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The generation of multiple co-existing *mal*-regulatory mutations through polygenic evolution in glucose-limited populations of *Escherichia coli*

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Summary

The multicomponent glucose transport system of *Escherichia coli* was used to study the polygenic basis of increased fitness in prolonged nutrient-limited, continuous cultures. After 280 generations of glucose-limited growth, nearly all bacteria in four independent chemostat populations exhibited increased glucose transport and contained multiple, stable mutations. Fitter bacteria increased outer membrane permeability for glucose through overexpression of the LamB glycoporin. Three classes of mutation influenced LamB levels as well as regulation of other *mal* genes. Low-level *mal/lamB* constitutivity resulted from *mlc* mutations acquired in all populations as well as changes at another uncharacterized locus. Larger increases in transporter content resulted from widespread acquisition of a regulatory *malT–con* mutation in fit isolates. The *malT* mutations sequenced from 67 adapted isolates were all single base substitutions resulting in amino acid replacements in the N-terminal third of the MalT activator protein. Analysis of *malT–con* sequences revealed a mutational spectrum distinct from that found in plate-selected *malT* mutants, suggesting that mutational pathways were affected by environmental factors. A second major finding was the remarkable allele diversity in *malT* within a population derived from a single clone, with at least 11 different alleles co-existing in a population. The multiplicity of alleles (as well as those found in adaptive *mgl* changes in the accompanying study) suggest that the periodic selection events observed previously in such populations are not a major factor in reducing genetic diversity. A simple model is presented for the generation of genetic heterogeneity in bacterial populations undergoing polygenic selection.

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Introduction

Prolonged continuous culture with glucose limitation leads to multiple mutations and alteration of protein expression patterns in chemostat-adapted *Escherichia coli* (Kurlandzka *et al*., 1991; Rosenweig *et al*., 1994). The contribution of these changes to fitness is largely unknown, but the single most frequently identified characteristic of nutrient-limited populations is the improved uptake of limiting nutrient (Dykhuizen and Hartl, 1983; Helling *et al*., 1987; Sonti and Roth, 1989). Mutations in glucose-limited chemostats leading to better transport have not been identified. The rapidity of takeover of chemostat populations by fitter bacteria is also well known (Helling *et al*., 1987), but the number and type of mutations in these evolved bacteria has never been defined. Also, the relationship of DNA changes under nutrient limitation to the contentious 'directed' or 'adaptive' mutations observed in agar plate selections of Cairns *et al.* (1988), Foster (1993), Sniegowski and Lenski (1995) and Benson (1997) is also unknown. In this and the accompanying study (Notley-McRobb and Ferenci, 1999), we identify some of the most commonly found mutations in glucose-limited populations.

Glucose limitation in a defined medium provides a seemingly simple selection condition, but the cellular response to this stress is necessarily multifactorial. As shown in Notley-McRobb and Ferenci (1999) (see Fig. 1), glucose uptake into *Escherichia coli* at low substrate concentrations involves two parallel pathways, nine proteins and at least five separate gene-regulatory inputs (Ferenci, 1996). Individual mutations affecting any of the transport steps could improve glucose uptake, but a more significant improvement must involve multiple changes to improve outer membrane permeability as well as transport through the cytoplasmic membrane. The evolution of bacteria in nature as well as in the chemostat must often result from multiple genetic changes, and the multifactorial adaptation of sugar transport provides an unexpectedly useful model for considering polygenic evolution. As seen below, a consequence of polygenic adaptation is the accumulation of genetic diversity in bacterial populations.

Outer membrane permeability is particularly important at low external substrate concentrations, and the LamB protein is the most significant contributor to glucose uptake with micromolar substrate (Death *et al*., 1993).

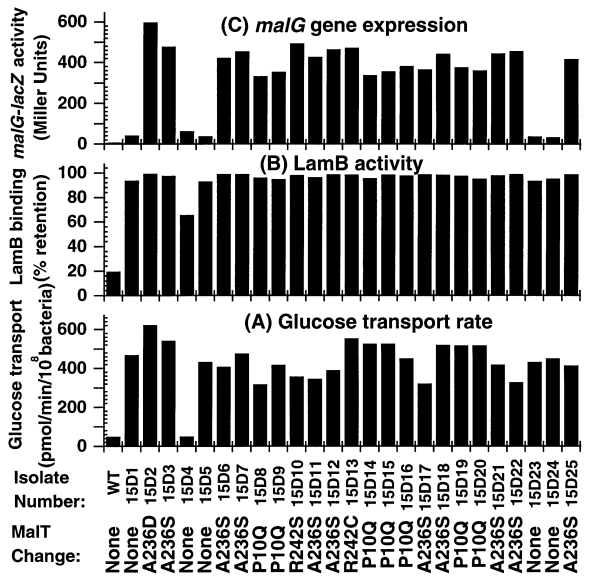

Fig. 1. Profile of chemostat-adapted bacteria after 280 generations of glucose limitation. Chemostats described in previous studies (Death *et al.*, 1993; Notley-McRobb *et al.*, 1997) were inoculated with BW2952, an MC4100 derivative with a *malG–lacZ* transcriptional fusion (Notley and Ferenci, 1995). A sample was plated onto non-selective nutrient agar after 4 weeks at a dilution rate of $0.3\,h^{-1}$, and 25 randomly separated colonies were tested for the following phenotypes.
A. The initial rate of 0.5 μM [^{14}C]-glucose uptake into isolates grown on glycerol minimal medium was measured as described previously (Death and Ferenci, 1993).
B. The LamB-dependent starch binding activity of glycerol-grown bacteria was assayed with starch–Sepharose columns as described previously (Ferenci and Lee, 1982).
C. The β-galactosidase activity resulting from the *malG–lacZ* fusion was determined with glucose-grown bacteria as described previously (Notley and Ferenci, 1995).

Bacteria have a short-term regulatory mechanism for boosting LamB expression under glucose limitation (Notley and Ferenci, 1995) but, as demonstrated below, extended limitation leads to more extensive genotypical changes. The LamB glycoporin is regulated as part of the *mal* regulon, which has been studied extensively (Schwartz, 1987; Notley and Ferenci, 1995). The central MalT activator protein regulates expression from at least five 'maltose box' promoters, most of which are also under Crp/cAMP control. Recently, it has been demonstrated that *malT*, and indirectly the Mal system, is also regulated by a global repressor called Mlc (Plumbridge, 1998). MalT protein requires intracellular maltotriose for the activation of transcription (Raibaud and Richet, 1987), and growth on maltose or other maltosaccharides is the classical means of eliciting *mal* gene induction. Maltose and maltodextrin transport functions are encoded in the *malEFG* and *malKlamBmalM* operons (Schwartz, 1987). The best-studied mutations affecting *mal* regulation are *malT–con* changes, which result in amino acid substitutions that reduce the dependence of MalT on inducer (Debarbouille *et al.*, 1978; Dardonville and Raibaud, 1990). Such mutants were obtained in agar plate selections starting with *lac*$^-$ *mal–lacZ* fusion strains, selecting for the improved, maltose-independent ability to ferment lactose. As seen below, adaptive evolution during growth on glucose in chemostats also selects for *malT–con* mutations, but with an interestingly different mutational spectrum.

Results

Selection and fitness in bacteria adapted to glucose limitation

The strain BW2952 (Notley and Ferenci, 1995) was inoculated into glucose-limited chemostats fed with medium at a dilution rate of $0.3\,h^{-1}$ as described by Notley-McRobb and Ferenci (1999). Growth was maintained with 2×10^{10} bacteria for 200–280 generations over a 3- to 4-week period. Preliminary experiments indicated that, by this time, nearly all bacteria in four separate populations exhibited much higher glucose transport activity, as shown in Fig. 1A for 25 randomly isolated members of one population. Changes in the properties in Fig. 1 were also obtained in three other populations after 200 generations of glucose-limited growth.

Increased lamB *and* mal *expression under glucose limitation*

As the outer membrane permeability of glucose under nutrient limitation involves LamB glycoporin (Death *et al.*, 1993), the level of protein in chemostat isolates was investigated. Individual isolates from chemostats were inoculated into batch cultures with high (0.2%) glucose or glycerol and assayed for LamB using immunoblots (Chan *et al.*, 1996), as shown in Fig. 2A, and by LamB-dependent starch binding as shown in Fig. 1B. There was a significant alteration in LamB regulation in isolates over the time course of these experiments, and the rapidity of LamB changes are shown in Fig. 2A. Within 1 week (about 70 generations), three out of 18 random isolates from a chemostat population showed higher LamB expression, but lower than that in fully maltose-induced, wild-type bacteria. By the second week, all isolates had higher LamB amounts, three with the intermediate level found in week 1, but 15 with a very high LamB content even after growth in glucose medium. These changes were found in all populations, and all 25 isolates after 280 generations (Fig. 1B) from another population also had higher LamB-dependent sugar binding resulting from higher protein levels (confirmed by immunoassay; not shown).

lamB expression is controlled by the *mal* regulon (Schwartz, 1987), and the expression of *mal* genes in chemostats was followed using a *malG–lacZ* transcriptional

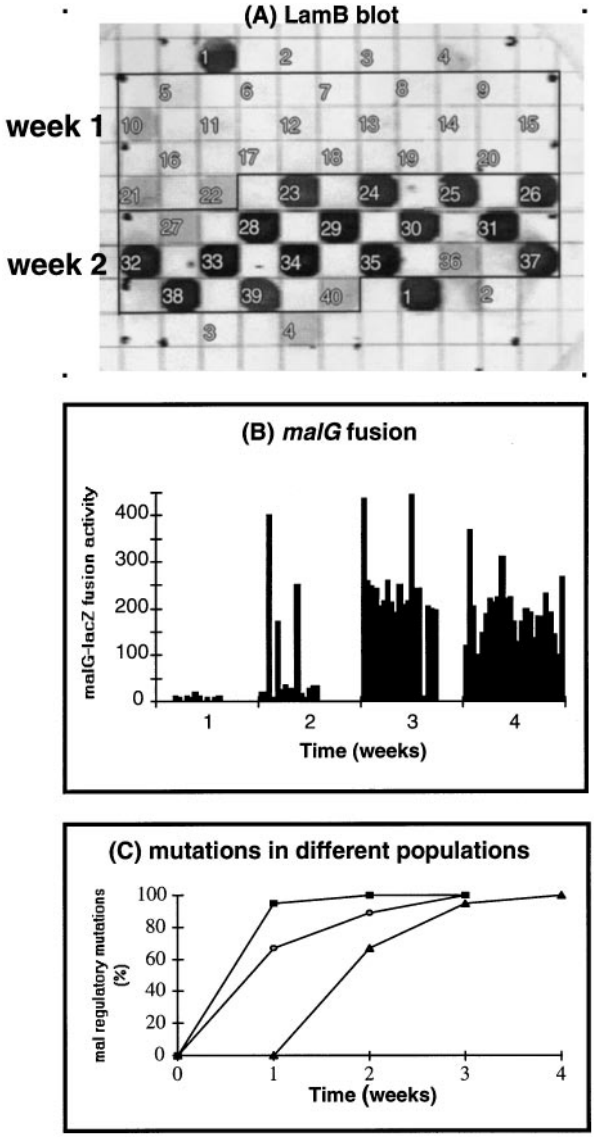

Fig. 2. Accumulation of *mal*-regulatory mutations in glucose-limited populations.
A. An immunoblot measuring LamB protein levels in bacteria isolated from a chemostat with glucose limitation after 1 and 2 weeks (70 and 140 generations). The samples from the chemostat were streaked for single colonies, and 18 randomly picked colonies from non-selective media were grown on glucose minimal medium in batch culture. Samples from these cultures were assayed according to the method of Notley *et al.* (1994), with controls including fully induced maltose-grown bacteria (no. 1), LamB$^-$ bacteria (no. 2) and glucose- and glycerol-grown uninduced wild-type bacteria (nos 3 and 4).
B. Increase in *malG–lacZ* fusion activity in chemostat isolates obtained after up to 4 weeks (280 generations) of glucose-limited selection. Isolates processed as in (A) were assayed for β-galactosidase activity using glucose-grown, batch-cultured bacteria. Results are expressed in Miller units (Miller, 1972).
C. The time course of *mal*-regulatory changes in isolates assayed as in (B) for three independent chemostat cultures, with 15–25 random isolates assayed at each time point.

fusion in the starting strain. As shown for one population in detail, the development of fitness over the 4 weeks of selection resulted in universal changes in *malG* expression, with low-level constitutivity developing early (Fig. 2B). As shown in Fig. 2C, all three populations analysed in this way eventually showed a 100% change in *mal* regulation, tending to high levels of constitutivity by 200 generations of selection. As shown in more detail in Fig. 1C, all 25 isolates contained *mal*-regulatory mutations as shown by *malG* fusion activity above wild-type levels.

The results in Figs 1 and 2 suggest that a near-universal response to glucose limitation is a regulatory change in *mal* expression. It should be noted that the fusion strain used to initiate selections was Mal$^-$ resulting from the *malG* insertion. The advantage of the constitutive *mal*/*lamB* regulon is therefore not attributable to maltose utilization during growth on glucose; the improved outer membrane permeability resulting from glycoporin function is the likeliest selective determinant under these conditions (Death *et al.*, 1993). The kinetic advantage to glucose transport of high *mal* expression is evident from the effect of a *malT* mutation abolishing *lamB* expression. The introduction of a *malT* mutation into one of the 'fit' isolates in Fig. 1 (isolate 15D2) increased the apparent K_m for glucose transport from 1.6 μM to 5 μM without significantly affecting the V_{max} (results not shown), which would be particularly important at the low glucose concentrations found in the chemostat (Senn *et al.*, 1994).

Three classes of mal-*regulatory changes*

As is clear from the results in Figs 1 and 2, there were two levels of *mal* constitutivity in the chemostat isolates. Genetic mapping with the transducing phage P1 localized one site of mutation in the 20 high-activity isolates in Fig. 1 near the *glgA* and *ompR* genes at 77 min on the chromosome, which are close to the *malA* region involved in *mal* regulation (Schwartz, 1987). Mutations in *malT* were the likeliest cause of constitutivity in the 77 min region (Debarbouille *et al.*, 1978), but the complexity of the chemostat population was revealed further by transduction results, which showed that another weak *mal*-regulatory mutation co-existed with *malT–con* mutations in most isolates. Transduction of the *glgA*-linked mutation to a wild-type background resulted in lower *mal* expression than in the original mutant, as shown for one example (15D2) in Fig. 3. Likewise, replacing the *malT* region in 15D2 with a wild-type *malT* gene resulted in a higher than uninduced residual *mal* expression. Both results suggested that a second low-level regulatory mutation was present. Twelve out of 12 chemostat *malT–con* isolates analysed had this additional *mal* mutation, as did 10 out of 10 isolates from two other populations. The weak *mal*-regulatory mutation was unlinked by transduction to either *lamB* or *malT*.

A third class of regulatory mutation was present in four out of the five low-activity *mal*-constitutive isolates in the same population in Fig. 1, also unlinked by transduction to either *lamB* or *glgA*. The constitutivity in 15D1, 15D5, 15D23 and 15D24 was caused by mutations at the *mlc* locus. As shown by Notley-McRobb and Ferenci (1999), the *mlc* type of mutation was present in each of four independent populations, each with a different type of mutation. The nature of the low-level constitutivity mutation in 15D4 is unidentified so far, but neither low- nor high-level constitutivity appeared to result from amplification of the *lamB* structural gene.

The nature of the DNA changes at *malT* were analysed by sequencing after polymerase chain reaction (PCR) amplification from each isolate. The promoter region as well as the structural gene were looked at, as *malTp* mutations could also cause *mal* upregulation (Chapon, 1982). Point mutations were found only within the structural gene as listed in Table 1, in every case resulting in a single amino acid substitution. The 21 independent replacements obtained in chemostats, at sites between residues 5 and 358 in the large, 901-amino-acid protein, extend the information available on the regions of MalT protein involved in inducer-independent induction. Many of the changes clustered around one of two regions (near residue 242) previously identified by Raibaud (Dardonville and Raibaud, 1990) as contributing to constitutivity, but many additional substitutions towards the N-terminal part of the activator protein also contributed to a *malT–con* phenotype.

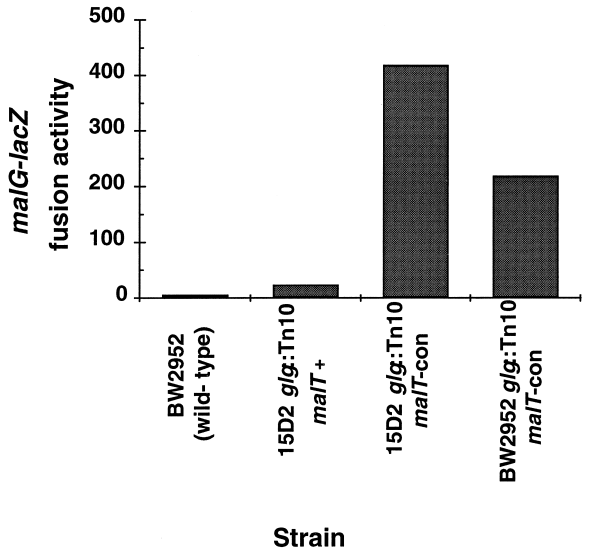

Fig. 3. The contribution of *malT* and other regulatory mutations to *mal* gene expression. P1 transduction with a *glgA*::Tn*10* (Decker *et al*., 1993) resulted in 70% co-transduction of the linked *malT* gene in isolate 15D2, resulting in two different levels of *malG* gene expression as shown. A P1 lysate grown on the high-activity transductant was used to transfer constitutivity back into a clean BW2952 background, without the other mutations accumulated by chemostat isolates.

Table 1. malT changes in chemostat isolates.

Amino acid/codon	Occurrence[a]
Chemostat 15 (42 *malT–con* sequenced)	
P10Q CCG → CAG	×7
A236D GCC → GAC	×1
A236S GCC → TCC	×25
A240E GCA → GAA	×1
A244E GCG → GAG	×1
R242S CGC → AGC	×1
R242C CGC → TGC	×2
M311I ATG → ATA	×1
M311I ATG → ATT	×1
W317P TGG → CGG	×1
S358I AGC → ATC	×1
Chemostat 2 (22 *malT–con* sequenced)	
S5L TCA → TTA	×6
R9S CGT → AGT	×2
T38R ACG → AGG	×1
D65E GAT → GAG	×1
A236D GCC → GAC	×8
A236S GCC → TCC	×2
R242S CGC → AGC	×2
Chemostat 22 (3 *malT–con* sequenced)	
A219T GCG → AGC	×1
R242S CGC → AGC	×1
E302D GAG → GAT	×1

a. Occurrence indicates the number of times each sequence was found in a population.

Extensive sequencing was carried out with two independent populations shown in Table 1 as well as less extensive analysis from one other population. Two common features were evident in all of the sequencing results. First, there was a considerable bias in substitutions at the DNA level, with the great majority of mutations arising from transversions at GC sites in the native sequence. Another unexpected feature of the sequence analysis was the multiplicity of mutations in each population. Five different alleles were found among the 20 *malT* mutants described in Fig. 1, and six additional alleles listed in Table 1 were among 22 other sequenced but less characterized members of the same population. As also shown in Table 1, another culture contained seven alleles in only 22 *malT–con* mutants analysed, and three out of three were different in a third population, highlighting the remarkable genetic diversity in these evolving populations. The significance of these results is discussed below.

Discussion

It is evident from the above results that every bacterium contained mutations influencing transport capability after 200–280 generations in a glucose-limited chemostat, with upregulation of the LamB component of the outer membrane universal within all populations tested. Combined with *mgl* mutations in the same isolates (Notley-McRobb and Ferenci, 1999), the high-affinity glucose transport pathway

in Fig. 1 of Notley-McRobb and Ferenci (1999) was significantly enhanced. Adaptation involved at least three classes of *mal*-regulatory mutations, with more than one frequently present in fit strains. The accumulation of these mutations occurred over a relatively short time frame and generated multiple, co-existing alleles of *mal*-regulatory changes. These results impinge on a number of commonly held notions on *mal* gene regulation, adaptive mutations and evolution of bacterial populations, so these aspects will be considered in turn.

MalT and mal *gene regulation*

E. coli possesses a mechanism for inducing *mal* genes under glucose limitation through endoinduction. Bacteria contain elevated LamB levels because of the accumulation of endogenously generated maltotriose in glucose-limited continuous culture. The extent of *mal* gene induction through endoinduction is only half-maximal (Notley and Ferenci, 1995), so there is a definite advantage to be gained in chemostats by increasing *malI lamB* expression through regulatory mutations. In this instance, increased gene dosage was not a major factor in *mal* upregulation.

The acquisition of *malT–con* mutations in chemostats was not surprising, given that such mutations were known to increase *malI lamB* expression (Debarbouille *et al*., 1978; Dardonville and Raibaud, 1990). Also, the recent description of *mlc* as a repressor of *mal* expression (Decker *et al*., 1998) provides an explanation of the prevalence of *mlc* mutations in each selected population. But the nature of the other, low-level constitutivity mutation is more puzzling. Some loci are known to affect *mal* regulation, including *malI*, *crp*, *lrp* and the *ompB* locus, but it is not obvious how mutations at these sites would lead to elevated expression. A *crp** mutation can be ruled out, as transductional experiments rule out the *crp* locus (experiments not shown). As the mutation is unlinked to *lamB*, it cannot affect the postulated MalK-dependent regulation of MalT (Kuhnau *et al*., 1991). Another possibility is that elevated expression is caused by higher pools of endogenous inducer (Reidl *et al*., 1989) improving *mal* induction, as high endogenous maltotriose levels can stimulate LamB expression (Notley and Ferenci, 1995).

Mutational processes under glucose limitation

Growth in a chemostat with glucose limitation reduces catabolite repression (Notley-McRobb *et al*., 1997) and induces stationary phase gene expression (Notley and Ferenci, 1996). Mutation/repair processes controlled by catabolite repression (MacPhee, 1985) starvation (Bridges, 1994) or RpoS (Gomez-Gomez *et al*., 1997) may well influence the nature and frequency of advantageous mutations. The presence of a low proportion of mutator variants in the population can also speed evolution (Sniegowski *et al*., 1997; Taddei *et al*., 1997), and it is well established that mutator variants have a selective advantage in chemostats (Gibson *et al*., 1970; Trobner and Piechocki, 1984). Hence, altered regulation of, or mutations in, particular mutator genes would increase mutation rates and bias the type of mutations obtained (Miller, 1996).

The spectrum of *malT* base changes shown in Table 1 and Fig. 4, with 19 out of 21 independent point mutations occurring at G:C basepairs, indeed suggests that particular mutational processes dominated under glucose limitation. An interesting contrast to the chemostat spectrum is provided by the *malT*-constitutive mutations obtained from plate selections (Debarbouille *et al*., 1978; Dardonville and Raibaud, 1990), which contain a more commonly observed distribution of A:T changes also found among spontaneous mutants of *lacI* and *crp* as shown in Fig. 4. The *mgl* point mutations in the accompanying study (Notley-

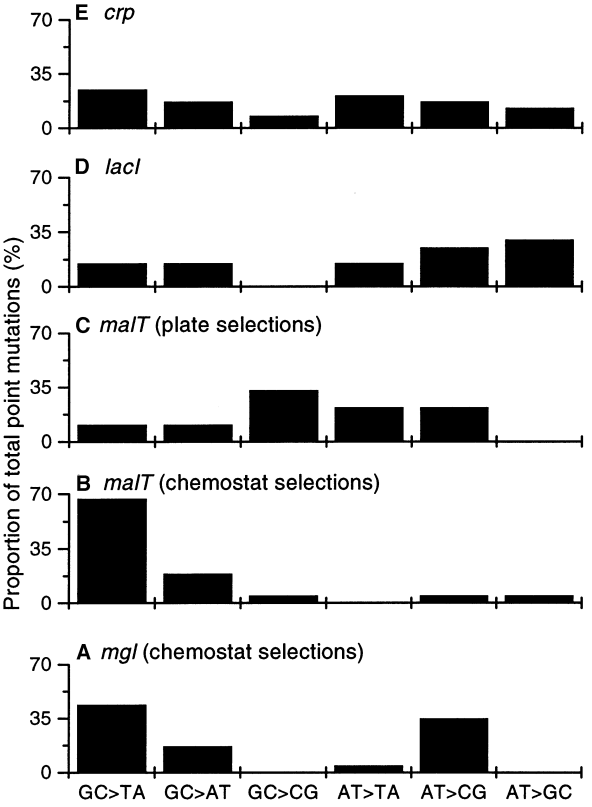

Fig. 4. Types of base changes in chemostat and plate selections. The sequence data for chemostat isolates are based on: (A) *mgl* sequences in Notley-McRobb and Ferenci (1999); (B) *malT* sequence changes in Table 1; (C) the sequences of mutants obtained in plate selections were published by Debarbouille *et al.* (1978) and Dardonville and Raibaud (1990). One additional plate-derived sequence, in strain pop3325 (Debarbouille *et al*., 1978), was obtained and contained a W317R substitution and a TGG → AGG codon change.
D and E. The *lacI* and *crp* point mutation data are based on studies by Schaaper *et al.* (1986) and Takimoto *et al.* (1997) respectively.

McRobb and Ferenci, 1999) also show a predominance of changes at G:C basepairs but contain a cluster of AT→CG changes in particular *mgl* operator mutants. Both *mgl* and *malT* point mutations derived in chemostats are biased towards transversion events, more so than the spontaneous mutations obtained by conventional agar plate selections in *malT* or other genes (Fig. 4).

It is an open question as to which 'environmental' factor(s) differ between selecting for growth on agar plates as against faster growth in chemostats to generate these mutational differences. As noted above, glucose limitation could itself be a factor. Also, possibly oxygen radical damage may be greater in air-saturated chemostats than inside a layer of static, microaerophilic bacteria or colonies on a plate. There is a tempting correspondence of the mutational spectrum in Fig. 4 in chemostat mutants with that seen in *mutY* mutants lacking oxygen damage repair (Miller, 1996). These factors will be investigated in subsequent studies, which offer the prospect of looking at the spectrum of mutations in the controlled environment of a chemostat.

Population genetics of glucose-limited chemostats

There was an amazing multiplicity of alleles in several populations looked at, and there must have been others missed by sampling only up to 42 out of 10^{10} members of one population. As noted in the accompanying study (Notley-McRobb and Ferenci, 1999), the diversity of clones in the chemostat needs to be reconciled with the classic periodic selection model of bacterial population shifts (Atwood *et al.*, 1951; Selander *et al.*, 1987). As discussed in more detail in (Notley-McRobb and Ferenci, 1999), our results indicate that there were enough co-existing alleles in a population to maintain a high level of genetic diversity, despite the possible purging effects of periodic selection. To resolve this problem, there is a definite need for more detailed kinetic studies to understand the contribution of mutations to population shifts. The identification of *mal* and *mgl* genes rapidly altered under glucose limitation will allow future work to concentrate on the fate of alleles during population shifts and, hence, illuminate the processes of bacterial population dynamics in more detail.

A possible source of allele diversity through polygenic selection

Even if environmental or other factors in the chemostat provide a 100- to 1000-fold increase in mutation rates as seen with strong mutator alleles, it is still unlikely that favourable *malT* + *mal*? + *mgl* or *mgl* + *mal*? + *mlc* combinations arose simultaneously in chemostats several times in the same population. More probably, sequential acquisition of mutations took place during continued polygenic selection under glucose limitation (Fig. 5). If an *mgl1* mutation

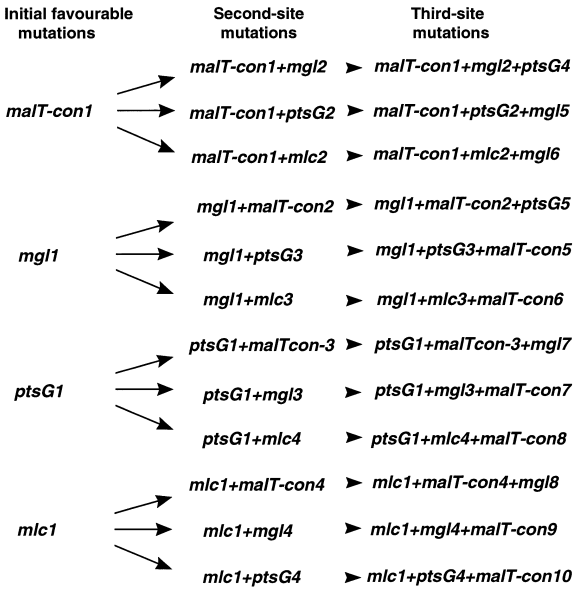

Fig. 5. A model for the co-evolution of multiple alleles during polygenic selection. The chemostat population consists initially of wild-type bacteria, but selection leads to the enrichment of bacteria with individual mutations at the four loci contributing to improved glucose transport. Two additional rounds of acquiring other advantageous mutations leads to the accumulation of 10 different *malT* alleles in progeny. Not all possible outcomes are shown for other loci.

arose independently of a *malT–con*1 change in another bacterium early in the history of a population, each mutant would have a growth advantage over the wild-type clone. After continued selection, a *malT–con*2 mutation arising in combination with the *mgl*1 mutation (as well as *mgl*2 with *malT–con*1) would provide the additive beneficial effects of both outer and inner membrane changes. Excluding lateral gene transfer, the *malT–con*2 mutation arising in this way is independent of *malT–con*1. Hence, starting from a single clone and assuming four separate and additive means of improving glucose transport as in Fig. 5, continued selection pressure must result in a fitter population with 16 independent *malT–con* alleles by the time all the isolates accumulated mutations in all four genes, with 24 possible allele combinations.

Of course, the quantitative outcome of polygenic selection as in Fig. 5 is more complex and influenced by a number of variables, such as mutation frequencies at different loci, the fitness properties of alleles as well as hitch-hiking effects. Nevertheless, diversity would be even more accentuated if a greater number of loci contribute to fitness, as is highly likely (e.g. porin regulation or structural gene adaptation). With more loci contributing sequentially to an increasingly fit state, the number of alleles of each would be even higher and explain why at least 11 different *malT* alleles co-exist in a small sample of an evolving population. Despite the complexity of the quantitative aspects, the random sequential acquisition of mutations

suggested by Fig. 5 provides one explanation for the establishment of the high diversity of *malT* alleles in the chemostat population.

Through the process outlined in Fig. 5, a high level of heterogeneity is predicted not only for *malT* mutations, but for any gene contributing to the multifactorial process. Indeed, a postulated consequence of polygenic selection is the accumulation of genetic diversity at each of the loci contributing to the multifactorial phenotype. The diversity of *mgl* alleles in Notley-McRobb and Ferenci (1999) is consistent with this notion. In any complex evolutionary selection, the important consequence of polygenic selection is that there is no unique winner clone, but a diverse collection of physiologically fit but genetically distinct organisms.

Experimental procedures

Media and growth conditions

Bacteria in continuous culture were grown in 80 ml chemostat cultures for prolonged selections using the minimal medium A–0.04% glucose medium and apparatus described previously (Death *et al*., 1993; Notley-McRobb *et al*., 1997). Batch cultures were in the same medium but with 0.4% carbon source specified for each experiment. The starting strain used for selections was an *E. coli* K-12 MC4100 derivative, BW2952 (Notley and Ferenci, 1995), which contains a *malG–lacZ* transcriptional fusion but was otherwise wild type for glucose transport. Growth was continued with four independently inoculated populations for 4 weeks at a dilution rate of $0.3\,h^{-1}$ and monitored as described previously (Death *et al*., 1993; Notley-McRobb *et al*., 1997). Samples taken from the chemostat at weekly intervals were streaked onto nutrient agar plates. Twenty-five independent colonies from these plates were repurified by further streaking and stored in glycerol stocks for assays described below.

Assay of transport, protein and enzyme activities

The initial rate of 0.5 μM [^{14}C]-glucose uptake into isolates grown on glycerol minimal medium was determined as described previously from four time points in the first 2 min after the addition of substrate (Death and Ferenci, 1993). Transport kinetics were measured in the same way, but by adding bacterial suspensions at $A_{580} = 0.1$ and using substrate glucose in the 0.05–4.5 μM range. The LamB protein content of bacteria was measured in two ways. First, the starch binding activity of bacteria, as assayed by Ferenci and Lee (1982), is a measure of LamB function. Secondly, a filter-based immunoassay was used as described by Chan *et al*. (1996). β-Galactosidase activity from gene fusions was assayed according to the method of Miller (1972).

Genetic mapping and DNA sequencing

Bacterial genetic techniques involving P1 (*cml clr*100) for mapping and strain construction was performed as described by Miller (1972). The *glgA*::Tn*10* marker used for mapping the *malT* mutants was obtained from strain KD261 (Decker *et al*., 1993). The *ompR*::Tn*10* came from BW2637 (Heine *et al*., 1988).

To sequence the 2703 bp *malT* gene as well as 160 bp of its upstream promoter region, oligonucleotide primers were used to generate five overlapping PCR fragments for automated DNA sequencing. The five fragments were flanked by AGTTTTTTGATTGCCGTCTGT (forward) and AACACCAACTGCGGATTTTCC (reverse), GCCTGGAGTCTGTTCAACCAT (forward) and GAGATCGCTCATCAACCG (reverse), AGCACGATGTCTGGCACTAC (forward) and GAGATCGCTCATCAACCG (reverse), TGCTGGGCGAGTTTGAACCG (forward) and CGTTATCCGGCTAAACTTACA (reverse) and GCGTCGTTTTAGGTGAGTTG (forward) and ATTAGCTGTAGTGCCGTCGC (reverse) primer sequences. Both strands of PCR products were sequenced after purification using Promega Wizard kits by dye-terminator technology on a Catalyst Robotic workstation.

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