Multiple Duplications of Yeast Hexose Transport Genes in Response to Selection in a Glucose-Limited Environment

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When microbes evolve in a continuous, nutrient-limited environment, natural selection can be predicted to favor genetic changes that give cells greater access to limiting substrate. We analyzed a population of baker's yeast that underwent 450 generations of glucose-limited growth. Relative to the strain used as the inoculum, the predominant cell type at the end of this experiment sustains growth at significantly lower steady-state glucose concentrations and demonstrates markedly enhanced cell yield per mole glucose, significantly enhanced high-affinity glucose transport, and greater relative fitness in pairwise competition. These changes are correlated with increased levels of mRNA hybridizing to probe generated from the hexose transport locus *HXT6.* Further analysis of the evolved strain reveals the existence of multiple tandem duplications involving two highly similar, high-affinity hexose transport loci, *HXT6* and *HXT7.* Selection appears to have favored changes that result in the formation of more than three chimeric genes derived from the upstream promoter of the *HXT7* gene and the coding sequence of *HXT6.* We propose a genetic mechanism to account for these changes and speculate as to their adaptive significance in the context of gene duplication as a common response of microorganisms to nutrient limitation.

Introduction

The adaptive flexibility of microorganisms, including yeasts, is evident in how quickly microbial populations respond to selection in the laboratory. Classic evolutionary models hold that random mutations, coupled with large population size and short generation time, sufficiently explain how microbes overcome the restrictions to adaptation posed by a predominantly asexual mode of reproduction (Muller 1932). A number of recent studies suggest that this adaptive flexibility may be enhanced by the occurrence of nonrandom mutations in response to specific stressors (Hall 1992; Wright 1996; but see also Lenski and Mittler 1993), as well as by the development of populations that remain stably differentiated over many generations (Rosenzweig et al. 1994; Shapiro 1995).

Microbial adaptation has been studied by examining how cells in batch or plate culture respond to the presence of toxins or novel substrates. This approach has demonstrated that selection pressures targeting specific metabolic pathways can favor a variety of responses, including changes in protein structure (Wills and Phelps 1978), gene duplication (Hartley 1984), and altered regulatory patterns (Hall 1984). The general result of such changes is that cells quickly develop resistance to toxins or improved ability to metabolize exotic carbon sources.

Microbial adaptation has also been studied in the context of continuous nutrient-limited culture (e.g., Horiuchi, Horiuchi, and Novick 1963; Hansche, Beres, and Lange 1978; Adams et al. 1985; Hartl, Dykhuizen, and Dean 1985; Helling, Vargas, and Adams 1987). This approach has allowed investigators to evaluate fitness in terms of the relative competitive abilities of ancestral and evolved strains (Lenski 1995), or strains that are isogenic except for allelic differences at one or a few loci (Hartl, Dykhuizen, and Dean 1985). Continuous culture studies have yielded a rich literature concerning the tempo and the trajectory of evolutionary change (Elena, Cooper, and Lenski 1996), as well as the extent to which the adaptative process is constrained by the intrinsic properties of metabolic networks (Dykhuizen and Dean 1990), chance (Lenski and Travisano 1994), habitat complexity (Dykhuizen and Dean 1994; Korana et al. 1994), and historical precedent (Travisano et al. 1995).

Paquin (1982) investigated the effect of ploidy on the rate of evolutionary change. Isogenic strains of either diploid or haploid baker's yeast were placed under glucose limitation (0.08% w/v), and propagated vegetatively under aerobic conditions. Population size was kept large enough to effectively eliminate the role of genetic drift. Experimental populations were followed for several hundred generations, during which time the appearance and proliferation of novel adaptive mutants was documented by monitoring fluctuations in the frequencies of rare neutral genetic markers. The resulting data indicate that diploid and haploid populations differ significantly in the number of cell generations between fixation of adaptive mutants (Paquin and Adams 1983*a*). Adams et al. (1985) further characterized a succession of adaptive clones from one such experiment. They found that, among other changes, cellular transport of xylose, a nonmetabolizable analog of glucose, increased 3.5 times in the terminal population relative to the parental population and that the surface-area-to-volume ratio was 28% greater in the cells from the terminal population relative to the parental.

We wished to ascertain the genetic mechanism(s) underlying fitness changes in one of Paquin's (1982) populations. The results of Adams et al. (1985) led us to hypothesize that, compared with the parental strain, evolved strains would reveal genetic changes at loci associated with high-affinity glucose uptake. We hypoth-

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Table 1
Amino Acid Sequence Identity Among the Glucose Transport Proteins of Yeast[a]

Gene	*HXT1*	*HXT2*	*HXT3*	*HXT4*	*HXT6*
HXT2 . . .	63.5				
HXT3 . . .	86.4	64.2			
HXT4 . . .	72.2	66.8	74.5		
HXT6 . . .	71.6	66.8	74.5	83.4	
HXT7 . . .	71.6	66.8	74.5	83.4	99.7

[a] From Kruckeberg (1996).

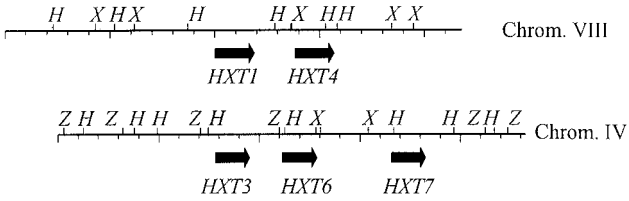

FIG. 1.—Genetic maps showing the relationships among the five HXT genes, *HXT1, HXT3, HXT4, HXT6,* and *HXT7* (modified from the Saccharomyces Genome Database). *HXT2* is on chromosome VIII. *H* = *Hin*dIII, *X* = *Xba*I, and *Z* = *Xho*I.

esized further that these changes would result in significantly increased levels of glucose transport gene expression. At least six genes participate in yeast hexose transport (table 1). Of these, *HXT6* and *HXT7* appear to encode high-affinity transporters required for growth at very low glucose concentrations (<0.1%) (Reifenberger, Friedel, and Ciriacy 1995). *HXT6* and *HXT7* share 99.7% nucleotide sequence identity, as well as 99.7% amino acid sequence identity, in their coding regions and are tandemly arrayed downstream of the *HXT3* locus on chromosome IV (fig. 1). It is reasonable to anticipate that *HXT6* and *HXT7* would become targets for natural selection when yeast are cultured under glucose limitation.

In this paper, we describe the genetic mechanism underlying one response to selection in a continuous nutrient-limited environment. We characterize at the populational, physiological, and molecular levels the ancestral and terminal yeast populations from one of Paquin's (1982) experiments. Specifically, we compare a clone propagated from the diploid yeast strain used to initiate this experiment with a clone isolated from the population in existence after 450 generations of culture under glucose limitation.

Materials and Methods

Strains

CP1AB is an isogenic diploid derived from the haploid *Saccharomyces cerevisiae* strain XC500A by Paquin and Adams (1982); its genotype is **a**/α, *gal2/gal2, mel/mel, mal/mal.* A chemostat was inoculated with CP1AB and propagated vegetatively under aerobic conditions at 30°C in glucose-limited media for 450 generations (Paquin 1982). Initial population size was held constant at approximately 4–5 × 10^9 cells by maintaining a constant dilution rate of 0.2/h. Alternative carbon sources were not available; however, all other nutrients, including nitrogen and phosphorus, were present in nonlimiting concentrations. At regular intervals, samples from the experimental population were placed in 15% glycerol and stored at −70°C. A sample from the terminal population was streaked onto YEPD agar. The large colonies which predominated on this plate were observed to be homogeneous with respect to yield and growth rate under nonlimiting nutrient conditions using a variety of fermentable or nonfermentable carbon sources (unpublished data). The few small colonies observed were subsequently shown to be *petite* mutants (J. Adams, personal communication). A large colony from this terminal population, hereafter designated strain 28–15L4, was characterized in comparison with the parent strain, CP1AB.

Media and Culture Conditions

Batch Culture

Cells were cultured aerobically at 30°C using synthetic minimal media (Sherman 1991) containing either 0.8% or 0.08% (w/v) glucose unless otherwise noted.

Continuous Culture

Cells were cultured continuously at 30°C in synthetic minimal medium containing 0.08% (w/v) glucose. Two-hundred-milliliter culture vessels were aerated using sterile-filtered humidified air delivered at a flow rate of 500 mm^3/min. Dilution rate was set at 0.2/h, as described by Paquin (1982). Monocultures of parental and evolved strains were fed by the same media reservoir.

Measurement of Yield and Fitness Parameters

Batch Culture

Frozen (−70°C) glycerol stocks of CP1AB and 28–15L4 were streaked onto YEPD agar (Sherman 1991) and cultured for 48 h at 30°C. Individual colonies were used to inoculate monocultures in 0.08% (w/v) glucose minimal liquid media. Cultures were sampled every 1–2 h after inoculation for 24 h. Optical density was measured at A_{600}, and cells were appropriately diluted and plated onto YEPD agar. YEPD plates were incubated for 48 h at 30°C, and colonies were enumerated in order to obtain viable cell counts per milliliter of culture (VCCs). Maximum growth rate under nonlimiting conditions, μ_{max}, was estimated from the slope of the linear regression of ln(VCC) versus time during logarithmic phase. A_{600} was measured at 30 and 36 h after inoculation in order to verify that cultures had attained stationary phase. Cultures were sampled at 36 h to determine yield cell number and dry weight per milliliter of culture. Cell number was estimated as VCC. Culture dry weight was estimated by filtration of ~40 ml culture onto tared 0.45-μm nylon filters, drying the filters overnight at 65°C, and then reweighing filters using a Mettler AE100 analytical balance. Cell yield was calculated as the number of cells/ml at 36 h divided by the amount of glucose initially present (800 μg/ml). Similarly, dry weight yield was calculated as mg dry weight/ml at 36 h divided by the amount of glucose initially present.

Continuous Culture

Single colonies of either CP1AB or 28–15L4 were used to inoculate batch cultures in 0.8% (w/v) glucose minimal media. Cells were grown overnight at 30°C, harvested by centrifugation, washed in 0.08% glucose minimal media, and used to inoculate 0.08% glucose-limited continuous cultures. These monocultures were allowed to come to equilibrium by operating chemostats 10–20 generations at a dilution rate of 0.2/h. Cells were harvested for estimation of yield cell number and yield dry weight per milligram of glucose as described for batch cultures. The concentration of glucose used in these calculations was the concentration in the media reservoir, 800 μg/ml, minus the equilibrium glucose concentration in the chemostat. The equilibrium substrate concentration, **s**, in the chemostat, was determined by spectrophotometric assay of 0.2-μm filtered chemostat cultures using the coupled hexokinase/glucose-6 phosphate dehydrogenase assay (Boehringer-Mannheim Biochemicals Kit No. 716251). In order to extend the detection limit of the assay, the basic protocol was modified to accomodate up to 2.5 ml of filtrate in a reaction mixture having 3.02 ml final volume.

Competition Experiments

Overnight batch monocultures in 0.8% glucose minimal media were inoculated with five colonies each of CP1AB and 28–15L4 from YEPD agar. Cells were harvested by low-speed centrifugation at room temperature and washed once in sterile 0.08% glucose (w/v) minimal media. Parental and evolved strains were then introduced into replicate chemostats running under the conditions described above. The density of the two strains upon their introduction was equal, at 1 OD/ml each. Cell density in the chemostats was monitored every 12 h for 4 days. At the same time, cells were appropriately diluted in 0.08% glucose (w/v) minimal media and then plated onto YEPD and 0.8% galactose (w/v) minimal agar. Early in our investigations we observed that 28–15L4 had reverted to the GAL+ phenotype. When plated on 0.8% galactose minimal agar and observed after 72 h, 28–15L4 forms large colonies, whereas, CP1AB forms very small colonies. We took advantage of this striking difference in their respective GAL phenotypes to score for differences in the frequency of parental versus evolved cells in co-culture. Galactose plates were therefore scored for the fraction of large versus small colonies for each sample. These values were compared with the total number of colonies on the YEPD plates and were found to accurately reflect the total number of cells present in the chemostats. The log-transformed ratio of the strains' frequencies was plotted as a function of the number of cell generations after time zero, and the selection coefficient was calculated by linear regression.

Tetrad Dissection

Frozen (−70°C) glycerol stocks of 28–15L4 were streaked onto YEPD agar and cultured for 72 h at 30°C. Individual colonies were then patched onto sporulation plates (Rose, Winston, and Hieter 1990) and incubated at 30°C for 2–3 days. Cells were resuspended in 1 M Sorbitol plus 50 μg/ml glucylase (Boehringer-Mannheim Biochemicals) and placed on ice for 24 h. Tetrads were dissected onto YEPD plates and incubated at 30°C for 48 h. From each of six tetrads analyzed, only two viable segregants were recovered. This result indicates either the presence of heterozygous recessive lethal mutations or loss of essential genes via unequal chromatid exchange. No such loss of viability is observed among CP1AB ascospores (unpublished data).

Molecular Techniques

DNA and RNA were extracted from yeast grown in batch culture (DNA), or in chemostats (RNA) using standard techniques for yeast (13.12.1 and 13.11.3 in Ausubel et al. 1992). Southern (Southern 1975) and northern hybridizations (4.9.2 in Ausubel et al. 1992) were performed using standard techniques. Probes used in the Southern hybridizations were fragments containing the coding regions of *HXT4* (Theodoris et al. 1994), labeled with digoxigenin and detected by antibody conjugation and autoluminometry or colorimetric detection (Boehringer-Mannheim Biochemicals). Hybridizations were in 5 × SSC at 65°C and washes were in 0.5 × SSC at 65°C. The *HXT6* and *HXT7* genes have only five nucleotide differences within their coding regions. The probes used for detection of *HXT6* and *HXT7* (Reifenberger, Freidel, and Ciriacy 1995) or *ACT1* (Gallwitz and Seidel 1980) in northern hybridizations were amplified from CP1AB genomic DNA using the primers HXT6/7 (forward: 5′-ATTGCAGAGCAAACTCCTG-TG-3′; reverse: 5′-GGCTTATCATCGTGAGCCATT-3′) and ACT1 (forward: 5′-CTACTGGTATTGTTTTGGA-TTC-3′; reverse: 5′-TCGTCGTATTCTTGTTTTGA-3′) and radioactively labeled by standard techniques. Filters were sequentially hybridized, because the conditions used did not separate the *ACT1* transcript and the *HXT6/7* transcript. Filters were prehybridized and then hybridized first with *ACT1*; the amount of radioactivity in the *ACT1* band was detected by an AMBIS radioanalytic imaging system (San Diego, Calif.). Filters were again prehybridized, a process that tended to strip off *ACT1* probe, then hybridized with the *HXT6* probe. The resulting *HXT6/7* band was detected by the radioanalytic imaging system. Hybridizations were conducted in 50% formamide, 5 × SSC at 42°C, and washes were in 0.5 × SSC at 65°C.

Glucose Transport Assays

Glucose transport kinetics were estimated by following the uptake of ^{14}C-glucose at 30°C in concentrated suspensions of cells cultured in glucose-limited chemostats. Transport assays were performed using a modification of the procedure described by Bisson and Fraenkel (1983). Yeast were withdrawn from chemostat monocultures using sterile 50-ml volumetric pipets and concentrated to an A_{600} of 9–12 in sterile 0.1 M KPO_4, pH 6.5. Twenty microliters of various glucose concentrations were added to 80 μl of cells, allowed to take up glucose for 5, 10, or 15 s before being vacuum filtered, and rinsed with 30 ml of ice-cold glass-distilled

Table 2
Means ± Standard Errors for Various Fitness Estimates

	N[a]	P (CP1AB)	E (28-15L4)	(df, P)[b]
Fitness parameter				
Continuous culture				
s (μg/ml)	4	0.078 ± 0.016	0.008 ± 0.003	(3.2,[c] 0.024)
K_s (μg/ml)	6	0.077 ± 0.005	0.008 ± 0.001	(5,[c] 0.0000)
Batch culture				
μ_{max} (h^{-1})	6	0.396 ± 0.012	0.414 ± 0.008	(10, 0.24)
Doubling time (min)	6	105 ± 3.2	101 ± 1.9	(10, 0.22)
Yield				
Continuous culture				
Y_1 dry weight (μg DW/μg glucose)	4	0.30 ± 0.05	0.60 ± 0.10	(6.0, 0.0375)
Y_2 cell number (cells/μg glucose)	4	$1.66e^4$ ± $0.13e^4$	$4.40e^4$ ± $0.42e^4$	(3.6,[c] 0.0055)
Batch culture				
Y_3 dry weight (μg DW/μg glucose)	6	0.32 ± 0.03	0.37 ± 0.02	(10, 0.168)
Y_4 cell number (cells/μg glucose)	6	$1.20e^4$ ± $0.12e^4$	$1.73e^4$ ± $0.08e^4$	(10, 0.004)

[a] Number of replicates per strain.

[b] The hypothesis that there is no difference in means between CP1AB and 28-15L4 was tested using a *t*-test at the $\alpha = 0.05$ level with the degrees of freedom (df) shown. The *P* value is listed.

[c] Due to unequal variances, Welch's *t*-test was used to test for differences between means.

water. Filters with washed cells were counted for 10 min in a scintillation counter set to detect ^{14}C. All assays were performed in triplicate. Uptake velocities were determined as fmoles per second per milligram dry weight. A two-way analysis of variance using strain versus glucose concentration was performed on the log-transformed data to test for significant differences among velocities. (A log transformation was necessary to equalize the variances among glucose concentrations.) Multiple *t*-tests for differences among strains at each glucose concentration ($\alpha = 0.006$ to correct for multiple comparisons) were performed because the ANOVA was significant.

Results

The Parental and Evolved Strains Differ Physiologically when Grown in Monoculture Under Glucose Limitation

Yeast were cultured in chemostats under conditions identical to those under which the strains evolved. The parental and evolved strains were run in pairs such that both populations received the same media at approximately the same dilution rate. Chemostats were sampled after 10 generations and can therefore be assumed to have reached steady state. The viable cell count per milliliter, dry weight per milliliter, and the amount of residual glucose in the chemostats were measured (table 2). The amount of residual glucose (**s**) present in the chemostat monocultures was 10-fold lower for the evolved strain than for the parental strain, indicating that the former was far better able to gain access to the limiting substrate. Assuming that the Monod (1942) equation, $\mu = \mu_{max} \times \mathbf{s}/(K_s + \mathbf{s})$, reasonably approximates the behavior of these cultures, substitution of the known variables (dilution rate, μ; growth rate under nonlimiting conditions, μ_{max}; and residual glucose concentration, **s**) predicts that the substrate concentration at which cells attain their half-maximal growth rates, K_s, is an order of magnitude lower in the evolved strain.

Viable cell count and dry weights were significantly greater in 28–15L4. When converted to yield per milligram of glucose, it is evident that when grown in continuous culture, the evolved strain produces 2.6-fold more cells and 2-fold greater mass than the parental strain (table 2). The magnitude of these differences indicates that the evolved strain has not only greater transport capacity, but enhanced metabolic efficiency in the processing of limiting nutrient.

Many of these fitness differences are not realized under nonlimiting conditions in batch culture. When the two strains are grown in 0.8% glucose minimal media, μ_{max}, doubling time, and yield dry weight per milligram of glucose are not statistically different (table 2). Cell yield per milligram of glucose was significantly greater in the evolved strain than in the parental strain; however, the magnitude of this difference was far less than when cells were cultured under nutrient limitation. Taken altogether, these results suggest that physiological tradeoffs are not required for the evolution of enhanced substrate uptake and assimilation efficiency in yeast.

The Evolved Strain Outcompetes the Parental Strain when they are Grown Together in Continuous Culture

Previous observations showed that the evolved strain had reverted to the GAL+ phenotype; 28–15L4 and CP1AB are therefore readily distinguished by colony size on 0.8% galactose minimal agar. A pair of chemostats was initiated with equal densities of the parental and evolved strains, and their relative frequencies were followed for 20 generations (fig. 2). The frequency of the evolved strain increased steadily in both chemostats until the parental strain could no longer be detected. Relative to its progenitor, the evolved strain realized a selection coefficient of 0.094 ($r^2 = 0.91$).

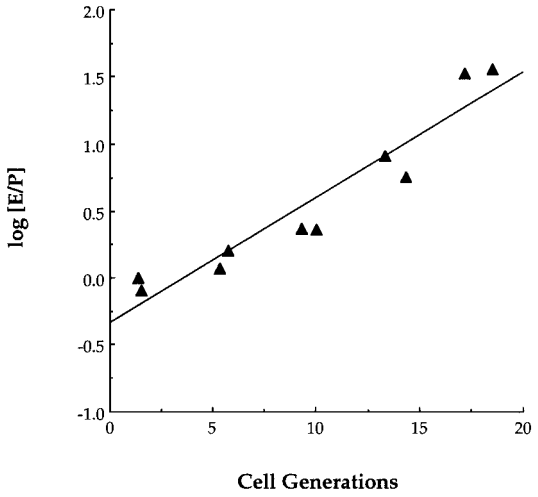

FIG. 2.—The evolved strain outcompetes the parent under glucose-limiting conditions. Log ratio of the frequencies of 28–15L4 to CP1AB was plotted as a function of cell generations. Data points are from replicate chemostat cultures fed from the same media reservoir under conditions described in *Materials and Methods.* The formula for the linear regression of log strain ratio against cell generations is $y = 0.094x - 0.338$, $r^2 = 0.909$.

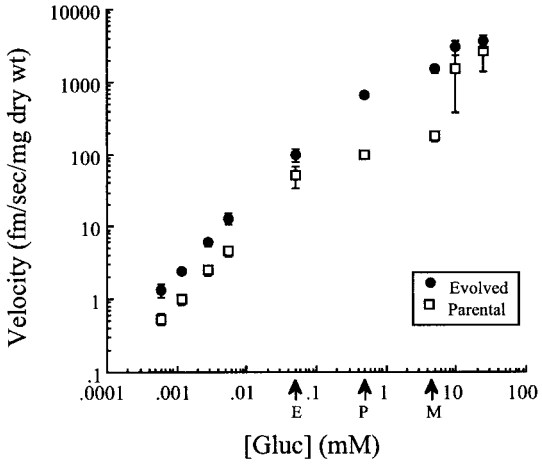

FIG. 3.—Glucose uptake velocity (with 95% confidence intervals) versus glucose concentration for the parental and evolved strains growing in continuous culture. Arrows mark average glucose concentrations seen in monoculture chemostats of the parental (P) and evolved (E) strains; M marks the concentration of the fresh media. Note that the axes are log scale. Velocity was measured by the uptake of ^{14}C-glucose by a protocol modified from Bisson and Fraenkel (1983).

The Evolved Strain Transports Glucose Two to Eight Times Faster than the Parental Strain

Given our observation that the two strains differ in **s** by an order of magnitude, the simplest explanation for their difference in competitive ability is that selection has favored the evolution of an improved mechanism for transporting limiting substrate. Figure 3 shows the results of glucose transport assays comparing uptake velocity at several glucose concentrations for cells grown in chemostat monoculture on 0.08% glucose at a dilution rate of 0.2/h. The evolved strain consistently demonstrates greater substrate uptake velocity than the parental strain. A two-way analysis of variance on the log-transformed values for velocity indicates that differences between strains are significant ($F = 295.62$, df = 1, $P < 0.0001$), as are differences among concentrations and the interaction between strain and glucose concentration. At 25 mM glucose, the difference in velocity is 1.4-fold, but this is not significant if we correct for multiple comparisons. For all other substrate concentrations, however, the results are significant at or below the 0.006 level.

Of greatest relevance in explaining differences in competitive ability are the transport estimates obtained within the range of fresh chemostat media and the steady-state concentrations of chemostat monocultures (fig. 3). At these glucose concentrations, the difference between strains ranges between six- and eightfold.

Expression of *HXT6* and *HXT7* is Elevated in the Evolved Strain Relative to the Parental Strain

Given that the evolved strain revealed lower steady-state glucose concentrations in chemostat monoculture and demonstrated enhanced glucose transport capacity, it was reasonable to hypothesize that the initial step in hexose transport had been a target of directional selection. *HXT6* and *HXT7* are two hexose transport genes that differ by only five nucleotide substitutions and are expressed at low (<4.4 mM) glucose concentrations (Reifenberger, Freidel, and Ciriacy 1995). Northern analyses were used to determine whether there is a difference between the parental and evolved strains with respect to their expression of these two genes. mRNA was prepared from two pairs of chemostats, and northern analysis using 10 μg of total RNA was performed as described in *Materials and Methods.* The amount of HXT mRNA detected by the probe was normalized to the amount of actin transcript on the same

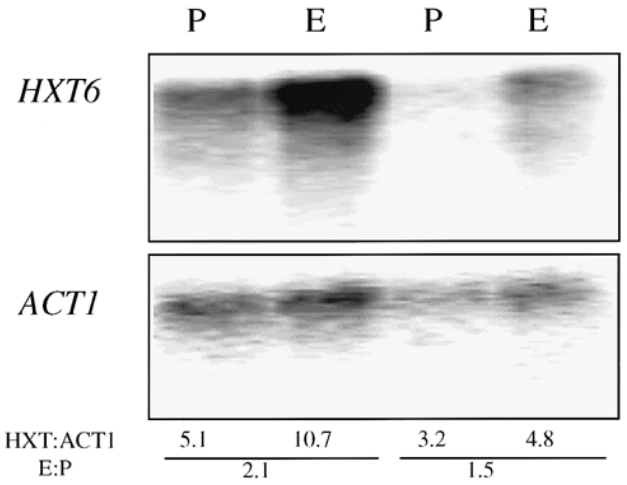

FIG. 4.—Detection of *HXT* mRNA from the parental (P) or evolved (E) strains growing in chemostats under steady-state conditions. Two pairs of chemostats are shown. The radioactive *HXT6* probe detects both *HXT6* and *HXT7,* and possibly other *HXT* genes. Intensity of hybridization was detected by the AMBIS radioanalytic imaging system. The amount of *ACT1* (actin) mRNA per lane was used to detect loading differences among the lanes on the gel. The normalized ratio of evolved to parental *HXT* mRNA for each pair of chemostats is shown on the last line.

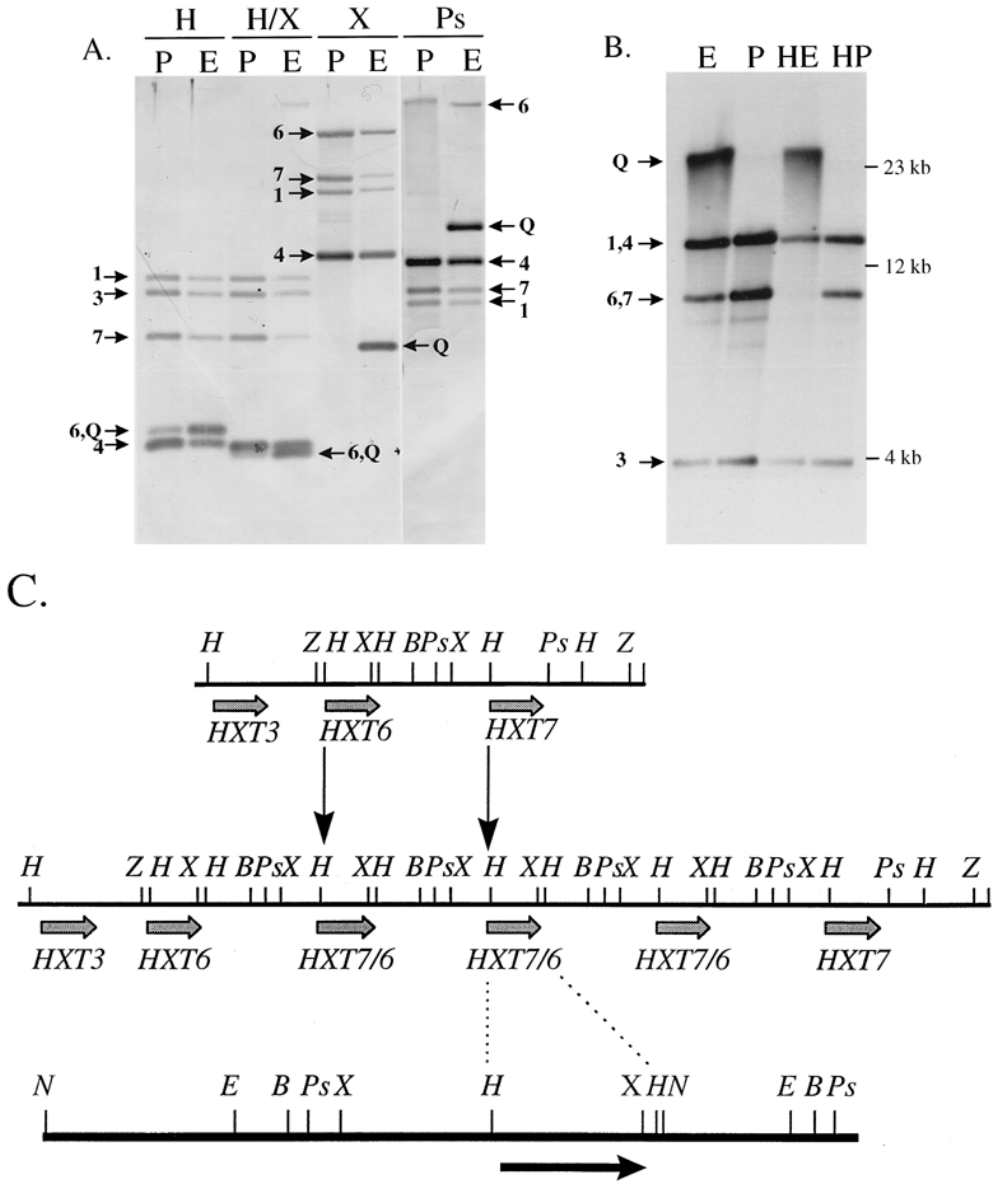

FIG. 5.—Restriction site analysis of the *HXT* loci from the parental and evolved strains. *A*, Genomic DNA from the parental (P) and evolved (E) strains was digested with *Hin*dIII (H), *Hin*dIII and *Xba*I (H/X), *Xba*I (X), or *Pst*I (Ps) and probed with the *HXT4* gene. Q refers to the band found in the evolved strain but not in the parental strain; numbers refer to *HXT* loci. *B*, Genomic DNAs from the evolved strain (E), the parental strain (P), and the haploid products of the evolved strain carrying either the duplicated genotype (HE) or the parental genotype (HP) digested with *Xho*I, electrophoresed on a gel, and transferred to a nylon membrane. The membrane was hybridized with a fragment of the *HXT4* gene labeled with digoxigenin, and hybridized bands were detected by autoluminography. Numbers on the left designate *HXT* loci; numbers on the right designate sizes in kilobases. *C*, Restriction maps of the *HXT6* and *HXT7* gene regions in the parental strain (top), the restriction map created by three tandem duplications of the region from the start of *HXT6* to the start of *HXT7* (middle), and the map for the fragment marked **Q** in A and B; the arrow indicates the region with sequence similarity to the *HXT* genes (bottom). *B* = *Bgl*II, *E* = *Eco*RI, *H* = *Hin*dIII, *N* = *Nco*I, *Ps* = *PstI*, *X* = *Xba*I, and *Z* = *Xho*I.

filter. On average, the transcripts of *HXT6* and *HXT7* are 1.8-fold (±0.3) greater in the evolved strain than in the parental strain (fig. 4).

The Evolved Strain Has Multiple Tandem Duplications of an *HXT6* and *HXT7* Chimera on One of its Chromosomes

Genomic DNAs from the parental and evolved strains were digested by various restriction enzymes and probed with the *HXT4* gene, which has sufficient sequence identity with *HXT1*, *HXT3*, *HXT6*, and *HXT7* to detect all of these genes in Southern hybridizations. For both strains, several bands corresponding to the *HXT1*, *HXT3*, *HXT4*, *HXT6*, and *HXT7* genes were visualized, with the *HXT4* bands having the strongest hybridization (fig. 5). In addition, the evolved strain revealed a strongly hybridizing band (marked **Q** in fig. 5*A* and *B*) from a unique restriction fragment (fig. 5*C*).

Inspection of restriction sites surrounding *HXT3*, *HXT6*, and *HXT7* showed that the restriction map of the duplicated region could be reconstructed by duplicating the DNA sequence from the start of the *HXT6* gene to

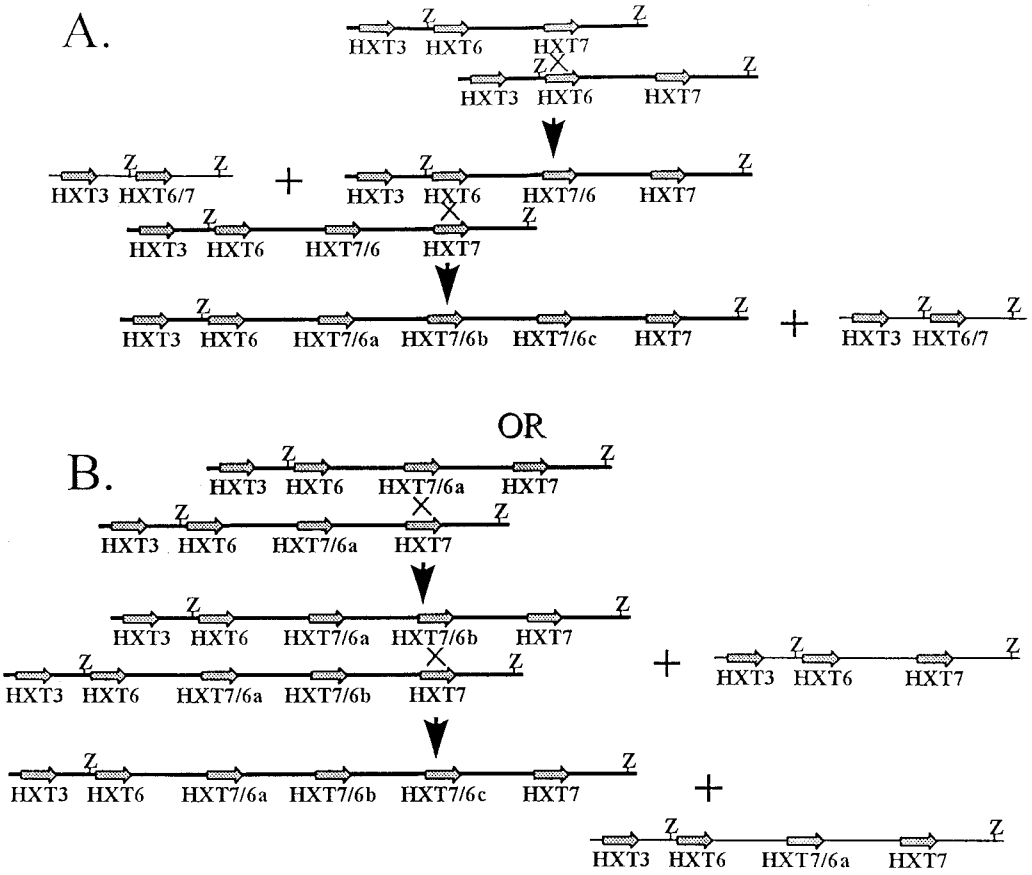

FIG. 6.—Hypothetical unequal crossover events leading to the quadruplication of *HXT7/HXT6.* The top diagram shows the orientation of the three *HXT* genes in the parental strain and of one chromosome of the evolved strain. Z designates the recognition sites for the restriction enzyme *Xho*I. X's designate the crossover events between nonhomologous sequences, and the arrows point to the duplicated products of the crossover events. The light line drawings designate the alternate products of the crossover events. *A,* The most parsimonious scenario, wherein two unequal crossovers between *HXT6* and *HXT7* on sister chromatids lead to three novel *HXT* genes (*HXT7/6a,b,c*). *B,* An alternate scenario beginning after the initial unequal crossover event between *HXT6* and *HXT7.* The initial event could have been between sister chromatids or homologous chromosomes, because the subsequent crossover events between *HXT7* and *HXT7/6a* on homologous chromosomes reproduce the parental chromosome. The final crossover event in this scenario would have to be between sister chromatids.

the start of the *HXT7* gene (fig. 5*C*) and inserting this duplication at the start of *HXT7.* This produces a duplicated gene that has the coding sequence of *HXT6* and the promoter of *HXT7.* The strong intensity of hybridization to this band by the *HXT4* gene cannot be easily explained by a single gene duplication; if there was only a single duplication, the intensity of hybridization should be the same as the hybridization to *HXT6* or *HXT7.* Thus, we reasoned that the region stretching from the start of *HXT6* to the start of *HXT7* had undergone multiple duplications.

The restriction enzyme *Xho*I digests chromosome IV on either side of *HXT6* and *HXT7,* producing a 10-kb fragment containing both of these genes. We confirmed that more than three regions of 5.2 kb each were inserted in the *HXT6*/*HXT7* gene region by detecting an increase in size from 10 kb to greater than 26 kb of this *Xho*I fragment in the evolved strain (fig. 5*B*). Additionally, we sporulated the evolved strain, dissected the tetrads to obtain haploid products, and conducted the same restriction site analyses on the haploids. We found that one chromosome carries the parental genotype in the *HXT6/HXT7* gene region, and the other chromosome carries the multiply duplicated genotype (fig. 5*B*).

Unequal Crossover Between Sister Chromatids Is the Simplest Explanation for the Tandem Duplications of *HXT7/6*

The multiple duplications that we have identified appear to result from unequal crossover events between *HXT6* and *HXT7.* The most parsimonious explanation for these duplications involves unequal crossover events between *HXT6* and *HXT7* on sister chromatids (fig. 6*A*). However, alternative scenarios can be postulated which involve unequal crossover events between homologous chromosomes; one such alternative is described in figure 6*B.* We hypothesize that these crossover events led to the formation of more than three new genes (*HXT7/6*) that have the upstream promoters of the *HXT7* gene and the coding sequence of *HXT6.*

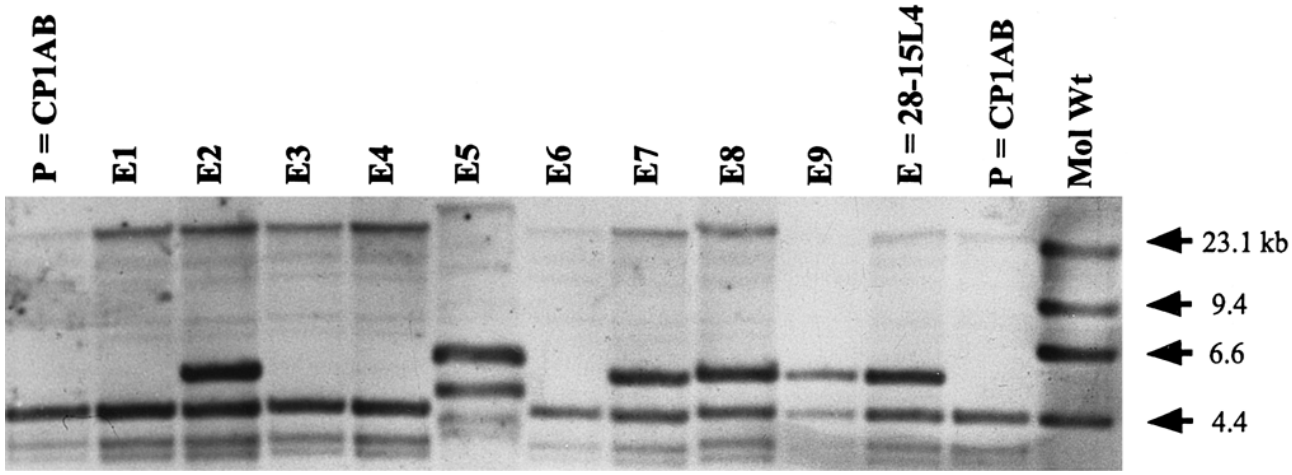

FIG. 7.—Southern analysis of random colonies from the terminal population from which 28–15L4 was isolated. *Pst*I genomic digests were probed with *HXT4.* The apparent difference between E5 and the banding pattern of the other evolved clones is due to high salt concentration in the sample.

Multiple Duplication of the High-Affinity Glucose Transporters Is Not the Only Response to Selection in this Experimental Population

The strain designated 28–15L4 was isolated as a single clone from the terminal population of a 450-generation evolutionary experiment. Previous work has shown that polymorphisms can arise among microbial populations evolving under glucose limitation (Helling, Vargas, and Adams 1987). Such polymorphisms can be stable and persist over many generations as a result of cross-feeding among adaptive clones (Rosenzweig et al. 1994). It is not therefore unreasonable to anticipate the existence of other adaptive clones within this experimental population. Genomic digests of DNA from colonies selected at random from the terminal population were subjected to Southern analysis using an *HXT4* probe. Our results (fig. 7) show that the *HXT7/6a,b,c* genotype is present in five of nine such clones, indicating the presence of other lineages in which gene duplication events involving *HXT6* and *HXT7* have not occurred.

Discussion

We show here that a strain of yeast that has evolved for 450 generations under glucose limitation has multiple tandem duplications involving the high-affinity hexose transport genes *HXT6* and *HXT7.* Restriction site analysis indicates that the duplicated genes have the upstream promoter of the *HXT7* gene and the coding sequence of the *HXT6* gene. These duplications were likely formed by unequal crossovers between the tandemly arrayed *HXT6* and *HXT7* genes during sister chromatid exchange. The change we observe in copy number is correlated with significant increases in mRNA detected with the *HXT6* probe. The multiply duplicated genes are heterozygous in the evolved strain; thus, four duplicate genes would result in twice as many high-affinity hexose transport genes in the evolved strain as in the parental strain. This correlates well with the 1.8-fold increase observed in *HXT7/6* transcript. The evolved strain of yeast has demonstrably greater fitness under glucose limitation than the parental strain from which it is derived, and this greater fitness appears to be due, at least in part, to a significant increase in its ability to scavenge glucose at low substrate concentrations. We have, therefore, identified the genetic basis of one response to selection in a glucose-limited environment.

Targets of Selection Among Microbes Evolving Under Nutrient Limitation

Our growth parameters may be compared with those of Adams et al. (1985). These workers estimated μ_{max}, K_s, and yield for seven adaptive clones that arose sequentially during the course of a 264-generation experiment. Consistent with our observations, they determined that highly significant differences between parental and adaptive yeast clones in K_s and **s** do not translate into significant differences in μ_{max}. Genetic changes that increase fitness under nutrient-limiting conditions do not appear to compromise performance under nonlimiting conditions. These observations suggest that there may be no physiological-genetic ''cost'' required for the evolution of enhanced glucose transport capacity—at least measured in the currency of growth rate on this substrate.

The evolved strain in our study achieves twofold greater yield biomass than its progenitor under substrate-limiting conditions. By contrast, Adams et al. (1985) observed that yield significantly increased among early adaptive clones but declined among those that arose later (>181 generations). Their observation is somewhat surprising. As demonstrated by Dykhuizen and Dean (1994), growth rate at steady state is a function of both the yield coefficient and the flux at which limiting nutrient is imported into cells. Under substrate-limiting conditions, increased fitness can therefore be expected to be achieved by either of two mechanisms:

increased uptake or increased cell yield with respect to limiting nutrient. The evolved strain we describe in this paper appears to utilize both mechanisms.

This discrepancy between our results and those of Adams et al. (1985) is difficult to reconcile theoretically. However, there is no reason a priori to expect the adaptive trajectories of two clonal lineages to be identical—even if they arise from the same genotype and evolve under identical conditions. Moreover, this incongruity may illustrate the importance of historical contingency in the evolutionary process, and would be concordant with the findings of Travisano and Lenski (1996). These workers analyzed replicate *Escherichia coli* populations derived from the same ancestor which evolved for 2,000 generations in an environment in which glucose served as the sole carbon source. Analysis of their terminal populations revealed marked among-strain differences in the physiological mechanisms of adaptation.

It is not surprising that one response to selection in a glucose-limited environment is increased high-affinity glucose transport. Glycolytic flux in yeast is generally considered to be rate-limited by sugar transport (Becker and Betz 1972; Gancedo and Serrano 1989). Any genetic change that relieves this rate limitation and does not have negative pleiotropic effects can thus be expected to be adaptive in a glucose-limited environment. Dykhuizen, Dean, and Hartl (1987) showed that selection in *E. coli* for growth on lactose resulted in an increase in lactose permease activity. Indeed, comparison of the fitness-activity landscape for alleles of *lac*-operon genes reveals that greater fitness differences are almost always realized among *lac* permease variants as opposed to those for β-galactosidase (Dean 1989). *Escherichia coli* evolving under lactose limitation may, however, follow alternative adaptive trajectories. Classic work by Novick and colleagues (Horiuchi, Tomizawa, and Novick 1962; Horiuchi, Horiuchi, and Novick 1963) demonstrated that hypersynthesis of β-galactosidase was achieved by multiple duplications of the *lac* operon.

In *S. cerevisiae,* duplication also affords a means by which the number of hexose transport molecules could be increased. Furthermore, duplication may be particularly facile given the relationship between *HXT6* and *HXT7.* The two genes are tandemly arrayed on chromosome IV and are 99.7% identical within their coding regions (table 1 and fig. 1). Thus, their organization and homology offer a ready mechanism for increasing copy number by unequal crossover. This mode of gene duplication presents an advantage over point mutations inasmuch as copy number can be easily reduced by the same mechanism when selection is subsequently relaxed. Preliminary analyses indicate that the number of duplicated *HXT7/6* genes can decrease when 28–15L4 is serially diluted in rich (YEPD) liquid media supplemented with 2% glucose. This response occurred in less than 75 generations (data not shown) among a subset of replicate cultures. Intergenic recombination between these loci has been noted by other workers. In seeking to identify glucose transporters still present in a s*nf3*Δ *hxt1*Δ *hxt2*Δ *hxt3*Δ *hxt4*Δ strain (CY280), Liang and Gaber (1996) discovered by colony hybridization a chimeric *HXT6/7* gene consisting of the 5′ half of *HXT6* and the 3′ half of *HXT7.* Subsequent analyses showed that most members of the CY280 population harbored the *HXT6/7* chimera and that regulation of high-affinity glucose transport among these cells was similar to wild type.

It is important to note that enhanced glucose transport capacity could be mediated by a variety of other genetic mechanisms. For example, substitutions in HXT coding regions could produce amino acid changes that decrease the Michaelis constant (K_m) or increase the turnover rate (k_{cat}) of such proteins. Whether such changes have also occurred and contribute to the changes we observe in glucose uptake kinetics will require sequencing of the region of chromosome IV that has undergone intergenic recombination between *HXT6* and *HXT7.* Evolutionary change at *HXT* promoters could also mediate increased transport molecule abundance. For example, the number of such molecules could be readily increased by the derepression of *HXT1, HXT3,* and *HXT4,* genes not normally expressed at low glucose concentrations (Ozcan and Johnston 1995; Reifenberger, Friedel, and Ciriacy 1995).

Gene Duplication May Be a Common Evolutionary Response to Selection in Yeast

The observation that multiple duplications involving *HXT6* and *HXT7* have arisen under selection has interesting implications concerning the evolution of the *HXT* gene family. It can be reasonably speculated that the entire hexose transport multigene family in yeast arose through a series of gene duplication and divergence events. *Saccharomyces cerevisiae* has 20 gene sequences that can be grouped together as hexose transport genes on the basis of amino acid sequence similarity, the conservation of 11 transmembrane domains, and the presence of 2 sugar-transport motifs that are conserved in eukaryotes (Kruckeberg 1996). *HXT8* through *HXT17* were detected solely by their appearance in the Saccharomyces Genome Database, and may be important in the transport of exotic hexoses encountered by yeast in natural environments. Within the known glucose transporters, a gene duplication event has been inferred leading to the tandemly arrayed *HXT1/3* and *HXT4/6* genes (fig. 1). Duplications of whole segments of chromosomes led to two sets of tandemly arrayed genes; *HXT3* and *HXT6* are paralogs of *HXT1* and *HXT4,* respectively. *HXT7* was then duplicated from *HXT6.* The proximity and homology of *HXT6* and *HXT7,* as well as their common function and pattern of expression, are likely to favor them as targets of selection in a glucose-limited environment.

The work of Hansche (1975) and Hansche, Beres, and Lange (1978) indicates, however, that proximity and homology are not prerequisites for evolution by gene duplication in response to selection. Their studies used yeast grown continuously under phosphate limitation, a condition that favors upregulation of the acid phosphatase genes (Francis and Hansche 1972). They found that almost half of the right arm of chromosome II was translocated to another chromosome in five separate selection

experiments (three experiments induced translocation by UV irradiation). The translocated chromosome II apparently segregated with the intact chromosome II, effectively doubling the copy number of the genes in the translocated segment. The translocations included the two acid phosphatase genes *PHO3* and *PHO5* (Bajwa et al. 1984) and all of the loci distal to these sequences.

Recent analyses of the complete yeast genome sequence provide compelling evidence for the role of gene duplication in the evolution of this species (Goffeau et al. 1996; Netter, Maillier, and Coissac 1996). Indeed, the entire *S. cerevisiae* genome appears to have undergone duplication in the distant past, and subsequent deletions and translocations have broken up the original chromosomal associations (Wolfe and Shields 1997). We can only speculate as to the role played by directional selection as opposed to other evolutionary forces in directing this process. Adams et al. (1992) screened for major chromosome length polymorphisms among replicate yeast populations maintained in phosphate-limited culture for up to 1,000 generations. They observed large-scale rearrangements involving 9 of 16 chromosomes in 9 of 13 terminal populations. Surprisingly, in only four instances did these changes result in increased copy number of structural genes for acid phosphatase. Nevertheless, the prevalence, the persistence, and the magnitude of these chromosomal accretions and deletions (10–390 kb) argue strongly for their adaptive value. Our study, as well as those by Hansche (1975), Hansche, Beres, and Lange (1978), and Adams et al. (1992) indicate that the yeast genome is surprisingly labile and responsive to directional selection.

Duplication of the *HXT7/6* Chimera Is Not the Only Adaptive Response of Yeast Evolving Under Glucose Limitation

Multiple duplications involving *HXT6* and *HXT7* are not the only responses to selection exhibited among cells of the terminal population. Although a majority of clones examined exhibit the *HXT7/6a,b,c* genotype, others are present whose *HXT* restriction pattern cannot be distinguished from that of the parent. Further study will be required to determine the mechanisms by which these lineages have responded to selection, as well as to resolve whether the observed polymorphism can persist over many generations. Even within that lineage leading to strain 28–15L4, multiple duplications involving *HXT6* and *HXT7* cannot be the only responses to selection. Paquin and Adams (1983*a*, 1983*b*) showed that yeast evolving under glucose limitation undergo adaptive shifts approximately every 50 generations. Thus, in this 450-generation experiment, we can expect approximately nine adaptive mutations to have accumulated within the lineage of the predominant terminal clone. The adaptive changes that we report in this paper only require the fixation of three independent mutations.

That other adaptive changes have occurred is clear from consideration of the following phenotypic differences between the parental strain and 28–15L4. Consistent with Adams et al. (1985), we observe that when cells of the evolved strain are grown in continuous culture, they are somewhat smaller and decidedly more elongated (data not shown) than are those of the parental strain. The evolved strain therefore shows morphological changes that result in a more favorable surface-area-to-volume ratio for substrate uptake. In addition, although the evolved strain consumes 10% more of the total available glucose than does the parent strain from which it was derived, its yields in cell mass and cell number are 2-fold and 2.6-fold greater, respectively. The evolved strain not only transports limiting substrate more rapidly and to lower steady-state concentrations, but also utilizes that substrate more efficiently in the production of biomass. Further work is needed to uncover the genetic bases for these differences in morphology and substrate utilization efficiency as well as for other, yet-to-be-identified, traits that affect fitness in a constant glucose-limited environment.

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