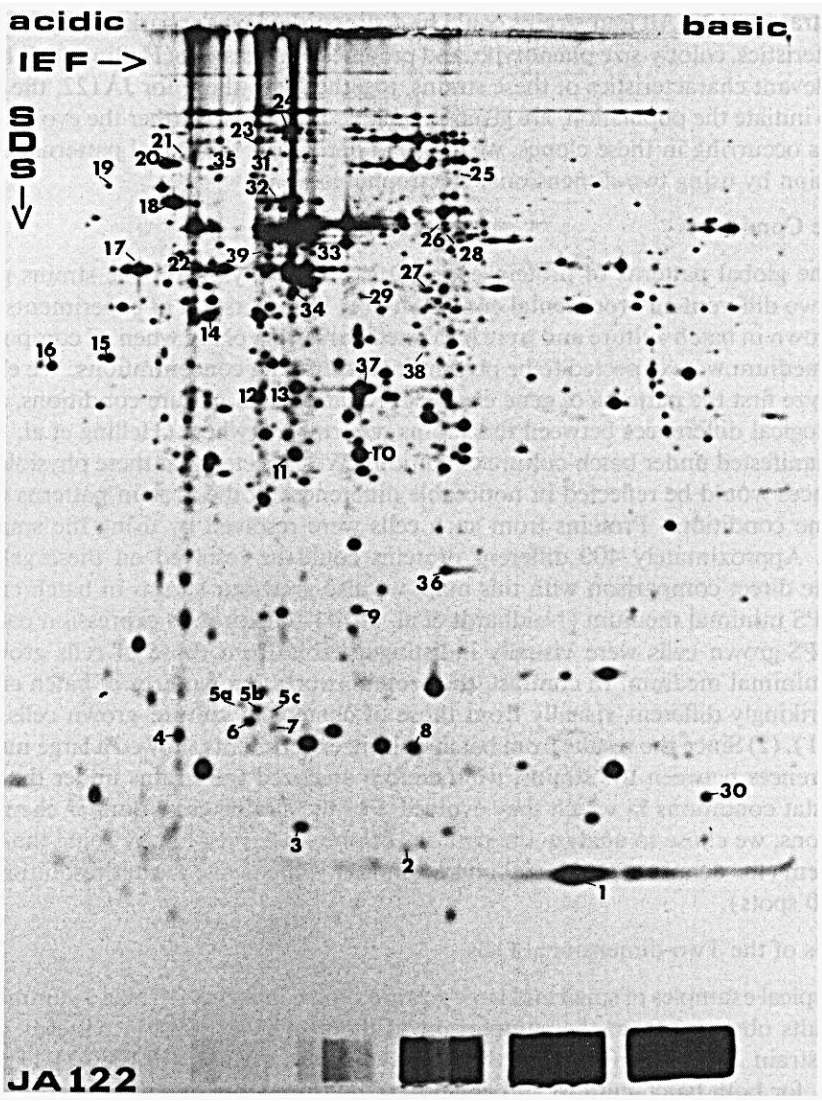

FIG. 1.

Correlated Changes in Protein Levels

If the majority of changes in protein expression levels is due to the pleiotropic effects of regulatory genes, then we may expect to find groups of proteins whose expression levels vary coordinately from strain to strain. To search for coordinated groups of proteins, we elected to use the data obtained from the chemostat gels, as these (*a*) were most representative of the conditions under which the clones had evolved and (*b*) provided the greatest degree of resolution.

Pairwise correlation coefficients were calculated between the 311 proteins (total 743) that exhibited significantly different levels of expression in at least two strains. The average of these correlation coefficients, 0.0065, indicated that there was no global correlation of changes in the whole group of 311 proteins. This large number of variables

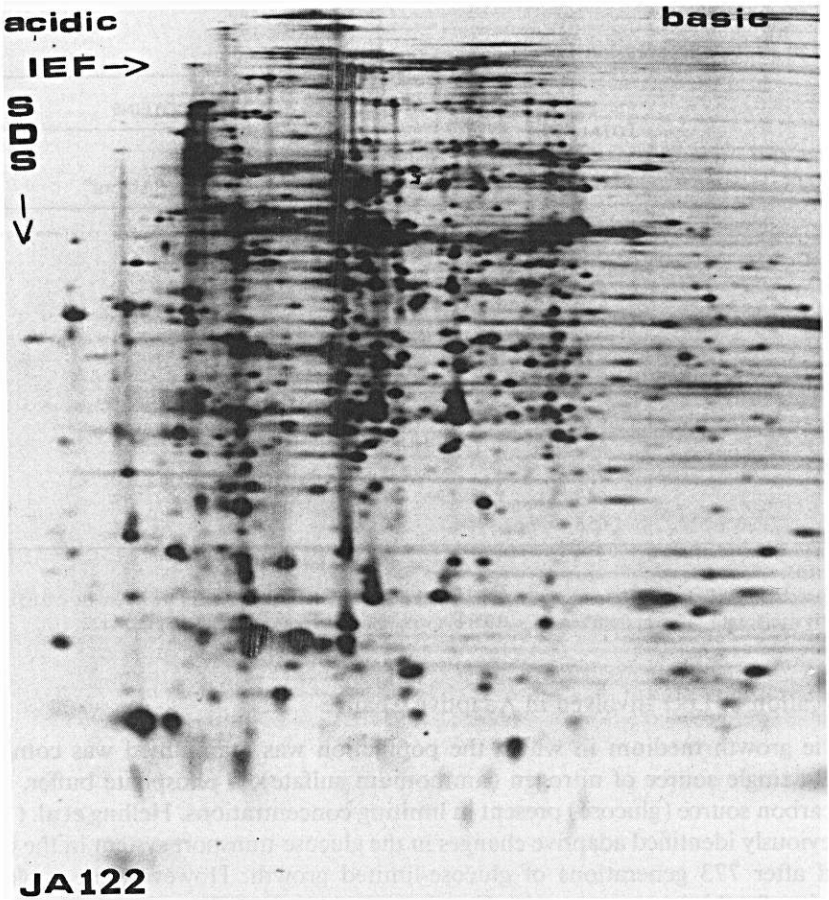

FIG. 1. *Opposite,* Two-dimensional fluorogram of proteins synthesized by strain JA122 grown in glucose-limited chemostat culture. This type of gel is referred to in the text as a "large" gel. Numbers indicate identified proteins, listed also in table 3. 1 = lpp; 2 = ptsH; 3 = F10.1; 4 = bacterioferritin; 5a, 5b, and 5c = rpsF(1), (2), and (3), respectively; 6 = groES; 7 = C14.7; 8 = G13.5; 9 = ssb; 10 = ompA; 11 = tufA; 12 = tsf; 13 = ompA; 14 = recA; 15 = ompF; 16 = ompC; 17 = lamB; 18 = groEL; 19 = nusA; 20 = dnaK; 21 = aceF; 22 = uncD; 23 = pheT; 24 = aceE; 25 = glnS; 26 = uncA; 27 = trpB; 28 = lpd; 29 = aspC; 30 = rplI; 31 = F84.1; 32 = D60.5 (lysU); 33 = sucB; 34 = sucC; 35 = C62.5; 36 = F21.5; 37 = ompA; 38 = htpR; and 39 = D48.5. *Above,* Two-dimensional autoradiogram of proteins synthesized by strain JA122 in glucose-limited batch culture, referred to in text as "small" gel.

precluded any conventional multivariate analysis of the data, because of both the dimensionality of the variance-covariance matrix and the requirement of approaches such as principal component analysis to invert such a matrix. We therefore elected to perform an agglomerative-cluster analysis of the data (UPGMA—unweighted average pair-group clustering; e.g., see Sneath and Sokal 1973), using the pairwise correlation coefficient as the distance metric. The resulting phenogram was characterized by a cophenetic correlation of 0.70 (Sneath and Sokal 1973), and from the phenogram we were able to identify 10 well-distinguished groups of proteins, with the average pairwise correlation coefficient being ≥ 0.94. The number of proteins in each group was 4–31, with the average number being 12.

Table 2
Changes in Protein Expression Levels

Culture and Strain	Total No. of Proteins	No. of Proteins		
		Significantly Different from JA122[a]	Absent[b]	New[c]
Batch:				
JA122	305	...	...	...
CV101	256	99	58	10
CV103	260	95	55	9
CV115	284	84	31	12
CV116	246	123	65	7
Chemostat:				
JA122	690	...	...	..
CV101	664	156	43	23
CV103	666	160	53	36
CV115	649	158	57	24
CV116	658	157	44	17

[a] $\alpha < 0.05$.
[b] Undetectable levels, but with expression levels in JA122 that are significantly different from zero ($\alpha < 0.05$).
[c] Levels significantly different from zero ($\alpha < 0.05$) but with undetectable expression levels in JA122.

Identification of Loci Involved in Adaptive Change

The growth medium in which the population was maintained was composed only of a single source of nitrogen (ammonium sulfate), a phosphate buffer, and a single carbon source (glucose) present in limiting concentrations. Helling et al. (1987) had previously identified adaptive changes in the glucose-transport system in the clones isolated after 773 generations of glucose-limited growth. However, the molecular mechanism for this improvement in glucose transport was not determined. The number of differences in gene expression levels shown in table 2 suggested that many other physiological and metabolic changes had also occurred in these clones. For example, adaptive changes involving modifications in the mechanisms regulating glucose-starvation response, amino acid–starvation response (stringent response), glucose transport, membrane modifications, and more efficient glucose catabolism may be expected, given the simple environment in which the cells were grown. To attempt to determine other mechanisms of adaptive change, we looked for changes in the proteins identified and listed in the *E. coli* gene protein index (Phillips et al. 1987). This index provides identifications for ~10% of the proteins resolved on the O'Farrel two-dimensional gels, and of these proteins a sizable fraction have also been identified on large gels. We have focused on the changes seen under chemostat conditions, since this was the environment in which the population evolved. Table 3 lists the proteins that were unambiguously identified on large gels. The identification of 37 of these proteins is shown in figure 1. The remaining two proteins were identified on the nonequilibrium gels (data not shown). A number of other tentative assignments were made but, because of their ambiguous nature, were excluded from further consideration. The identified proteins were grouped into seven categories according to their function. In each group at least one protein had strongly modified expression in one or more strains. None of these seven categories correspond to the 10 groups identified above by the cluster analysis. However, this is not unexpected, as only ~5% of the protein spots were

unambiguously identified. Furthermore, coordinate changes of the proteins within each of the seven categories are not necessarily expected.

Three of the evolved strains revealed no detectable protein at the migration coordinates corresponding to dihydrolipoamide acetyltransferase; the fourth showed an apparent 10-fold increase in expression over the parental strain. In addition, one strain (CV103) demonstrated no detectable protein at the coordinate corresponding to lipoamide dehydrogenase. It is unlikely that such changes are regulatory in nature, as *aceE* (pyruvate dehydrogenase) and *aceF* (dihydrolipoamide acetyltransferase), components of the pyruvate dehydrogenase complex, constitute an operon (Langley and Guest 1978; Guest et al. 1981) and would be thus expected to vary coordinately. Since lack of a functional pyruvate dehydrogenase complex could have major consequences related to the conversion of carbon to energy, we directly assayed the activity of this complex, as well as four other enzymes whose relative activities are sensitive to intracellular pyruvate. The results (see table 4) show that all the strains possess activity for the pyruvate dehydrogenase complex, although the activity in CV103 is two- to threefold lower than the activity in the initial strain, JA122. We therefore conclude that structural changes have occurred in these two proteins and have resulted in changes in activity with concomitant alterations in mobility, rendering the *aceF* and *lpd* gene products unrecognizable on the gels.

From the data in table 3 it can be seen that in some of the evolved strains the level of a given protein was very low, whereas in another strain the level of the same protein was much higher than that in the parent, JA122. This lack of similarity between levels of proteins confirms a high degree of differentiation between analyzed clones.

Discussion

The results described in the present paper document the adaptive changes occurring in clones isolated from a population of *Escherichia coli* maintained for almost 800 generations in a simple defined laboratory environment. The results provide information on the types of adaptive changes occurring in populations, as well as on the mechanisms of the adaptive changes involved. Three aspects of these results deserve further comment.

1. Importance of Regulatory Changes in Adaptive Responses to Growth under Glucose Limitation

Incorporation of two favorable mutations into the same individual in an asexual population is expected to occur only by the occurrence of the second mutation in a descendant of the first (Muller 1932). Helling et al. (1987) had estimated that on the order of 10 adaptive mutations had become incorporated into a population evolving for ~800 generations, and we estimate that a similar number would be incorporated into the population from which the four adaptive clones (CV101, CV103, CV115, and CV116) had been isolated. The estimates of the number of significant changes in protein expression level are at least an order of magnitude larger (see table 2) and lead us to conclude that adaptation has involved mutations with regulatory and/or highly pleiotropic effects—such as changes in transcription, translation, and membrane composition—rather than changes in structural genes having minimal secondary or pleiotropic effects on other genes. The large number of proteins in some groups suggests that major adjustments of some basic cellular processes have occurred during adaptive change.

Three considerations are important in interpreting these data. 1. This type of

Table 3
Levels of Identified Proteins in Original Strain JA122 and Four Adaptive Clones Isolated[a]

	Level in Strain or Clone (dpm)				
	JA122	CV101	CV103	CV115	CV116
Group I—outer-membrane proteins:					
Porins, amino acid transport:					
ompA (1)	1,118	929	112	1,128	435
ompA (2)	1,136	2,853	2,511	2,539	2,631
ompA (3)	888	1,276	3,114	2,158	1,262
ompA[b]	3,142	5,058	6,745	4,697	4,328
Porins, regulation of diffusion:					
ompF	577	695	95	2,996	1,180
ompC	637	2,342	281	1,106	816
lamB	1,257	4,055	2,143	8,577	10,498[c]
Lipoprotein, outer-membrane-building particle:					
lpp	721	6,317	8,025	3,923	721
Group II—ribosomal subunits:[d]					
30S ribosomal subunit protein S6:					
rpsF (1)	95	399	22	329	166
rpsF (2)	198	150	84	118	192
rpsF (3)	56	0	14	0	0
rpsF[e]	349	549	120	447	358
50S ribosomal subunit protein L9:					
rplI	893	403	163	459	304
Group III—t-RNA synthetases:[f]					
pheT	113	229	345	64	169
aspC	43	188	0	69	100
trpA	51	105	203	172	68
Group IV—heat-shock, stringent response:[g]					
D48.5	276	1,052	207	264	361
D60.5 (*lysU*)	181	0	260	481	0
F10.1	294	23	67	15	13
F84.1	81	0	52	0	40
G13.5	365	38	113	28	23
groEL	1,543	2,900	3,627	3,378	2,942
groES	185	303	607	390	123
Group V—λ-phage life cycle:[h]					
nusA	118	27	40	24	40
recA	902	2,451	797	2,557	2,338
lamB	1,257	4,055	2,143	8,577	10,498[c]
groEL	1,543	2,900	3,627	3,378	2,942
groES	185	303	607	390	123
Group VI—carbohydrate metabolism, TCA cycle[i]					
Pyruvate dehydrogenase:					
aceE	323	326	202	660	286
Dihydrolipoamide acetyltransferase:					
aceF	23	0	0	0	223
Lipoamide dehydrogenase:					
lpd	103	83	0	149	173
Succinyl CoA synthetase:					
α Subunit, *sucB*	586	847	511	922	2,511
β Subunit, *sucC*	300	674	306	238	542

Table 3 (*Continued*)

	Level in Strain or Clone (dpm)				
	JA122	CV101	CV103	CV115	CV116
Group VII—transport:					
Hexose phosphotransferase:					
ptsH	100	16	31	5	14
H^+-translocating ATPase:					
α Subunit, *uncA*	120	410	165	264	233
β Subunit, *uncD*	318	1,672	186	1,536	768
Iron storage and transport:					
Bacterioferritin	290	24	61	28	22

[a] Protein levels are only listed for those proteins for which at least one significant difference existed among the five strains.

[b] Total level of ompA protein. This protein is seen as three discrete spots on the gels. The relative levels of the different forms may reflect different posttranslation modifications and different degrees of these modifications.

[c] The level of the lamB protein in CV116 (10,498) is a minimum estimate, as the fluorograms of the gels, at all exposures, were saturated for this protein spot.

[d] Also identified in this group but not exhibiting any significant differences among strains were 30S ribosomal subunit protein S2 (*rpsB;* identified from nonequilibrium gels) and translation elongation factor (*tsf*).

[e] Total level of rpsF protein. This protein is seen as three discrete spots on the gels. The relative levels of the different forms may reflect different posttranslation modifications and different degrees of these modifications.

[f] Also identified in this group but not exhibiting any significant differences among strains were *trpB* and *glnS*.

[g] Also identified in this group but not exhibiting any significant differences among strains were *htpR*, C14.7, C62.5, F21.5, and *dnaK*.

[h] Also identified in this group but not exhibiting any significant differences among strains were *ssb* and *dnaK*.

[i] Also identified in this group but not exhibiting any significant differences among strains was *araC* (identified from nonequilibrium gels).

analysis will specifically detect changes in expression level and will not necessarily identify nonpleiotropic structural changes. A protein structural change resulting in a large mobility change will be manifested in our analysis as the disappearance of a protein spot in one strain and as the appearance of a new protein spot in a new position. Analysis of the identified proteins provides evidence for two such occurrences (*aceF* and *lpd* gene products; see above). Of course, the occurrence of a coupled disappearance/appearance of spots when strains are compared can also result from a repression (or loss in function)/derepression pair of changes. The data in table 2 also show the number of "new" proteins and the number of "missing" proteins in the adaptive clones, when compared with JA122. The smaller of each pair of numbers therefore provides an estimate of the maximum number of structural changes resulting in altered mobility. The figures suggest that the proportion of such changes is not large. In addition, not all amino acid changes in primary structure can be detected by gel electrophoresis—only those resulting in charge and size differences and, occasionally, those changing the conformation of the proteins. Consideration of the ionic properties of amino acids suggests that as few as one-third of all amino acid replacements are detectable by gel electrophoresis (Selander and Whittam 1983). 2. It is possible that changes in the apparent level of a protein may result from structural changes which alter the stability of the protein. Thus, increased levels of a protein may reflect an increased stability, whereas decreased levels may reflect increased rates of degradation. 3. Finally, it is possible that an increased level of a protein may not reflect an increased level of *functional* protein. A mutation that attenuates function may stimulate

Table 4
Activities of Pyruvate Dehydrogenase Complex and Enzymes Related to TCA Cycle

Enzyme	Mean + SE Activity in Strain (μmol/min/g soluble protein)				
	JA122	CV101	CV103	CV115	CV116
Pyruvate dehydrogenase complex	125 ± 22	109 ± 9	46 ± 5	127 ± 15	190 ± 68
Malate dehydrogenase	4,419 ± 1,143	4,984 ± 1,130	3,713 ± 698	2,407 ± 26	4,382 ± 974
Isocitrate dehydrogenase	358 ± 26	472 ± 41	474 ± 70	414 ± 49	727 ± 106
Isocitrate lyase	5.3 ± 0.7	3.1 ± 0.8	3.4 ± 1.6	3.5 ± 0.4	8.6 ± 1.9
PEP carboxykinase	61 ± 21	90 ± 14	45 ± 12	58 ± 17	52 ± 14

Note.—Each reported value represents duplicate assays of three independent samples, except for the values of PEP carboxykinase for CV101 and CV115, which are duplicate assays for single samples.

selection of a regulatory mutation resulting in a compensatory increase in level. Although we cannot exclude this possibility from consideration, we do not regard it as likely.

2. Adaptation to Glucose Limitation Involves a Reduced Number of Proteins Expressed

The data in table 3 show that all adaptive clones, whether grown in batch culture or chemostat culture, exhibit a decreased number of proteins synthesized to detectable levels, when compared with the parent strain, JA122. The data do not allow us to distinguish between lack of expression of a gene and deletion of that gene. However, pulse-field electrophoretic separation of *Not*I fragments of total genomic DNA revealed no discernable difference between the parent strain and the four evolved strains (data not shown), indicating that no large deletions had occurred in the evolved strains. Small deletions would not be detected on such gels.

A reduced number of expressed genes is consistent with the so-called energy conservation hypothesis, first postulated by Lwoff (1944) in reference to microorganisms but alluded to by Darwin (1859, p. 454). This hypothesis—which postulates that organisms can evolve to increase, by the loss of nonessential functions, efficiency of energy utilization—is easy to rationalize. However, a number of tests of this hypothesis by using microorganisms (Zamenhof and Eichhorn 1967; Baich and Johnson 1968; Andrews and Hegeman 1976; Dykhuizen 1978; James 1978; Koch 1983) have given equivocal results, because of questions concerning either the isogenicity of the strains or secondary effects of the mutations studied.

3. Mechanism of Adaptation to a Glucose-limited Environment

Although the number of proteins with assigned metabolic functions identified in the present study was small in comparison with the number of proteins analyzed, the proteins that were identified permit a number of inferences to be made concerning the mechanisms of adaptation to a glucose-limited environment. The proteins identified have been grouped into seven categories as shown in table 3. The protein levels shown in this table illustrate the strong degree of differentiation among the four adaptive clones but also show some consistent trends, a number of which may be explained from a consideration of the nature of the environment in which the clones evolved. Of course it is possible, given the large number of changes observed, that the modulation

in the levels of the identified proteins is simply a consequence of other, more complex regulatory changes and not a direct result of selection.

The first group in table 3 includes a number of outer-membrane proteins coded by the genes, *ompA, ompC, ompF, lamB,* and *lpp.* With the exception of the ompF and ompC proteins in CV103, all adaptive clones show increased level of expression of these outer-membrane proteins. Particularly striking are the increased levels of lamB protein in the adaptive clones. In CV115 and CV116, this protein has become the most common cellular protein.

The proteins coded by *ompC, ompF,* and *lamB* genes are porins which allow the diffusion of low-molecular-weight compounds, such as monosaccharides, disaccharides, amino acids, and nucleosides. Although the ompC and ompF proteins form apparently nonspecific channels, the lamB protein exhibits a marked preference for maltose and maltodextrins (Schwartz 1983). However, cells deficient in the lamB protein are selected against in a glucose-limited chemostat environment (Szmelcman and Hofnung 1975). Thus, we may expect that increases in the expression of this protein would result in an increased level of adaptation to a glucose-limited environment. The level of lipoprotein (*lpp*)—the outer-membrane structural protein—is also increased in all the adaptive clones, with the exception of CV116. Increased levels of the lpp protein may be a consequence of increased levels of porin proteins and may serve to stabilize the structure of the outer membrane (Nikaido and Vaara 1987).

Selection for changes in the structure of the outer membrane are certainly expected in substrate-limited environments. Increases in the number and structure of porins should facilitate diffusion of the limiting substrate through the outer membrane, allowing increased access to the periplasmic and cytoplasmic membranes where the active transport proteins are localized. However, they should also result in increased release of extracellular proteins and metabolites. In this regard it is interesting to note that the plasmid-carrying adaptive clone CV103 shows a greatly increased rate of secretion of β-lactamase (encoded by the plasmid chromosome) into the medium, as compared with the original strain (JA122) or the other plasmid-carrying strain, CV101 (Modi 1990). In addition, CV103 has been shown to secrete a metabolite which supports the growth of other strains in the population (Helling et al. 1987).

The second and third groups in table 3 list changes in genes coding for ribsosomal proteins and tRNA synthetases. In general, changes involving a modulation of protein synthesis would be expected under carbon and energy starvation. In all evolved clones the levels of ribosomal proteins coded by genes *rpsF* and *rplI* were changed in comparison with those in the original strain, JA122. It is now generally accepted that the *E. coli* ribosome contains 52 different proteins (Noller and Nomura 1987). Although in vitro ribosome reconstitution experiments indicate that most of the ribosomal proteins are required for assembly and/or activity of ribosomes, some ribosomal proteins appear to be dispensable. It has been found, for instance, that protein S6 (coded by the gene *rpsF*) is not needed in the reconstitution of active 30S (Nomura and Held 1974) subunit. The level of this protein varied significantly among the four adaptive clones. In addition, L9 (*rplI*), to which a specific metabolic function has not been assigned, exhibited high strain-to-strain variability. However, it is now known that ribosomes and ribosomal proteins have functions other than in protein synthesis. For instance, some free ribosomal proteins function as translational repressors. At least one protein, S10 (unidentified on our gels), has been shown to participate in antitermination of transcription during lytic growth of phage lambda (Friedman et al. 1981). When not engaged in protein synthesis, ribosomes also apparently function as a negative

regulator in the synthesis of rRNA. Thus, ribosomal proteins whose functions have not been demonstrated by in vitro reconstitution may have some unknown important functions in vivo. Thus we can suppose that changes in the levels of ribosomal proteins observed in our strains could subsequently result in the modulation of the level of many other proteins. Also, with regard to the levels of some tRNA synthetases, we noticed significant differences (group III) between the original and evolved strains (particularly in CV103), differences which, we believe, could be pleiotropic in effect.

The largest group of proteins listed in table 3 comprise those which are synthesized in response to heat shock (group IV). However, no consistent trend toward increasing or decreasing levels of expression can be seen. Of the 12 proteins listed in group IV, seven had levels which varied significantly among the four adaptive clones and the original strain, JA122. In four cases a protein was missing or had its electrophoretic mobility changed to the extent that it could no longer be identified. It is known that several so called heat-shock proteins are in fact synthesized in response to other stimuli. For example, ethanol and oxidation stresses can result in the induction of some heat-shock proteins (Neidhardt and Van Bogelen 1987), and Matin and co-workers have described the induction of a number of shock proteins synthesized during early stages of carbon starvation (Groat et al. 1986). Thus, the expression of these proteins in all strains was not surprising, because, under conditions of glucose limitation, the concentration of glucose in the chemostats at equilibrium was on the order of 2–8 nmol/ml (H. Ikuma and J. Adams, unpublished data). The strain-to-strain changes in some shock-protein levels can reflect the flexibility of the response to low-glucose chemostat conditions. However, since proteins coded by *groEL* and *groES* are characteristic both of shock response and of λ-phage life cycle, the differences in these protein levels can result from the mutations related to the presence of the λ prophage in the genome.

The fifth group listed in table 3 includes proteins involved in the bacteriophage-λ life cycle. Since the original strain, JA122, was lysogenic for λ, and the prophage was still present in all the adaptive clones (data not shown), the changes in the levels of the proteins involved in the λ life cycle may have occurred as a result of evolutionary adaptation to the presence of a prophage. Several reports have described an increase in reproductive fitness due to the presence of a λ prophage in the genome of *E. coli* (Edlin et al. 1975; Lin et al. 1977; Dykhuizen et al. 1978). However, interpretation of the changes in the levels of proteins listed in this group (group V), as well as those in group IV, is difficult, because many of these proteins have multiple functions and because any changes involving these proteins may be expected to have highly pleiotropic effects. Thus, the proteins listed in group V are also involved in the structure of the outer membrane (*lamB;* Schwartz 1983), heat- and cold-shock response (*groES, groEL,* and *recA;* Neidhardt and Van Bogelen 1987; F. C. Neidhardt, personal communication), SOS response (*recA;* Walker 1987), recombination (*recA;* Walker 1987), movement of transposable elements (*recA;* Walker 1987), and transcriptional pausing and termination (*nusA;* Friedman et al. 1984), as well as in stringent response (Cashel and Rudd 1987). Nevertheless, the large increase in the level, in all the adaptive clones except CV103, of the recA protein, a key regulatory gene in the SOS response, would not be predicted in cells lysogenic for λ and remains refractory to explanation. An increase in the level of the recA protein resulting in cleavage of the λ repressor and induction of the lytic response would be expected to be strongly selectively disadvantageous.

The sixth group (table 3; also see table 4) comprises proteins involved in carbohydrate metabolism and in the TCA cycle. Three proteins, coded by genes *aceE,*

aceF, and *lpd*, form the pyruvate dehydrogenase complex, which catalyzes the multistep oxidation of pyruvate to acetyl-CoA. Lipoamide dehydrogenase is also a component of the 2-oxoglutarate dehydrogenase complex; its integration into these complexes is largely dependent on the growth conditions. Between-strain differences in the activity of the pyruvate dehydrogenase complex (see table 4) appear to be consistent with the changes in protein expression levels and/or mobility, shown in table 3. CV103, which lacks spots corresponding to both dihydrolipoamide acetyltransferase (aceF) and lipoamide dehydrogenase (lpd), demonstrates activity threefold lower than that of the parental strain JA122 and two- to fourfold lower than those of the other evolved strains. CV116, which exhibits an apparent 68% increase in the level of lpd over that in JA122, demonstrates a 50% increase in pyruvate dehydrogenase–complex activity, compared with both the parental strain and strains CV101 and CV115.

Altered activity at a major step in pyruvate dissimilation could have a variety of metabolic consequences related to differences in yield and in growth rate that previously have been observed among these evolved strains (Helling et al. 1987). For example it has been noted that pyruvate is a major effector of the bifunctional regulatory protein isocitrate dehydrogenase kinase/phosphatase (LaPorte and Koshland 1983; Nimmo and Nimmo 1984; El-Mansi et al. 1986). Pyruvate stimulates isocitrate dehydrogenase phosphatase which, in turn, converts isocitrate dehydrogenase into its active form. Isocitrate dehydrogenase activity has been demonstrated to be the controlling factor in the regulation of carbon flux at the isocitrate dehydrogenase/isocitrate lyase branch point (LaPorte et al. 1984). Thus, pyruvate pool size indirectly modulates the ratio of flux through the TCA cycle versus that through the glyoxylate bypass and thereby determines the allocation of carbon to energy versus intermediates for the biosynthesis of cellular components (Koshland et al. 1985). Accordingly, as well as measuring pyruvate dehydrogenase–complex activity, we assayed the activities of two TCA cycle and of two glyoxylate bypass–associated enzymes. Variation in pyruvate dehydrogenase–complex activity does not appear to modulate pyruvate pool size to an extent which markedly perturbs the relative activities of glyoxylate bypass–associated enzymes (isocitrate lyase and PEP carboxykinase) versus those of TCA-cycle enzymes (isocitrate dehydrogenase and malate dehydrogenase). This finding is consonant with current theories of metabolic regulation (Kacser and Burns 1973, 1979, 1981; Savageau 1976; Crabtree and Newsholme 1985) which predict that a complex network would be intrinsically buffered against such variation in pyruvate dehydrogenase–complex activity as seen here. The adaptive significance of changes in pyruvate dehydrogenase–complex activity therefore remains difficult to explain.

The last group (group VII) shown in table 3 includes proteins involved in transport. Two subunits of H-translocating ATPase were identified on our gels [α(*uncA*) and β(*uncD*)]. This ATPase is the central ion pump of bacterial membrane and is composed of at least eight distinct polypeptides. This enzyme is responsible for ATP hydrolysis-coupled import of amino acids, lactate, pyruvate, succinate, and gluconate but is not responsible for the transport of glucose, the carbon source in our experiments. However, we found that the level of α and β subunits was increased in CV115 and CV116 and that in CV101 the level of β subunit was increased approximately 10-fold. Moreover, the increased level of the α and β subunits is seen in both batch-culture and chemostat conditions. The increased level of ATPase in CV101 can explain the cross-feeding observed between CV101 and CV103. Helling et al. (1987) reported that CV103 excreted into medium an unidentified metabolite which supported growth of CV101. It is reasonable to suppose that this metabolite can be one of those trans-

ported through the H-substrate system. An increased level of ATPase allows the maintenance of increased proton and substrate concentration gradients (Maloney 1987). Collins et al. (1976) have shown that such an increased gradient was selectively favored during substrate-limited (lactate) growth. The increased levels of H-translocating ATPase in CV101 but not in CV103 may explain the preferential uptake by CV101 of the intermediary metabolite released by CV103.

Table 3 also shows that the levels of hexose phosphotransferase decreased in all the adaptive clones, compared with those in the initial strain, JA122. Since hexose phosphotransferase is a key enzyme of the phosphotransferase system, involved in the active transport of glucose (Postma 1987), this result is completely contrary to expectation and remains unexplained.

The numerous proteins discussed above span a variety of cellular functions including membrane transport, translation, phage-λ expression, stress response, glucose metabolism, and energy production. Most changes observed can be explained by the adjustment of many metabolic pathways and of the cell architecture to chemostat conditions and in many cases may be predicted by consideration of the environment in which the cells were growing. In some cases, however, the changes could not be predicted and were in fact contrary to expectation. It is hoped that a fuller analysis of some of these anomalous changes may lead to a better understanding of the mechanisms both of adaptation and of global regulation of metabolism of the bacterial cell.

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