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The roles of starvation and selective substrates in the emergence of *araB–lacZ* fusion clones

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The *araB–lacZ* fusion system has been a key case in the 'directed mutation' controversy. Fusions did not occur detectably during normal growth but formed readily after prolonged incubation on selective Ara–Lac medium. To distinguish the roles of starvation stress and selective substrates in coding sequence fusions, we applied sib selection and PCR technologies. Sib selection of the prefusion strain, MCS2, starved under aerobic conditions permitted us to isolate active fusion clones which had never been in contact with arabinose or lactose. Hence, a directive role for selective substrates is not essential. Aerobiosis was necessary for fusions to appear in glucose-starved cultures. The difference in fusion formation between normal and starved conditions is best explained by the response of a signal transduction network to physiological stimuli to activate Mu prophage joining of *araB* and *lacZ* sequences. PCR analysis revealed that direct plating on selective Ara–Lac agar yielded mostly a single class of 'standard' fusions, while sib selection yielded a broader spectrum of fusion structures. Standard fusions were found to occur within a narrow 9 bp window in *lacZ*. The high frequency of standard fusions in glucose-starved cultures suggested efficient and/or specific Mu action.

Key words: genetic fusion/hybrid coding sequences/PCR analysis/sib selection/signal transduction

Introduction

The realization that proteins evolve (at least in part) by the rearrangement of distinct functional domains is one of the major findings to come out of sequence database analysis (e.g. Stock *et al.*, 1990; Wild and Wales, 1990; Miklos and Campbell, 1992). The original Casadaban technique for isolating hybrid coding sequences directing the synthesis of *lacZ* fusion proteins has provided an experimental model for analyzing how such rearrangements occur (Casadaban, 1976). This technique has been used to isolate *lacZ* fusions to many upstream coding sequences (Silhavy *et al.*, 1984; Shapiro, 1987). Figure 1 schematizes the construction of strain MCS2, which has been used to analyze the formation of *araB–lacZ* coding sequence fusions (Shapiro, 1984). A detailed kinetic analysis of *araB–lacZ* fusions provided the first thoroughly documented example of selection-induced genetic change in *Escherichia coli* (Shapiro, 1984). The *araB–lacZ* fusion system attracted interest because of the dramatic kinetics with which fusion clones appeared on selective plates containing arabinose and lactose as sole carbon sources. No colonies appeared within the first 2 days of plating, indicating that no fusions were formed during normal growth prior to selection, but colonies appeared rapidly after a delay of between 4 and 19 days after plating (Shapiro, 1984; Cairns *et al.*, 1988; Mittler and Lenski, 1990).

The role of a transposable element in the *araB–lacZ* system was central to the phenomena observed. The Casadaban technique utilized a Mu*cts62* prophage as genetic homology to align the 5′ domain of any *E.coli* cistron upstream of a decapitated *lacZ* sequence which had no active promoter and which also carried the *lacZU118 ochre* mutation at codon 17. The resulting strains, such as MCS2, thus had a complete Mu*cts62* prophage separating the 5′ and 3′ domains to be fused (Figure 1). Recovery of β-galactosidase activity depended upon fusing *lacZ* to another transcribed coding sequence in-frame 3′ to the G → T transversion at nucleotide 49 (Kalnins *et al.*, 1983). Such fusions could remove as much as the first 25 *lacZ*-encoded amino acids without destroying enzymatic function (Fowler and Zabin, 1983; Silhavy *et al.*, 1984). This meant that enzymatically active *araB–lacZ* fusions could be formed with a *lacZ* endpoint within the 26 bp interval from nucleotides 49 to 75. Genetic studies of the *araB–lacZ* system have demonstrated a requirement for the Mu A transposition function in fusion formation (Shapiro and Leach, 1990). This result helped explain a peculiar feature of hybrid coding sequences obtained by the Casadaban procedure. They frequently contained inverted Mu right-end nucleotides forming a linker between the 5′ upstream sequence and the 3′ *lacZ* sequence (Shapiro, 1987). Figure 1 illustrates such an inverted Mu linker in an *araB–lacZ* fusion. A molecular model based on the known Mu A-dependent strand transfer process could explain how such linker structures formed (Shapiro and Leach, 1990).

In addition to Mu A function, cellular regulatory proteins including IHF, HU and ClpPX protease have been shown to play a role in fusion formation (Shapiro and Leach, 1990; Shapiro, 1993a). Supplementation of the selective Ara–Lac medium with limiting concentrations of glucose accelerated the fusion response in a part of the selected population, indicating a role for carbohydrate depletion in activation of the fusion events (Shapiro, 1984). All of these results supported the idea that an essential step in fusion formation was Mu prophage activation in response to a physiologically regulated cellular signal transduction network.

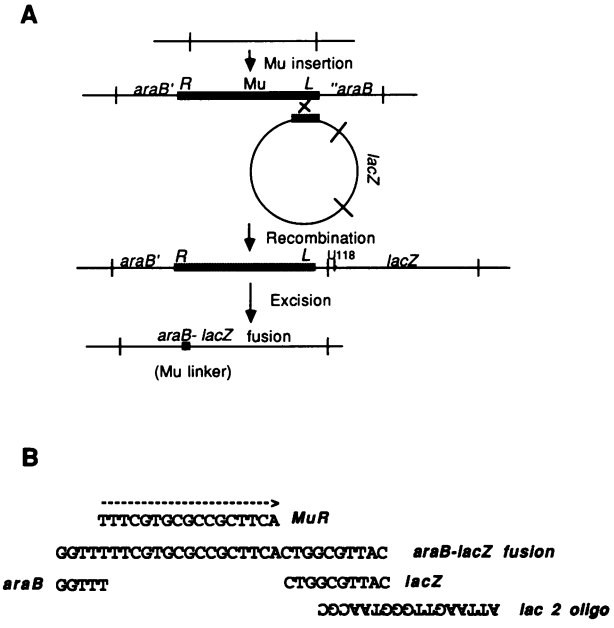

Fig. 1. The Casadaban (1976) technique for isolating *araB–lacZ* coding sequence fusions and the sequence of a fusion illustrating the inverted Mu linker. (**A**) Homologous recombination substrates for aligning *araB* and *lacZ* are the end of a Mu prophage inserted into *araB* and a terminal fragment of Mu located upstream of a decapitated *lacZ* cistron in a λ*plac* bacteriophage. The reciprocal recombination event depicted integrates the λ*plac* and positions *lacZ* downstream of *araB* to generate the prefusion structure. There is no promoter for *lacZ* transcription in the λ*plac*, and *lacZ* has the U118 *ochre* triplet at codon 17, so that neither transcription nor translation can occur without a fusion to upstream sequences. An appropriate excision event removes all blocks to transcription and translation between the start of *araB* and the region that contains the sequence for the catalytically significant domain of β-galactosidase downstream of *lacZ* codon 17. (**B**) As seen in this fusion, a small number of Mu-derived nucleotides are frequently found in the hybrid coding sequence and constitute the 'Mu linker' between the *araB* and *lacZ* domains. Note that the *MuR* terminal sequence adjacent to *lacZ* nucleotides in the fusion was originally adjacent to *araB* nucleotides in the prefusion strain MCS2.

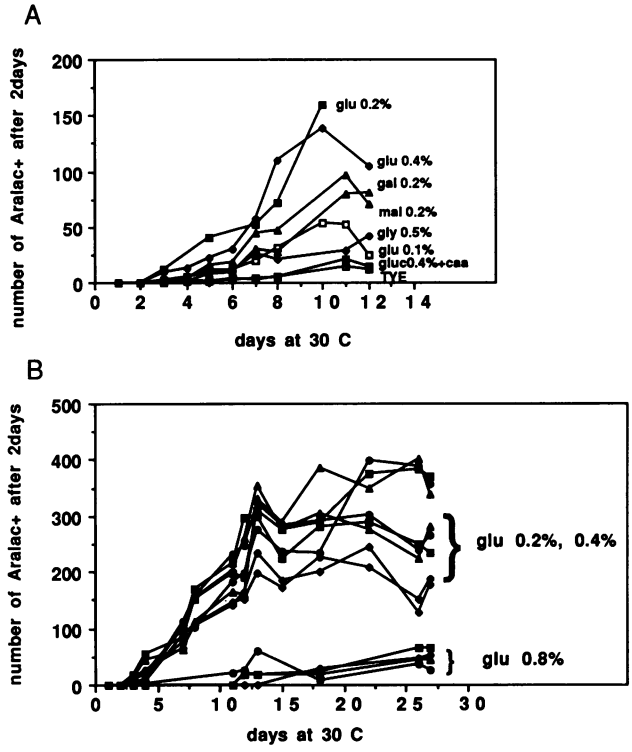

Fig. 2. The effect of carbon source on fusion formation during prolonged aeration. The ordinate gives the number of colonies appearing in 2 days on Ara–Lac selection plates (0.1 ml of cultures plated) and the abscissa gives the number of days of incubation on the roller drum at 30°C.

The kinetics of *araB–lacZ* fusion clone appearance posed the problem of explaining why there was such a sharp transition in the process of genetic change when prefusion cells were transferred from non-selective to selective conditions. Two different scenarios were suggested to account for the absence of fusion cells in unselected cultures: (i) formation of the precise DNA rearrangements needed to generate functional fusions somehow involved the arabinose and lactose substrates (Cairns *et al.*, 1988), or (ii) starvation *per se*, even in the absence of selective substrates, was sufficient to trigger Mu activity that would produce the appropriate DNA rearrangements (Mittler and Lenski, 1990). Mittler and Lenski supported their argument by demonstrating that prolonged incubation of unselected prefusion cultures under aerobic conditions resulted in the appearance of *araB–lacZ* fusion colonies within 2 days of plating. The ability to produce these colonies was maintained through subculturing, and thus involved a stable heritable change. Foster and Cairns (1994; accompanying paper) extended these observations by carrying out a fluctuation analysis of starvation-induced fusions. However, all these experiments relied on plating (i.e. contact with arabinose and lactose) to detect fusion-bearing bacteria and did not rigorously exclude all alternative possibilities, such as the formation of non-specific intermediate DNA structures which rapidly converted to active fusions in the presence of the substrates on the detection plates. P.Sniegowski (personal communication) has carried out recently replica-plate tests which likewise indicated that *araB–lacZ* fusion clones or their precursors appeared at specific locations in unselected lawns of the prefusion strain.

We decided to pursue these questions further by studying the DNA changes accompanying the appearance of *araB–lacZ* fusion clones under conditions where there was no direct exposure to arabinose and lactose substrates. We used sib selection and PCR technologies to detect and classify fusions. Our results confirmed the conclusion of Mittler and Lenski (1990) and demonstrated unambiguously that functional fusions formed at high frequencies in the absence of arabinose and lactose substrates after starvation in well-aerated, but not in static, culture conditions. To our surprise, we found that the structures of the fusions we detected were strongly influenced by the procedure used to identify the fusions. We also noted that fusions formed at remarkably high frequencies using a limited interval of the potential *lacZ* target sequence.

Results

Induction of fusions by starvation in cultures of MCS2

We repeated the experiments of Mittler and Lenski (1990), who had studied the appearance of presumptive fusion

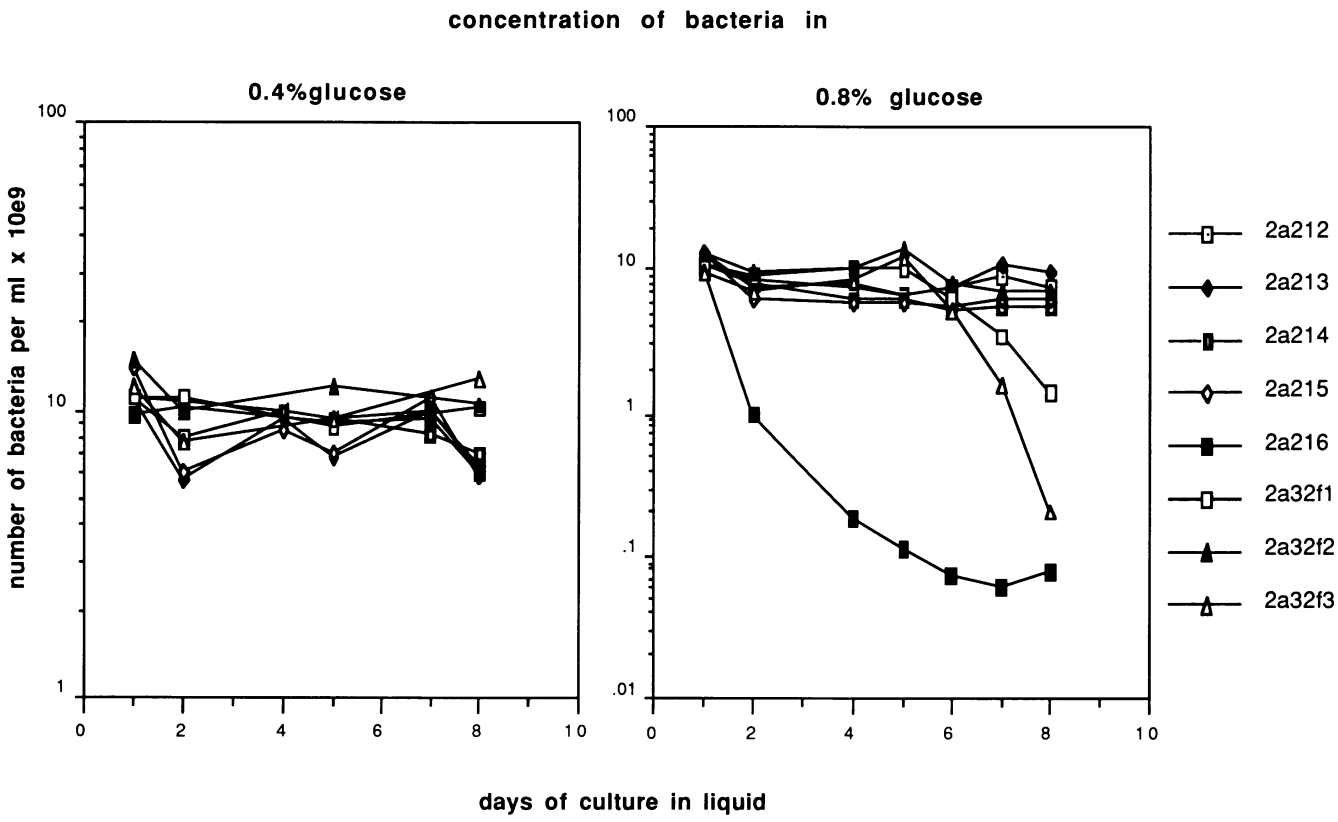

Fig. 3. Survival with prolonged aerobic incubation. Replicate MCS2 subclonal cultures were incubated on the roller drum at 30°C in minimal medium with different concentrations and sampled periodically for survival.

bacteria in well-aerated 0.1% glucose cultures. We varied three experimental parameters: carbohydrate substrate, glucose concentration and whether or not the cultures were subject to aeration (i.e. continual agitation on a roller drum). Our results agreed with and extended their report. We found that the yield of bacteria forming Ara−Lac$^+$ colonies within 2 days on selective Ara−Lac medium increased with glucose concentration up to peak yields at 0.2 and 0.4%, and then fell at 0.8%; other carbon sources (glycerol, galactose and maltose) gave qualitatively similar results but lower yields of fusions (Figure 2). Since we observed 2-fold increases in overnight colony-forming unit (c.f.u.) titers when we diluted and plated cultures containing 0.05, 0.1, 0.2 and 0.4% glucose, we assume that these were limiting concentrations. No increase in overnight c.f.u. titer was observed in going from 0.4 to 0.8% glucose. We obtained particularly low fusion colony yields with starved cultures in medium enriched with casamino acids and in tryptone−yeast extract medium, which may help explain why Cairns *et al.* (1988) failed to detect fusions in the absence of arabinose and lactose since they used rich medium. In the experiments with glucose as carbon source, there was no decrease in the survival of c.f.u. for 8 days' incubation in replicate 0.2 and 0.4% cultures, but there was sporadic loss of viability from occasional 0.8% glucose cultures in this time interval (Figure 3). After prolonged starvation, the frequency of bacteria forming 2 day colonies in 0.2 and 0.4% glucose cultures generally dropped upon subculturing into the same medium, but the total fusion colony yield increased because fresh subcultures had higher c.f.u. titers (Table I). By examining the kinetics of colony appearance after plating the starved cultures, it could be seen that the formation of putative *araB−lacZ* bacteria in these starved cultures was not accompanied by any acceleration of the fusion process in the remaining population. Once the early Ara−Lac$^+$ colonies had emerged, there was a plateau of many days before fresh fusion clones appeared (data not shown).

One possible discrepancy between our results and those of Mittler and Lenski (1990) was the observation that holding MCS2 cultures in buffer for several weeks did not lead to earlier fusion colony emergence (Shapiro, 1984). Since continued aeration and static incubation could lead to quite different conditions for the bacteria, we compared well-aerated and static MCS2 cultures. Subclones were grown to saturation overnight with aeration on a roller drum in 0.2% glucose medium (4×10^9 c.f.u./ml) and then split into two aliquots, continuing incubation for one set of samples on the roller drum and leaving the other set in the same 32°C incubator without agitation. Daily assays for 2 day Ara−Lac$^+$ colonies confirmed that incubation with aeration did stimulate the fusion process within 5 days, while incubation without aeration only yielded fusion colonies after 8 days (Figure 4). The real difference between aerated and static cultures may be even greater than seen here because it is possible that sporadic aeration of the static cultures during sampling contributed to the late-appearing fusions.

To begin addressing the question of whether aerobic starvation merely acted to stimulate Mu derepression, we also activated the Mu*cts62* prophage in MCS2 cultures by 42°C heat-shocks of different durations. Thermal induction did not stimulate the appearance of early Ara−Lac$^+$

Table I. Results of starvation in producing *araB–LacZ* fusion clones

MCS2 subclone	Carbon source	Primary starved culture				Overnight subculture		
		days/32°C	c.f.u./ml (glu)	AL^+/ml	fusion/c.f.u.	c.f.u./ml (glu)	AL^+/ml	fusion/c.f.u.
212	0.4% glucose	7	10.0×10^9	850	8.5×10^{-8}	8.0×10^9	530	6.5×10^{-8}
213	0.4% glucose	7	9.9×10^9	510	5.1×10^{-8}	8.7×10^9	320	3.7×10^{-8}
214	0.4% glucose	7	8.3×10^9	880	10.6×10^{-8}	8.5×10^9	380	4.5×10^{-8}
215	0.4% glucose	7	11.0×10^9	930	8.5×10^{-8}	12.0×10^9	260	2.2×10^{-8}
216	0.4% glucose	7	9.2×10^9	990	10.8×10^{-8}	12.0×10^9	530	4.1×10^{-8}
212	0.2% glucose	7	5.2×10^9	810	1.6×10^{-7}	4.8×10^9	480	10.0×10^{-8}
213	0.2% glucose	7	4.6×10^9	670	1.4×10^{-7}	5.6×10^9	450	8.0×10^{-8}
214	0.2% glucose	7	5.5×10^9	740	1.3×10^{-7}	4.9×10^9	430	8.8×10^{-8}
215	0.2% glucose	7	5.7×10^9	840	1.5×10^{-7}	5.3×10^9	690	13.0×10^{-8}
216	0.2% glucose	7	2.7×10^9	1120	4.1×10^{-7}	4.9×10^9	1200	24.0×10^{-8}
212a	0.4% glucose	13	7.4×10^9	1530	2.1×10^{-7}	1.3×10^{10}	2530	1.9×10^{-7}
212b	0.4% glucose	13	7.5×10^9	1540	2.1×10^{-7}	2.3×10^{10}	2000	0.9×10^{-7}
212c	0.4% glucose	13	7.4×10^9	1730	2.3×10^{-7}	1.8×10^{10}	2310	1.3×10^{-7}
214a	0.4% glucose	13	6.0×10^9	1760	2.9×10^{-7}	2.0×10^{10}	2700	1.4×10^{-7}
214b	0.4% glucose	13	5.2×10^9	1660	3.2×10^{-7}	3.3×10^{10}	2310	0.7×10^{-7}
214c	0.4% glucose	13	5.2×10^9	1580	3.0×10^{-7}	1.5×10^{10}	2000	1.3×10^{-7}
2121	0.4% glucose	19	6.0×10^8	1000	1.6×10^{-6}	1.1×10^{10}	5900	5.3×10^{-7}
2122	0.4% glucose	19	1.3×10^8	300	2.3×10^{-6}	0.9×10^{10}	1500	1.6×10^{-7}
2123	0.4% glucose	19	1.1×10^9	1120	1.0×10^{-6}	1.5×10^{10}	8800	5.8×10^{-7}
2142	0.4% glucose	19	6.4×10^8	1060	1.6×10^{-6}	1.1×10^{10}	10 900	10.0×10^{-7}
2121	0.4% glucose	19	9.3×10^7	1500	1.6×10^{-5}	3.9×10^9	32 000	8.2×10^{-6}
2122	0.4% glucose	19	4.0×10^7	800	2.1×10^{-5}	6.8×10^9	37 000	5.1×10^{-6}
214a	0.4% glucose	19	7.6×10^7	880	1.1×10^{-5}	5.2×10^9	38 000	7.3×10^{-6}
2161	0.4% glucose	19	8.0×10^7	1500	1.9×10^{-5}	6.5×10^9	59 000	9.1×10^{-6}
2162	0.4% glucose	19	5.0×10^7	820	1.6×10^{-5}	2.8×10^9	19 000	6.8×10^{-6}
2122	0.4% glucose	25	0.5×10^8	2100	4.4×10^{-5}	0.9×10^{10}	1500	1.6×10^{-7}
2123	0.4% glucose	25	3.0×10^8	1290	4.3×10^{-6}	1.3×10^{10}	22 500	17.0×10^{-7}
2142	0.4% glucose	25	1.0×10^8	1260	1.3×10^{-5}	1.8×10^{10}	25 000	14.0×10^{-7}

Independent subclonal cultures of MCS2 were grown with aeration at 30°C in 0.2 or 0.4% glucose medium and sampled periodically for plating. Dilutions were plated on glucose agar to estimate c.f.u./ml, and aliquots were plated on Ara-Lac selective agar to determine the number of Ara–Lac^+ (AL^+) colonies that appeared within 2 days. Overnight subcultures were prepared by diluting into fresh medium with the same concentration of glucose.

colonies in cultures where Mu derepression was clearly evident by both loss of viability (Table II) and the appearance of novel junction fragments in Southern blots (data not shown). Thus, it appears that derepression of the Mu*cts62* prophage in MCS2 is not by itself sufficient to lead to fusion formation.

We should note here that our results did differ from those of Mittler and Lenski (1990) in one respect. We incubated our Ara–Lac selection plates at 30 or 32°C, while they incubated theirs at 37°C. This difference in incubation temperature had two consequences. First, we systematically found a lower yield of fusion colonies when plating at 37 than at 30 or 32°C (Table III). Second, they reported the formation of satellite colonies surrounding early fusion colonies on their plates, and suggested that proliferation on the selective Ara–Lac medium due to cross-feeding played an important role in the rapid increase phase of fusion colony kinetics (Mittler and Lenski, 1990). We too observed this satellite phenomenon on 37°C selection plates, but it did not occur on our plates under our standard incubation conditions at 30 or 32°C (see also figures in Shapiro, 1984).

Sib selection analysis

As described in the next section, our attempts to use PCR technology to monitor directly the formation of active *araB–lacZ* fusions in glucose-starved cultures were unsuccessful. Consequently, we decided to monitor fusion formation by the sib selection procedure (Cavalli-Sforza

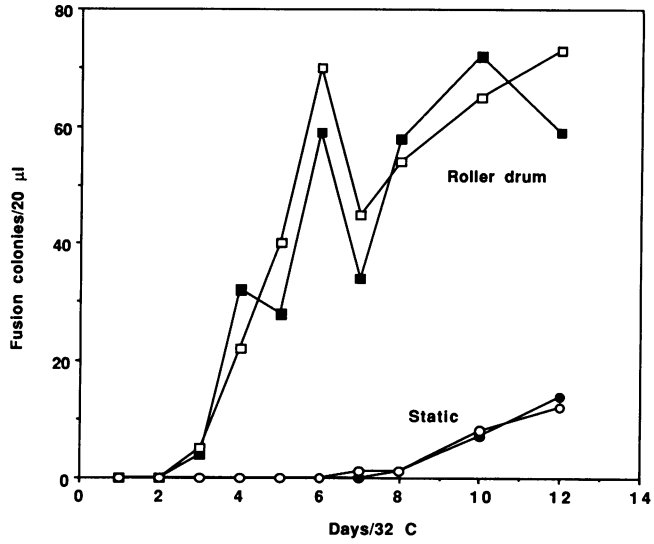

Fig. 4. Comparison of aerated and static cultures after glucose depletion. Sibling MCS2 subclonal cultures were grown overnight in 0.2% glucose medium at 32°C and then each was split into two samples. One sample of each culture was aerated on the roller drum while the other was left stationary in the same 32°C incubator. The cultures were sampled periodically and the number of colonies that appeared on Ara–Lac plates after 2 days' incubation were counted. There was little loss of viability in the first week of incubation at 32°C (4.7×10^9/ml for the roller drum aliquot and 4.8×10^9/ml for the static aliquot of one culture at day 7), but viability declined in the following period (0.3×10^9 for the roller drum aliquot and 1.06×10^9 for the static aliquot of the same culture at day 15).

Table II. Effects of thermal Mu prophage induction on fusion formation

MCS2a	Day first Ara−Lac$^+$ colony seen after:			Survival after:	
	30 min at 30°C	20 min at 42°C	30 min at 42°C	20 min at 42°C	30 min at 42°C
2121	7	7	6	1	0.32
2122	8	7	7	1	0.52
214	6	8	8	0.84	0.57
2161	6	7	8	0.73	0.55
2162	7	5	7	0.44	0.69

Independent MCS2 subclonal cultures were grown in CeriaB plus 0.4% glucose at 30°C to an optical density (600 nm) of 0.15 (2–3×10^8 c.f.u./ml). The cultures were divided into three samples and incubated at 30 or 42°C for 20 or 30 min. 0.2 ml of undiluted culture were plated on Ara−Lac selection medium and incubated at 30°C; the day the first colony appeared is indicated. Dilutions were plated on glucose agar and survival is given as the fraction of colonies obtained after incubation at 42°C compared with the same culture incubated at 30°C. Allowing the 42°C-induced cultures to recover overnight at 30°C before plating on selective Ara−Lac agar gave comparable results.

and Lederberg, 1956) which permitted the indirect isolation of pure cultures containing bacteria that formed 2 day Ara−Lac$^+$ colonies (Figure 5). Table IV summarizes the results of one set of sib selection experiments from which eight independent unselected fusion clones were isolated. Control cultures with non-starved, thermally derepressed MCS2 cultures failed to yield any positive wells in the first cycle, and samples from wells which tested negative in the first or second cycle continued to test negative in subsequent cycles.

Sib-selected fusion clones were characterized by PCR analysis after single colony purification. The sib-selected cultures were tested for β-galactosidase expression by streaking on XGal agar and assaying enzyme activity after arabinose induction (Table V). The XGal tests and enzyme assays demonstrated that these cultures indeed contained sequences encoding functional arabinose-inducible hybrid β-galactosidase proteins. Four of the 13 independent sib-selected fusions had lower levels of activity and produced clearly visible colonies on Ara−Lac medium only after 3 days incubation at 30°C.

PCR analysis

Our initial plan had been to monitor the formation of *araB−lacZ* fusions directly by PCR amplification of hybrid coding sequences using *ara* and *lac* oligonucleotide primers (Figure 6). Reconstruction experiments with the *ara2* and *lac2* primers indicated sufficient sensitivity to detect one fusion chromosome among 10^7 prefusion chromosomes (giving a 1.25 kb product), but PCR of DNA from glucose-starved cultures displayed a broad smear of novel amplification products, ~1.6 kb, and no clear evidence of fusion products at the 1.25 kb position (data not shown). Since the presence of multiple starvation-induced products made it impossible to discern fusion structures unambiguously, we adopted sib selection as the method of choice for demonstrating fusions without direct selection.

PCR analysis with the *ara2−lac2* primer pair usually yielded 1.25 kb amplification products, with DNA extracted from purified fusion clones and from mixtures of fusion colonies selected by plating fresh MCS2 cultures directly on Ara−Lac medium (18/23 tested; Table V). In retrospect, we see now that this should have been a rather surprising result because the 3′ end of the *lac2* oligo is at nucleotide 59 of *lacZ*, only 10 bases from the U118 *ochre* mutation at position 49. Consequently, PCR amplification with the *lac2* oligo will only succeed with fusions that have a *lacZ* endpoint within 9 bp (nucleotides 49–58) out of the genetically available window of 26 bp (nucleotides 49–75). Using the *ara7−lac6* primer pair which gave smaller and therefore more easily resolved PCR products, 21/23 directly selected fusions gave a 0.53 kb product (Figure 7 and Table V). The presence of 0.01% or 0.04% glucose in the Ara−Lac selection medium accelerated fusion colony emergence (Shapiro, 1984) but did not affect the sizes of the amplification products from Ara−Lac-selected fusions (Table V). Thus, almost all the fusions directly selected on Ara−Lac plates from fresh cultures had a similar structure which we have termed the 'standard' *araB−lacZ* fusion.

When we tried to amplify the fusions identified by sib selection with this same pair of primers, we were surprised to find that many of them failed to amplify with the *ara2−lac2* primers (eight out of 19 were negative; Table V), and the PCR products obtained from sib-selected fusions with primers further 3′ in *lacZ* displayed a spectrum of different sizes, ranging from 0.53 to 0.67 kb with the *ara7−lac6* pair (Figure 7 and Table V). In other words, different *araB−lacZ* fusion products were obtained from direct selection on Ara−Lac plates and from sib selection. The reasons for this difference remain unclear. When we selected fusions by plating fresh subcultures of glucose-starved MCS2 cultures directly on Ara−Lac medium, the

Table III. Effect of plating temperature on fusion recovery

MCS2 subclone	c.f.u./ml (×10^{-8}) at:		Ara−Lac$^+$/ml at:	
	30°C	37°C	30°C	37°C
0.2% glucose				
212	15	17	2970	1640
213	15	13	1500	1210
214	19	24	2600	1940
216	16	14	2470	1960
0.4% glucose				
212	30	41	1890	1710
213	22	30	1650	1170
214	33	34	2050	1010
216	37	50	1780	950

Independent subclonal cultures of MCS2 were incubated for 12 days at 30°C with aeration in 0.2 or 0.4% glucose medium and assayed for c.f.u. and 2 day *Ara−Lac*$^+$ colonies by plating replicate samples at 30 and 37°C.

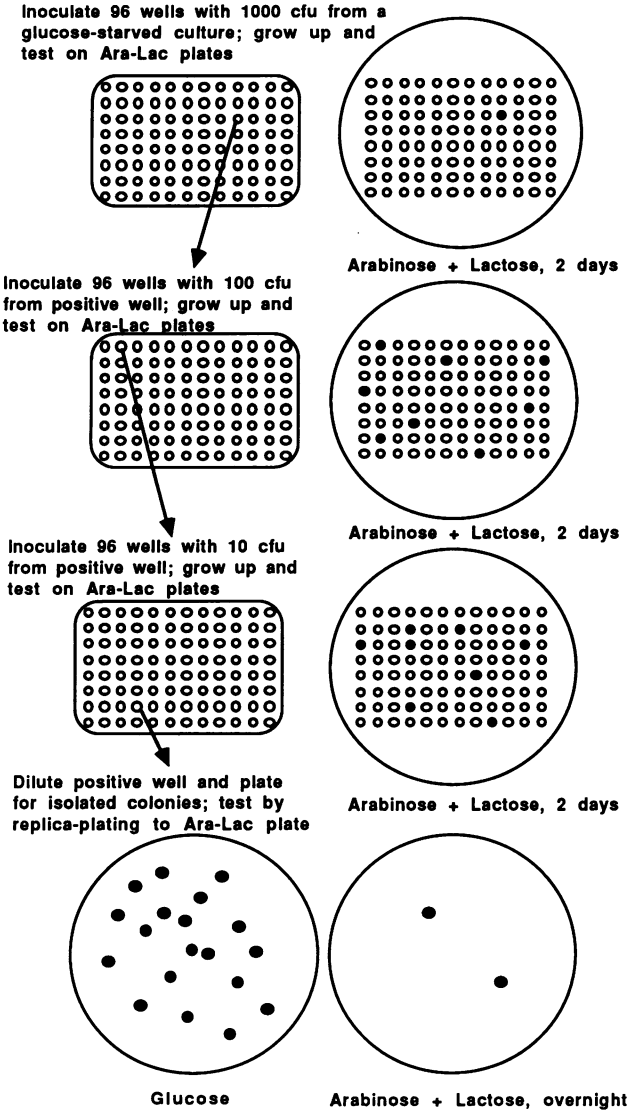

Fig. 5. An idealized sib selection experiment. Aliquots of 10^3 c.f.u. were taken from a glucose-starved MCS2 culture containing 10^{-5} fusion cells and were distributed in each of 96 wells in a microtiter dish before growing overnight to saturation. Aliquots were taken from the wells and spotted on an Ara−Lac selection plate. After 2 days, the well that initially harbored a single fusion cell was identified. It now contained ~10^{-3} fusion cells; aliquots of 10^2 c.f.u. from this well were distributed in fresh microtiter dish wells and grown overnight to saturation. When these were spotted on selection plates, ~10 wells were expected to score positive and contain ~10^{-2} fusion cells. Aliquots of 10 cells from one of these wells were then put through the same procedure, and 10 wells were again expected to score positive. Each of these should contain about one fusion cell per 10 c.f.u., and these fusions could be identified by replica-plating isolated colonies from glucose agar onto selective Ara−Lac agar.

2 day fusion colonies contained DNAs giving a narrow spectrum of PCR amplification products similar to those from fusion colonies appearing many days after direct plating of fresh MCS2 cultures (five out of seven were 1.25 kb with *ara2−lac2* and six out of seven were 0.53 kb with *ara7−lac6* primers; Figure 7 and Table V). Reconstruction experiments were performed by plating a small number of non-standard fusion cells with a 10^5-, 10^6- or 10^7-fold excess of MCS2 cells on Ara−Lac selection medium. Within 2 days, most of the non-standard fusions produced Ara−Lac$^+$ colonies under these conditions with efficiencies of 40–100% compared with plating corresponding dilutions on glucose medium in the absence of prefusion cells. To exclude the possibility that glucose starvation produced fusion precursor clones with structures that were changed rapidly to 'standard' fusions after growth on selective Ara−Lac medium, we characterized DNA extracted from five sib-selected fusions after growth on Ara−Lac medium. There was no change in the size of the PCR amplification products (Figure 7 and Table V). In an attempt to mimic the glucose starvation/sib selection protocol as closely as possible with starved cells on Ara−Lac medium, we examined 21 day-old selection plates originally seeded with ~500 MCS2 c.f.u. and picked microcolonies that had not proliferated into Ara−Lac$^+$ colonies (Shapiro, 1984). These starved but unproliferated microcolonies were inoculated on TYE agar plates and replica-plated to test for growth on Ara−Lac medium. Twenty-nine per cent of the clones tested (21/72) contained fusions; 12 such fusions were examined by PCR with the *ara7−lac6* primers: 11 gave the standard 0.53 kb amplification product while one gave a 0.67 kb product.

Discussion

Our results unambiguously demonstrate the formation of *araB−lacZ* cistron fusions encoding active hybrid β-galactosidase molecules in the absence of arabinose and lactose substrates. The fusions were obtained by the sib selection technique (Cavalli-Sforza and Lederberg, 1956) and characterized by PCR analysis and β-galactosidase assay (Table V). This demonstration means that there was no requirement for selective substrates (arabinose and lactose), and that the sharp transition (observed by three different laboratories) from normal growth conditions, where no fusion formation by MCS2 was detectable, to selection conditions, where fusion formation was observed at surprisingly high frequencies, was a response to carbon source depletion. Foster and Cairns (1994) have come to the same conclusion from their fluctuation analysis, as has P.Sniegowski from replica-plate tests (personal communication). The experiments reported here are the only ones which have identified functional *araB−lacZ* fusions that have never been in contact with selective substrates.

The fusion response was non-linear: it took several days to develop, either on plates or in liquid culture, and specific conditions associated with aeration were required in liquid culture (Figures 2 and 4; Shapiro, 1984; Mittler and Lenski, 1990). Together with genetic data indicating roles in fusion induction for the Mu A protein, Mu repressor, IHF and HU proteins and ClpPX protease (Shapiro, 1984, 1993a; Shapiro and Leach, 1990), the results reported here indicate that control of the fusion process resides in a signal transduction network sensitive to multiple physiological inputs.

This view of how selection-induced mutations are triggered in the *araB−lacZ* system is an example of what we have described previously as the operation of natural genetic engineering systems (Shapiro, 1992, 1993b). There are two main implications of the natural genetic engineering concept for evolutionary theory. The first is that major, abrupt changes in the frequency of genetic change are to

Table IV. Results of a sib selection experiment

MCS2a culture	Selection cycle 1		Selection cycle 2		Selection cycle 3 positive wells[e]
	Ara–Lac$^+$/ml[a]	positive wells[b]	*Ara–Lac*$^+$/ml[c]	positive wells[d]	
214a	830	A2	3.9×10^4	F8	F11, H1
214b	320	B8	1.0×10^5	E4 + 10 others	C4, C5, B12, E8
2121	280	H5	5.0×10^4	F4 + 1 other	D3, G6
2122	530	none			
2161	830	A4	1.0×10^5	A2 + 5 others	
		B6	7.5×10^4	10 wells	
		G8	7.3×10^4	A3 + 7 others	B11, E10, G6, G12
		H7	5.0×10^4	A2 + 7 others	A1, D6, D12, E3, E4, E10, E11, F10
2162	430	A11	6.1×10^4	C8	A3, H8, H9
		G2	3.7×10^4	B2 + 4 others	B5, C3, C4, D6, D8, E4, E10, E12, F4, F5, F9, G1, H5, H8
		H10	2.5×10^4	C10 + 1 other	A5, A12, B1, D11, E8, E11, F1, F11, G4, H12

This experiment is the 8/93 experiment of Table V. The MCS2 cultures were starved for 11 days in 0.4% glucose medium before dilution into microtiter wells.

[a]The starved cultures were tested for 2 day Ara–Lac$^+$ colonies.

[b]For selection cycle 1, 0.2 ml of a 10^{-4} dilution of each of the starved cultures were distributed into each of the 96 wells of one microtiter dish. After overnight incubation with aeration, 5 µl aliquots of the wells were spotted onto Ara–Lac selection plates, incubated for 2 days and scored for growth. The wells were identified by their coordinates on the microtiter plate. The well identified in cycle 1 or cycle 2 was used as the inoculum for the succeeding cycle. When more than one well from cycle 1 was further selected, they are listed individually in this column.

[c]Data for the positive wells in cycle 1.

[d]For selection cycle 2, 0.2 ml aliquots of a 10^{-6} dilution of a cycle 1 positive well were distributed into 96-well dishes and tested as for cycle 1.

[e]For selection cycle 3, 0.2 ml aliquots of a 4×10^{-8} dilution of a cycle 2 positive well were distributed into 96-well dishes and tested as for cycles 1 and 2.

be expected because DNA reorganization often results from the action of sophisticated biochemical 'machines' (Alberts, 1984) which are assembled and brought into play under specific conditions, such as aerobic carbon source depletion and other stress regimes (see also McClintock, 1984). In other words, much genetic change is not a stochastic ongoing process, controlled at the level of selection or mutation fixation (e.g. Stahl, 1992; Lenski and Mittler, 1993), but instead involves the regulated assembly of specific cellular complexes. The physical and biochemical events needed to produce an *araB–lacZ* fusion are too elaborate to occur by an accidental breakdown in the normal replication process (Shapiro and Leach, 1990).

The second evolutionary implication is that different conditions stimulating genetic variation will lead to distinct kinds of DNA rearrangement because each natural genetic engineering system operates in its own characteristic fashion. Some systems (like Mu) are capable of joining sequences encoding protein domains or mediating a variety of chromosomal rearrangements (Pato, 1989). Other systems may stimulate point mutations, as apparently happens with the RecA–RecBCD complex during induced frameshift mutagenesis (Cairns and Foster, 1991; Harris *et al.*, 1994). It is interesting to note that the Mu system (Shapiro, 1994), RecA (F.Taddei, I.Matic and M.Radman, personal communication) and spontaneous mutation of *E.coli* to valine resistance (MacPhee, 1992) are all subject to control by glucose repression and the cAMP–CRP regulatory complex. Since cellular regulatory networks have evolved to coordinate biochemical events appropriate to survival and proliferation under different conditions, it should not be surprising to find genetic change (the result of biologically controlled DNA biochemistry) frequently occurring when useful or necessary for reproduction. The natural genetic engineering concept also makes it easier to understand how discrete, reproducible sequences of genetic change can occur in non-evolutionary situations where cellular environments and selective conditions change in a regular fashion, as in tumor progression (Vogelstein and Kinzler, 1993).

We were surprised to find that two regimes for selecting *araB–lacZ* fusion colonies from glucose-starved cultures yielded different spectra of PCR products, and that direct plating appeared to reveal only a subset of the fusion products formed (Figure 7 and Table V). The significance of a greater variety of fusion structures in the sib-selected group is not yet clear. Our data do show, however, that we cannot make a simple extrapolation from colony counts to DNA rearrangements. The finding that direct plating on Ara–Lac medium failed to reveal classes of fusions detected by sib selection was an indication that the Ara–Lac$^+$ colonies appearing on selective Ara–Lac agar represented only a lower limit of functional DNA rearrangements. This is in agreement with earlier results of examining isolated MCS2 microcolonies on selective Ara–Lac medium, which indicated that not all cells containing *araB–lacZ* fusions proliferated into colonies (Shapiro, 1984).

Even though we can only measure its lower bounds, the quantitative aspect of glucose starvation-induced fusions is intriguing. The formation of a functional *araB–lacZ* fusion is a very specific kind of DNA rearrangement. It requires the in-phase alignment of two coding sequences plus (in many cases) a segment of the Mu R terminus (Figure 1; Shapiro, 1987). The natural expectation was that such precise events would be rare (Casadaban, 1976), and it was a surprise to find that they formed so regularly after prolonged selection (Shapiro, 1984). It was a further surprise to realize that this population consisted almost entirely of fusions which joined *araB* to *lacZ* within the nine nucleotide region between the first base of the *ochre* triplet at position 49 and the end of the *lac2* oligo at position 59 (Figure 6 and Table V). Given the reading

Table V. Properties of *araB–lacZ* fusions

MCS2a				PCR fragments produced (kb)[c]			XGal stain[d]		β-Galactosidase[e]	
expt	subclone	starved[a]	selection[b]	*ara2–lac2*	*ara7–lac6*	*ara6–lac9*	−Ara	+Ara	−Ara	+Ara
11/93	2122 B24	–	AL (8d)	(1.25)[g]		1.4				
	214 N31	–	AL (5d)	1.25	0.53	1.4				
	214 R32	–	AL (6d)	–	0.53	1.4				
	214 V33	–	AL (7d)	1.25	0.53	1.4				
	214 B34	–	AL (8d)	1.25	0.53	1.4				
	2161 R42	–	AL (6d)	1.25	0.53	1.4				
	2121	–	AL (11d)	1.25	0.53	1.4				
	2122	–	AL (11d)	1.25	0.53	1.4				
	214	–	AL (11d)	1.25	0.53	1.4				
	2161	–	AL (11d)	–	0.53	1.4				
	2162	–	AL (11d)	1.25	0.53	1.4				
	2121	–	AL (5–7d)	1.25	0.53					
	2122	–	AL (5–7d)	–	0.60					
	214	–	AL (5–7d)	1.25	0.53					
	2161	–	AL (5–7d)	–	0.53					
	2121	–	AL 0.01% glu (5–7d)	1.25	0.53					
	2122	–	AL 0.01% glu (5–7d)	1.25	0.53					
	214	–	AL 0.01% glu (5–7d)	1.25	0.53					
	2161	–	AL 0.01% glu (5–7d)	1.25	0.53					
	2121	–	AL 0.04% glu (5–7d)	1.25	0.53					
	2122	–	AL 0.04% glu (5–7d)	–	0.53					
	214	–	AL 0.04% glu (5–7d)	1.25	0.53					
	2161	–	AL 0.04% glu (5–7d)	1.25	0.53					
8/93	2121	11d	sib H5F4G6[f]	1.25	0.55	nt	LB	DB	62	2050
	214a	11d	sib A2F8F11	1.25[h]	0.55	nt	W/LB	DB	<5	1510
	214b	11d	sib B8E4C4	(1.25)[g,h]	0.60[h]	1.45	W	DB	6	463
	214b	11d	sib B8E4C5	–[h]	0.60[h]	1.45	W	DB	<5	556
	2161	11d	sib H7A2E3	1.25	0.55	nt	LB	DB	18	2082
	2161	11d	sib G8A3G6	–	0.53	nt	LB	DB	<5	2019
	2162	11d	sib H10F5F8	(1.25)[g,h]	0.60	1.4	LB	DB	43	2255
	2162	11d	sib G2B2C3	1.25	0.60	nt	LB	DB	108	3046
	2162	11d	sib A11C8H9	1.3[h]	0.67[h]	1.5	W/LB	DB	<5	1333
	2162	11d	sib H10C10B1	–	0.60		nt	nt	nt	2909
10/93	2121	19d	sib H4C5E8	–	0.65	1.45	W	DB	<5	419
	2121	19d	sib H4C5F8	–	0.65	nt	W	DB	<5	306
	2122	19d	sib D9E1E12	–	0.53	1.40	LB	DB	41	1859
	2122	19d	sib D9E1C8	–	0.53	1.40	LB	DB	45	1770
	214	19d	sib D10G1H11	–	0.60	nt	W	DB	19	740
	2161	19d	sib C1B1D10	1.25	0.62	1.45	W/LB	DB	<5	839
	2161	19d	sib C1B1H4	1.25	0.62	1.45	W/LB	DB	16	727
	2162	19d	sib A3H2C2	1.25	0.65	nt	LB	DB	23	1340
	2162	19d	sib A3H2B4	1.25	0.65	nt	LB	DB	11	1247
1/94	2122a	8d	AL	1.25	0.53					
	2161a	8d	AL	1.25	0.52					
	2162a	8d	AL	–	0.53					
	2162b	8d	AL	–	0.53					
	2121	9d	AL	1.25	0.53					
	2122b	9d	AL	1.25	0.53					
	2161b	9d	AL	1.25	0.53					

[a]'–' means no starvation; *x*d means starvation in aerated 0.4% glucose culture for *x* days.
[b]AL means selected on Ara–Lac agar. Sometimes the selective agar was enriched with the indicated concentration of glucose. Although the added glucose accelerates fusion formation in a subpopulation of the plated cells (Shapiro, 1984), this enrichment is not starvation in liquid glucose medium prior to plating. *x*d means that the fusion colony tested appeared after *x* days of selection at 30 or 32°C. The identity of sib-selected cultures is given by the identities of the cycle 1, 2 and 3 positive wells (see Table IV).
[c]The fragment sizes were estimated from gels similar to those illustrated in Figure 7. The accuracy of the fragments amplified with the *ara2–lac2* and *ara6–lac9* primers is good to the closest 0.05 kb; the accuracy of the fragments amplified with the *ara7–lac6* primers is good ±0.01 kb. Reproducible negative results are indicated (–).
[d]Only the sib-selected fusions were tested for XGal staining. LB, light blue; DB, dark blue; W, white after 2 days' incubation on minimal medium glycerol (0.5%) XGal agar plates with or without Ara (0.2%).
[e]Only the sib-selected fusions were assayed for β-galactosidase activity. Assays were performed in CeriaB-glycerol medium ±0.2% arabinose.
[f]All the results on sib-selected populations were obtained with a single colony isolate after purification of the population in the cycle 3 well.
[g]The parentheses indicate a weak PCR result. We suspect that the fusion may include only a portion of the sequence complementary to the *lac2* primer, and so give reduced amplification.
[h]Same result obtained after growing this fusion on arabinose + lactose agar.

frame requirements, we would expect active fusions to occur quite rarely in such a restricted window. Nonetheless, we found that glucose-starved cultures gave lower limits of up to four fusions per 10^7 c.f.u. after 7 days, i.e. before significant loss of viability (Table I and Figure 3). We find these frequencies to be surprisingly high, indicative

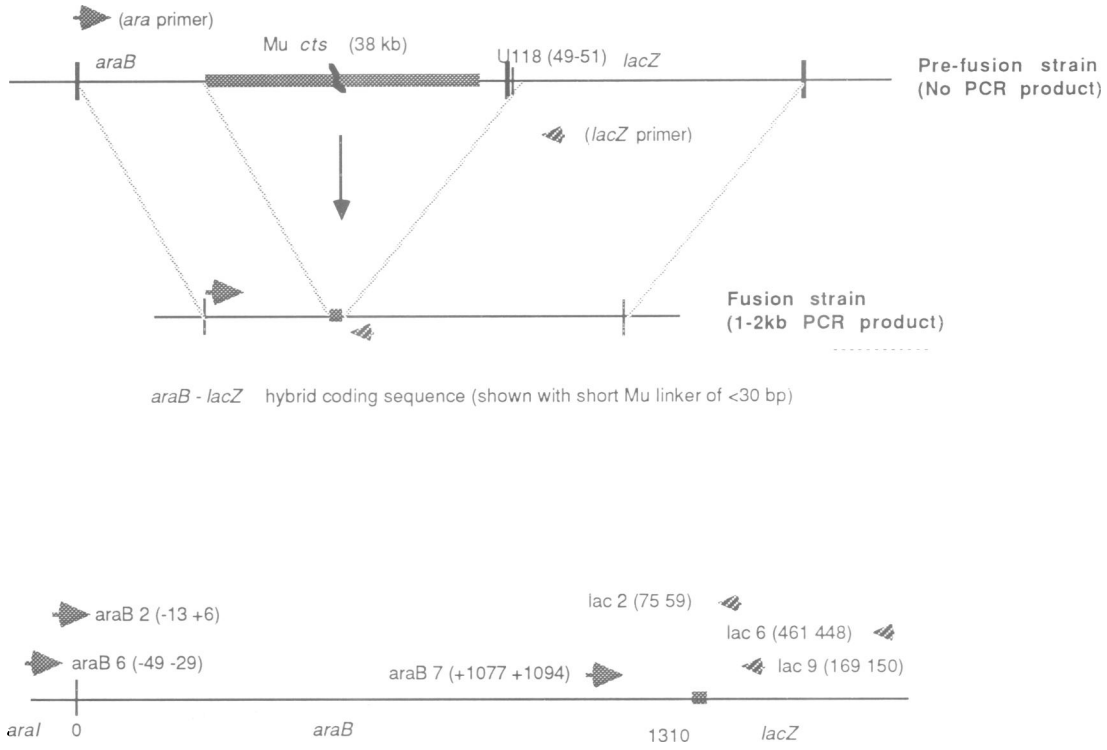

Fig. 6. PCR scheme. The top two horizontal lines schematize the configurations of the *araB–lacZ* regions before and after fusion formation. The position of the *U118 ochre* mutation is indicated in the prefusion strain chromosome. The bottom horizontal line schematizes the position of different primers on the chromosome of a fusion strain. PCR analysis indicates that the Mu insertion in MCS2 is located at *araB* nucleotide 1218 (numbering according to Greenfield *et al.*, 1978). The arrows indicate the positions of the primers used in this paper with their 5′ and 3′ coordinates in parentheses.

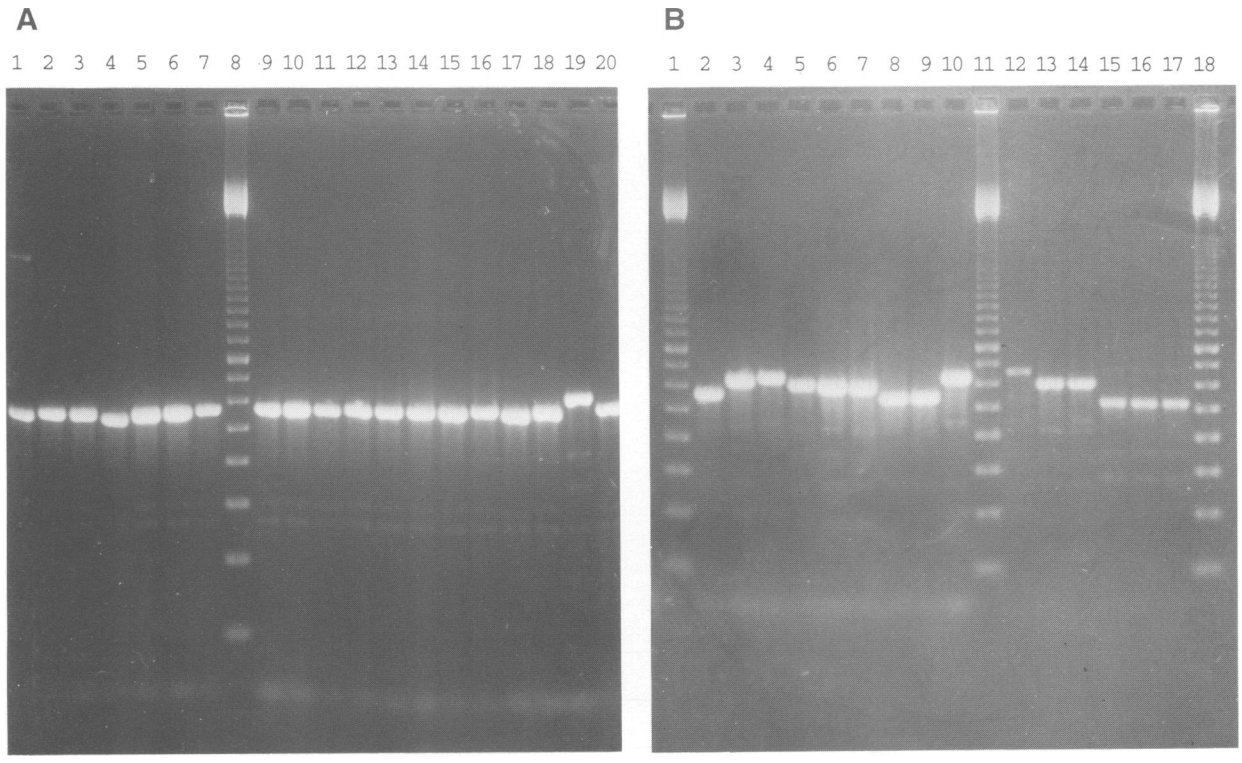

Fig. 7. PCR of DNA from fusions with *ara7–lac6* primers. (**A**) Fusions obtained from fresh and glucose-starved MCS2 cultures plated directly on Ara–Lac selection plates. Some of the selection plates were enriched by glucose. Lanes 1–4: 8 days of starvation. Lanes 5–7: 9 days of starvation. Lane 8: 100 bp ladder. Lanes 9–12, no starvation, 0.04% glucose enrichment. Lanes 13–16, no starvation, 0.01% glucose enrichment. Lanes 17–20, no starvation, no enrichment. (**B**) Sib-selected fusions and controls selected directly on Ara–Lac plates. Lane 1: 100 bp ladder. Lane 2: direct-selected fusion. Lanes 3–10: sib-selected fusions. Lane 11: 100 bp ladder. Lanes 12–14: sib-selected fusions grown on Ara–Lac plates prior to DNA extraction. Lanes 15–17: direct-selected fusions. Lane 18: 100 bp ladder.

of rather efficient Mu activation and/or some target specificity in glucose-starved cultures. To appreciate how high these frequencies are, let us assume no specificity in the fusion process. The 9 bp window constitutes 2×10^{-6} of all potential internucleotide targets in the genome (4.5×10^{6} bp). If Mu attacked all targets with equal probability, if 50% of all attacks occurred in the proper orientation and if 33% of all attacks yielded products in the proper reading frame, then prophage activation and rearrangement in every cell in a starved culture would be required to produce a fusion frequency of 3×10^{-7}, which is close to experimental observation for the standard fusions alone (Table I).

The possibility that there is target specificity as Mu acts to form *araB–lacZ* fusions is consistent with our previous observation that arabinose-independent *lacZ* fusions were almost completely undetectable when selecting MCS2 cultures on lactose medium (Shapiro and Brinkley, 1984). It may be that the Mu prophage preferentially attacks only nearby sequences under starvation conditions. An important field for future investigation is the influence of cellular physiology at different phases of the bacterial life cycle on spatial organization of the genome, and how this organization affects the accessibility of particular sequences to rearrangement by systems like phage Mu. Starvation may also affect other aspects of Mu activity. We found that thermal activation of the Mu prophage in MCS2 did not induce fusion colony appearance (Table II). During exponential growth, almost all Mu strand transfer products are probably converted to replication complexes (Pato, 1989), which create lethal rearrangements of the genome and thus preclude the emergence of fusion clones. Under starvation conditions, however, strand transfer complexes may have different fates and so be more readily processed into fusions (Shapiro and Leach, 1990).

The ability of a Mu prophage to join protein coding domains provides a useful starting point for understanding how such domains came to be reassorted during evolution (Stock *et al.*, 1990; Wild and Wales, 1990; Miklos and Campbell, 1992). If Mu can carry out coding sequence fusions with unexpectedly high efficiency, it will be of great interest to examine the ability of other DNA rearrangement systems in both prokaryotes and eukaryotes to make hybrid protein coding sequences. Allgood and Silhavy (1991) found that *xonA* mutations facilitated *ompR–lacZ* fusion formation by the deletion of sequences between microhomologies in the two ORFs. One interpretation of this observation is that exonuclease I activity disrupted potential intermediates in a process of reciprocal recombination at those microhomologies. Studies on reverse transcription and transduction of cellular sequences by retroviruses provide other models for systems capable of creating novel hybrid coding sequences (Derr *et al.*, 1991; Swain and Coffin, 1992). There is evidence that such systems have operated during natural evolution (Brosius, 1991; Long and Langley, 1993). Comparing DNA-based systems with retroposons will be particularly interesting because we may find that quite different molecular processes lead to similar genetic results, just as we have found to be the case with the movements of mobile genetic elements through the genome (Berg and Howe, 1989).

Materials and methods

Bacterial strains and culture conditions

The prefusion strain MCS2 has been described (Shapiro, 1984), and Figure 1 schematizes the genetic events in the *ara* region involved in its construction. For these experiments, cultures were prepared fresh from single colonies or by dilution of frozen cultures containing 50% glycerol. Each culture had a unique subclonal designation, receiving an additional digit after each single colony isolation step (Shapiro, 1984). Thus, subclones MCS2a212 and MCS2a213 are sibling single colony cultures obtained by plating culture MCS2a21. Complex media contained tryptone (1%), yeast extract (0.5%) and NaCl (0.5%). Synthetic media contained either PA salts (Nieder and Shapiro, 1975; Shapiro, 1984) or CeriaB salts (Glandsdorff, 1965) supplemented with the appropriate sugar(s) as carbon source(s). We have not observed any difference using the two salt solutions. XGal plates were prepared with CeriaB plus 15 g agar, 50 μg/ml XGal (Boehringer) supplemented with 0.5% glycerol with or without 0.2% arabinose. In general, 2 ml cultures were grown for various lengths of time at either 30 (Rhode St Genese) or 32°C (Chicago) with aeration on a roller drum before plating on selective Ara–Lac medium containing 0.2% L-arabinose and 0.2% lactose. The exceptions are noted in the text.

Sib selection protocol

The basic theory of sib selection is schematized in Figure 5. Starting with starved cultures displaying frequencies of between 5×10^{-7} and 2×10^{-5} putative fusion bacteria, 96 aliquots containing between 10^{3} and 4×10^{4} c.f.u. were distributed into microtiter dish wells containing glucose medium, grown overnight to $1–5\times10^{9}$/ml and then tested for Ara–Lac^{+} colony formation by spotting 5 μl on selective Ara–Lac medium. The few positive wells which contained between 2.5×10^{-5} and 10^{-3} putative fusion bacteria were then diluted so that the wells could be inoculated with 100–200 c.f.u. and put through successive sib selection cycles until pure single colony cultures could be established by testing isolated colonies from glucose salts agar.

Enzyme assays

Bacteria were grown overnight in CeriaB containing 0.5% glycerol with or without 0.2% arabinose. 0.1 ml aliquots from these cultures were assayed for β-galactosidase activity as described (Miller, 1972).

DNA extraction

1.5 ml aliquots of overnight or starved cultures were centrifuged, and the pellet was kept frozen at −20°C until extraction. The pellet was resuspended in 100 μl of Tris–HCl (10 mM, pH 8), EDTA (1 mM); 5 μl of 10 mg/ml lysozyme were added. The sample was incubated for 30 min at 37°C. 12 μl 10% SDS were added and the sample was then incubated for 30 min at room temperature and 30 min at 65°C before 5 μl of RNase (10 mg/ml) were added and the sample was again

Table VI. Oligonucleotide primers

Primer	Sequence	Coordinates (5′ → 3′)
ara2	ACTGTTTCTCCATACCCGTT	*araB* −13 +6
ara6	GCGGATCCTACCTGACGCTTT	*araB* −49 −29 (*Bam*HI site)
ara7	TGGCCGCTGGAACAGCTT	*araB* 1077 1094
lac2	ATTAAGTTGGGTAACGC	*lacZ* 75 59
lac6	GCGAATTCCACCACAGATGAAA	*lacZ* 461 448 + *Eco*RI site
lac9	CGCCATTCAGGCTGCGCAAC	*lacZ* 169 150

incubated for 2 h at 37°C. 2 µl of proteinase K (10 µg/ml) were then added followed by incubation for several hours (or overnight) at 65°C. The proteinase digestion was followed by two or three phenol/chloroform extractions and one chloroform extraction. The DNA was precipitated with 2 M ammonium acetate and an equal volume of isopropanol at room temperature. The pellet was washed twice with 70% ethanol and dried overnight at room temperature. The DNA was resuspended in sterile water. A rapid DNA extraction protocol was also used employing the Isoquick nucleic acid extraction kit from Microprobe.

PCR technology

The Mu-dependent fusion process was ideally suited to this procedure because the priming sites were separated by >38 kb in the prefusion strain (thereby preventing amplification), but were only hundreds of base pairs apart in the fusion products (Figure 6). The sequences of the primers used are given in Table VI. Our standard PCR protocol used the following ingredients: 10 mM Tris–HCl pH 8.7 (Sigma); 50 mM KCl; 2 mM $MgCl_2$; 1 µM of each primer (Bioscience and Pharmacia); 200 µM of each dNTP (Perkin Elmer Cetus); 2 U of *Taq* polymerase (Perkin Elmer Cetus); 0.1–1.0 µg DNA in a total volume of 50 µl layered with mineral oil. To improve the specificity of amplification, hot PCR using wax in place of oil was performed, starting with two layers (Perkin Elmer Cetus). The temperature of annealing was changed according to the pairs of primers used. For primers *lac2* and *ara2*, we used the following cycling regime: three cycles with 1 min at 94°C, 1 s at 57°C, 1 min at 55°C and 1 min at 72°C, followed by 32 cycles with 45 s at 94°C, 1 s at 57°C, 2 min at 55°C, 2 min plus 5 s of extension at 72°C, followed by 10 min at 72°C for completing primer extension and a final soak at 4°C. For primers *ara7* and *lac6*, the conditions were three cycles with 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, followed by 27 cycles with 45 s at 94°C, 1 min at 55°C, 1 min at 72°C plus 5 s of extension, followed by 10 min at 72°C.

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