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Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon

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Summary

To facilitate efficient allelic exchange of genetic information into a wild-type strain background, we improved upon and merged approaches using a temperature-sensitive plasmid and a counter-selectable marker in the chromosome. We first constructed intermediate strains of *Escherichia coli* K12 in which we replaced wild-type chromosomal sequences, at either the *fimB–A* or *lacZ–A* loci, with a newly constituted DNA cassette. The cassette consists of the *sacB* gene from *Bacillus subtilis* and the neomycin (kanamycin) resistance gene of Tn*5*, but, unlike another similar cassette, it lacks IS*1* sequences. We found that sucrose sensitivity was highly dependent on incubation temperature and sodium chloride concentration. The DNA to be exchanged into the chromosome was first cloned into derivatives of plasmid pMAK705, a temperature-sensitive pSC101 replicon. The exchanges were carried out in two steps, first selecting for plasmid integration by standard techniques. In the second step, we grew the plasmid integrates under non-selective conditions at 42°C, and then in the presence of sucrose at 30°C, allowing positive selection for both plasmid excision and curing. Despite marked locus-specific strain differences in sucrose sensitivity and in the growth retardation due to the integrated plasmids, the protocol permitted highly efficient exchange of cloned DNA into either the *fim* or *lac* chromosomal loci. This procedure should allow the exchange of any DNA segment, in addition to the original or mutant allelic DNA, into any non-essential parts of the *E. coli* chromosome.

Introduction

The advent of techniques that permit site-specific mutagenesis of cloned DNA allows detailed studies of DNA function. Mutations constructed *in vitro* on plasmids can be studied without further manipulation, but factors such as copy number and DNA supercoiling can make the interpretation of data difficult. These problems can be overcome by moving the altered sequences into their native location on the bacterial chromosome. Once this is accomplished it becomes possible to construct more complex strains by P1 transduction or by selecting compensatory or other mutations.

A variety of allelic exchange procedures have been reported for *Escherichia coli*. Many techniques employ a host strain or vector that is conditional for replication. Strains containing an integrated plasmid (plasmid integrates) can be selected by growing ColE1-based vectors in a temperature-sensitive *polA* strain at 42°C (Gutterson and Koshland, 1983). In wild-type strains, integrates can be isolated using a temperature-sensitive derivative of plasmid pSC101 or M13mp bacteriophage (Blum *et al.*, 1989; Hamilton *et al.*, 1989). Another strategy employs *recBC* and, more recently, *recD*, mutants which can be transformed with non-replicating linear DNA (Jansin and Schimmel, 1984; Russel *et al.*, 1989). The reciprocal nature of homologous recombination has been exploited to exchange vector sequences for chromosomally located, counter-selectable markers (Reid and Collmer, 1987; Russell and Dahlquist, 1989). This technique requires the prior construction of an intermediate strain, but allows the selection of recombinants without the inclusion of a selectable marker.

The rapid analysis of cloned mutations demands the use of efficient allelic exchange procedures. To achieve optimal practicability and versatility, the procedures must permit the transfer of non-selectable alleles into the chromosome of wild-type strains. These requirements rule out the use of *polA*, *recBC*, and *recD* strains, and exclude the use of the *rpsL* locus as a counter-selectable marker. Moreover, although M13mp bacteriophage vectors can be used in wild-type strains, the instability of large inserts restricts the use of this system (Zinder and Boeke, 1982). Bearing in mind the limitations of existing procedures, and the requirements outlined above, we have developed a novel allelic exchange procedure. The procedure, which combines the use of a temperature-sensitive vector with an intermediate strain that contains

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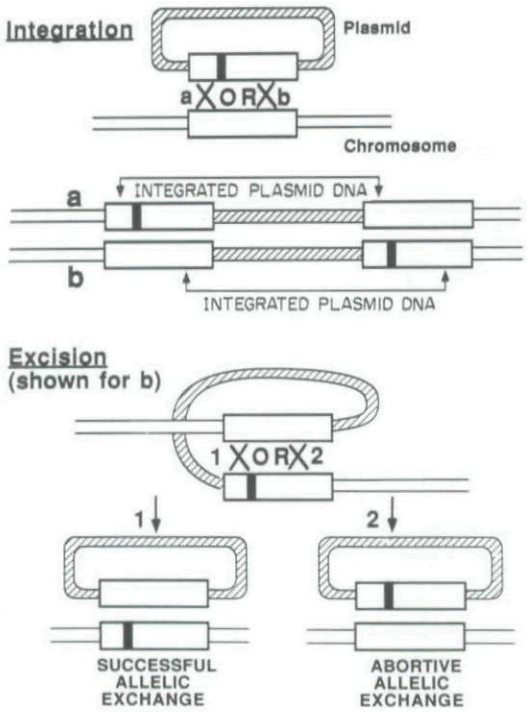

Fig. 1. The transfer of a plasmid marker into the chromosome requires two recombinations leading to plasmid integration followed by excision. Integration can occur by recombination on either side of the plasmid allele of interest (a or b). For excision to lead to a successful allelic exchange, recombination must occur in the second region of homology. Note that strains that have undergone the successful allelic exchange may retain the excised plasmid.

the *B. subtilis sacB* gene as a counter-selectable marker, should permit the efficient manipulation of any non-essential loci.

Results

Rationale

Positive selection for allelic exchange, resulting from plasmid integration followed by excision at a second site (Fig. 1), can sometimes be achieved using a vector whose replication is temperature dependent. In the first step, bacteria containing an integrated plasmid (plasmid integrates) are isolated by selecting transformants that express the vector-encoded antibiotic resistance at temperatures that inhibit plasmid replication (42°C to 44°C) (Blum *et al.*, 1989; Cunningham and Weiss, 1985; Gutterson and Koshland, 1983; Hamilton *et al.*, 1989). Since the replication of an integrated plasmid often inhibits cell growth (Cunningham and Weiss, 1985; Hamilton *et al.*, 1989; Yamaguchi and Tomizawa, 1980), passage of the plasmid integrates at 30°C frequently allows selection for the second step, i.e. the isolation of bacteria that have excised the integrated plasmid. However, recombination between short repeats in the bacterial chromosome is rare (Blum *et al.*, 1989; Cunningham and Weiss, 1985; Hamilton *et al.*, 1989; Mahan and Roth, 1988), and in our experience isolating bacteria that have excised an integrated plasmid is often difficult (our unpublished observations and see below). In addition, unless recombinants acquire an antibiotic-resistance marker, it is always necessary to screen colonies to distinguish recombinants from isolates that resemble the parent strain (Fig. 1). Thus the number of false-positive isolates often greatly outnumbers the desired recombinants, considerably limiting the scope of this approach.

In a different approach to allelic exchange, several workers have used an intermediate strain that contains a counter-selectable (and selectable) marker replacing the target chromosomal region (Reid and Collmer, 1987; Russell and Dahlquist, 1989). This technique, which requires the prior construction of an intermediate strain, allows the direct selection of recombinants, and in principle eliminates the need to screen colonies for the desired isolates. In practice, however, potential recombinants include mutants with lesions in the counter-selectable marker (Reid and Collmer, 1987; Russell and Dahlquist, 1989), and in our experience the application of this technique is also severely restricted.

We have developed a protocol that combines the use of the two different approaches to allelic exchange outlined above, i.e. the employment of a temperature-sensitive plasmid together with an intermediate strain that contains a counter-selectable (and selectable) marker. A outline of the procedure is shown in Fig. 2. By modifying the growth media and temperature, we are able to select for each step in the allelic exchange process (i.e. plasmid integration and excision) and final strain construction (plasmid curing). In contrast to other approaches, the procedure is highly efficient, with the majority of isolates comprising the desired recombinants.

Characterization of the sucrose sensitivity of the sacB*-containing strains*

The expression of the cloned *B. subtilis sacB* (Lepesant *et al.*, 1972) gene in *E. coli* (and in other Gram-negative bacteria) is lethal in the presence of sucrose at 37°C (Gay *et al.*, 1985). We have constructed two intermediate strains: one contains a *sacB*–Neo^R cassette replacing several of the type 1 fimbriae genes (strain AAEC064), and the other contains the cassette replacing the *lacZYA* genes (strain AAEC090). (The construction of these strains is described in the *Experimental procedures*.)

To assess the utility of using the *sacB* gene as a counter-selectable marker in the proposed allelic exchange procedure, it was first necessary to determine the sucrose sensitivity of strains AAEC064 and AAEC090 at 30°C. Both strains, grown to late log phase in LB broth,

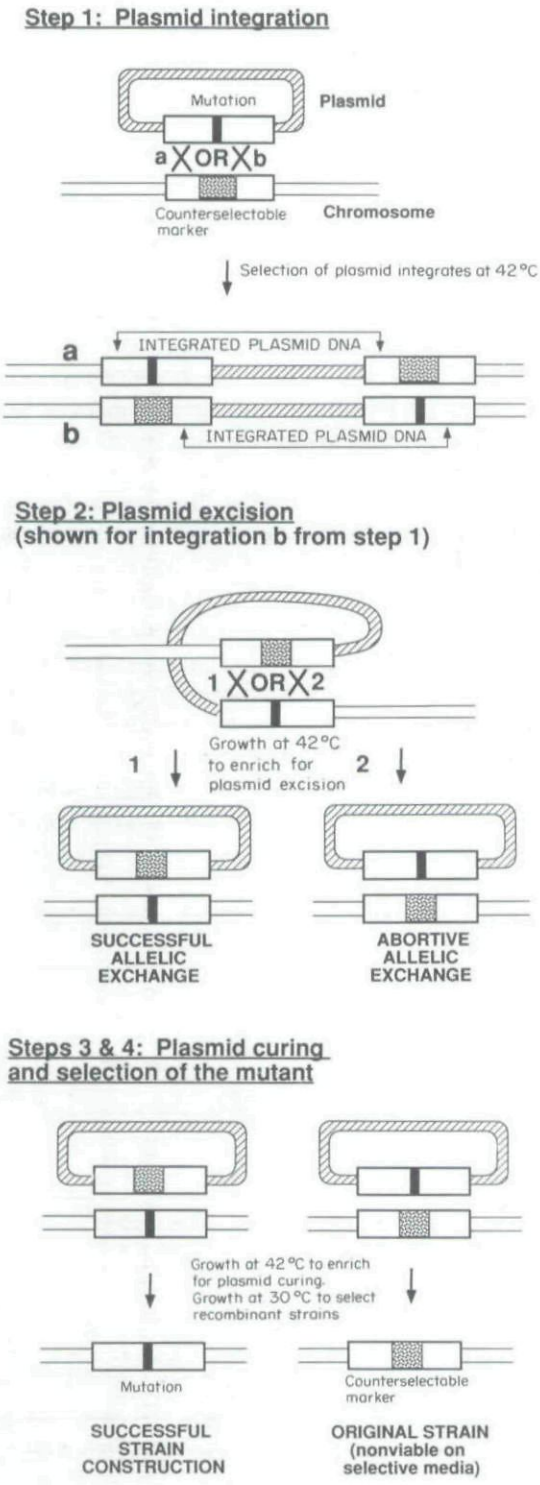

Fig. 2. Allelic exchange procedure and strain construction. Step 1: plasmid integrate derivatives of the intermediate strain are selected at the non-permissive temperature for plasmid replication (42°C). Steps 2 and 3: plasmid integrates are grown at 42°C in the absence of antibiotics to enrich for bacteria that have excised (Step 2) and later cured (Step 3) the integrated plasmid. Step 4: the culture is inoculated onto media that select the desired recombinants. Incubation at 30°C prevents the growth of background mutants of the plasmid integrate that contain lesions in the counter-selectable marker.

were spread onto LB agar containing 5% sucrose and incubated at either 30°C or 37°C. At 37°C the strains formed a lawn of bacterial growth (data not shown), although strain AAEC090 showed markedly greater sucrose sensitivity at 30°C (Table 1). We tested strain AAEC064 for growth inhibition on higher sucrose concentrations (up to 16%), but observed little or no effect.

This unexpected result led us to examine an additional factor that might influence sucrose sensitivity, i.e. sodium chloride concentration. Omission of sodium chloride from the media was found to effect a marked increase in the sucrose sensitivity of both strains, particularly at 30°C (Table 1). As expected, strain MG1655, the parent of strains AAEC064 and AAEC090, grew normally on media containing sucrose up to and including 10%. In the light of these results, we adopted the use of LB-agar media that contained 6% sucrose but lacked sodium chloride in all further experimentation.

As part of our study to assess the use of *sacB* as a counter-selectable marker, we also measured the spontaneous mutation frequency of strains AAEC064 and AAEC090 to sucrose resistance. We found that with strain AAEC064 spontaneous sucrose resistance occurred at a frequency of ~10^{-5}, of which only a small minority (~1/100) were also sensitive to kanamycin. Spontaneous sucrose resistance in strain AAEC090 occurred at a lower frequency (~10^{-6}), of which ~1/10 were also sensitive to kanamycin. These values were potentially low enough to permit the use of *sacB* as a counter-selectable marker.

The temperature-dependent growth properties of plasmid integrates containing derivatives of pMAK705

Plasmid integrates of the various strain/plasmid combinations listed in Table 2 were isolated as described before

Table 1. Sucrose-inhibition of growth of strains AAEC064 and AAEC090 at different temperatures and salt concentrations.

	Concentration of Sucrose in Media (%)						
Strains[a]	0.5	1.0	2.0	4.0	6.0	8.0	10.0
	LB agar lacking NaCl at 37°C						
AAEC064	++++b	++++	++++	++++	++++	+++	+
AAEC090	++++	++++	++++	+	+	NT[c]	NT
	LB agar at 30°C						
AAEC064	++++	++++	++++	++++	++++	+++	+++
AAEC090	++++	+++	+	+	+	NT	NT
	LB agar lacking NaCl at 30°C						
AAEC064	++++	+++	+++	+	+	+	+
AAEC090	+	+	+	+	+	NT	NT

a. Initial inoculum of 10^6 bacteria.
b. Growth measured as follows: ++++, confluent lawn; +++, reduced lawn; ++, more than 10 single colonies; +, fewer than 10.
c. Not tested.

Table 2. Plasmid integrate frequencies.

Strain	Plasmid[a]	Chromosome-plasmid homology (kbp)	Plasmid integrate frequency (10^{-4})[b]
AAEC064	pIB308	1.43 + 2.17	21.58
AAEC090	pIB308	6.55 + 0	100.76
AAEC090	pRR2	0.22 + 1.09	1.67
AAEC064	pRR2	0.22 + 1.09	3.12

a. All plasmids are derivatives of pMAK705 (Hamilton *et al.*, 1989).
b. Calculated as the average from two independent experiments as the ratio of transformants at 42°C to 30°C isolated on chloramphenicol agar.

(Hamilton *et al.*, 1989), except that transformed cells were grown on chloramphenicol at 42°C rather than at 44°C. The frequencies of plasmid integrate formation (Table 2) are comparable with those reported previously, and are strongly influenced by the extent of homology between the vector and chromosome.

As noted earlier, plasmid integrates containing derivatives of pMAK705 are reported to grow poorly on agar at 30°C (Hamilton *et al.*, 1989). In our experience, however, the growth inhibition of plasmid integrates that contained insertions in *lac* was minimal (see the *Experimental procedures*). To investigate this phenomenon in more detail, plasmid-integrate derivatives of AAEC064 containing insertions in *fim* (integrated plasmid: pIB308), or AAEC090 derivatives containing insertions in *lac* (pRR2), were grown at 42°C to late log phase in the absence of antibiotics. Dilutions of the cultures were spread onto LB agar and incubated at either 30°C or 42°C. Whereas the viability of pRR2-containing plasmid integrates was indistinguishable at 30°C or 42°C, counts for the pIB308-containing plasmid integrates at 30°C were considerably lower than those at 42°C (Table 3). We repeated these experiments with plasmid integrates containing pIB308 in strain AAEC090 and pRR2 in strain AAEC064. These results confirmed the position-dependent growth characteristics of the plasmid integrates (Table 3).

Many of the bacteria isolated from the pIB308-containing plasmid integrates grown at 30°C were sensitive to chloramphenicol, suggesting that these survivors have excised (and cured) the integrated plasmid. It seems likely, therefore, that an integrated copy of pIB308 in *fim* is lethal at 30°C, suggesting that the relationship between cell viability and the presence of an integrated plasmid is dependent on the position of integration.

Use of the exchange procedure to reconstruct the wild-type fim *genes in strain AAEC064*

The allelic exchange was undertaken following the rationale outlined in Fig. 2, using the optimized selective conditions for sucrose sensitivity. A schematic representation of the expected recombinant is shown in Fig. 3. Two independent pIB308-containing plasmid integrates of AAEC064 were grown at 42°C to late log phase in LB broth. Dilutions of the cultures were spread onto sucrose agar and incubated at 30°C. The resulting colonies were then tested for sensitivity to both kanamycin (the chromosomal marker) and chloramphenicol (the plasmid marker). For each of the two plasmid integrates examined, 86 isolates were tested; all 172 were sensitive to both antibiotics. These results allowed us to estimate the frequency of excision, and subsequent loss, of the *sacB*–Neo^R cassette from strain AAEC064 (Table 4), assuming a low frequency of background mutations.

The approximate frequency of background mutants selected on the sucrose agar was expected to be comparatively low (~10^{-8}), calculated as the product of two independent events: (i) the development of spontaneous sucrose resistance, and the (ii) excision of the integrated plasmid. We determined the magnitude of each of these two events experimentally as ~10^{-5} for the frequency of mutation to spontaneous sucrose resistance in strain AAEC064, and ~10^{-3} for the excision frequency of pIB308 (see Table 3). To estimate the background mutation frequency more directly, at least for one class of plasmid integrates, we repeated our experimentation with a second plasmid, pIB312. Like pIB308, pIB312 contains a

Table 3. Relative growth of plasmid integrates on LB agar incubated at 30°C vs. 42°C[a].

Plasmid	Integrate no.	Strains	
		AAEC064	AAEC090
pIB308[b]	1	2.8×10^{-3}	1.93×10^{-2}
	2	1.8×10^{-3}	2.05×10^{-2}
pRR2[c]	1	~1.0	~1.0
	2	~1.0	~1.0

a. The values represent the ratio of viable counts obtained on LB agar at 30°C to those obtained at 42°C.
b. Plasmid integrates via *fim* homology.
c. Plasmid integrates via *lac* homology.

Table 4. Frequency of sucrose-resistant isolates of AAEC064-plasmid integrates containing either pIB308 or pIB312.

Plasmid[a]	Integrate no.	Frequency of sucrose resistance
pIB308	1	4.18×10^{-4}[b]
	2	8.30×10^{-5}
pIB312	1	8.40×10^{-9}
	2	2.21×10^{-8}

a. Plasmids integrate via *fim* homology.
b. The values are calculated as the ratio of viable counts on agar containing 6% sucrose (without NaCl) at 30°C to growth on LB agar at 42°C.

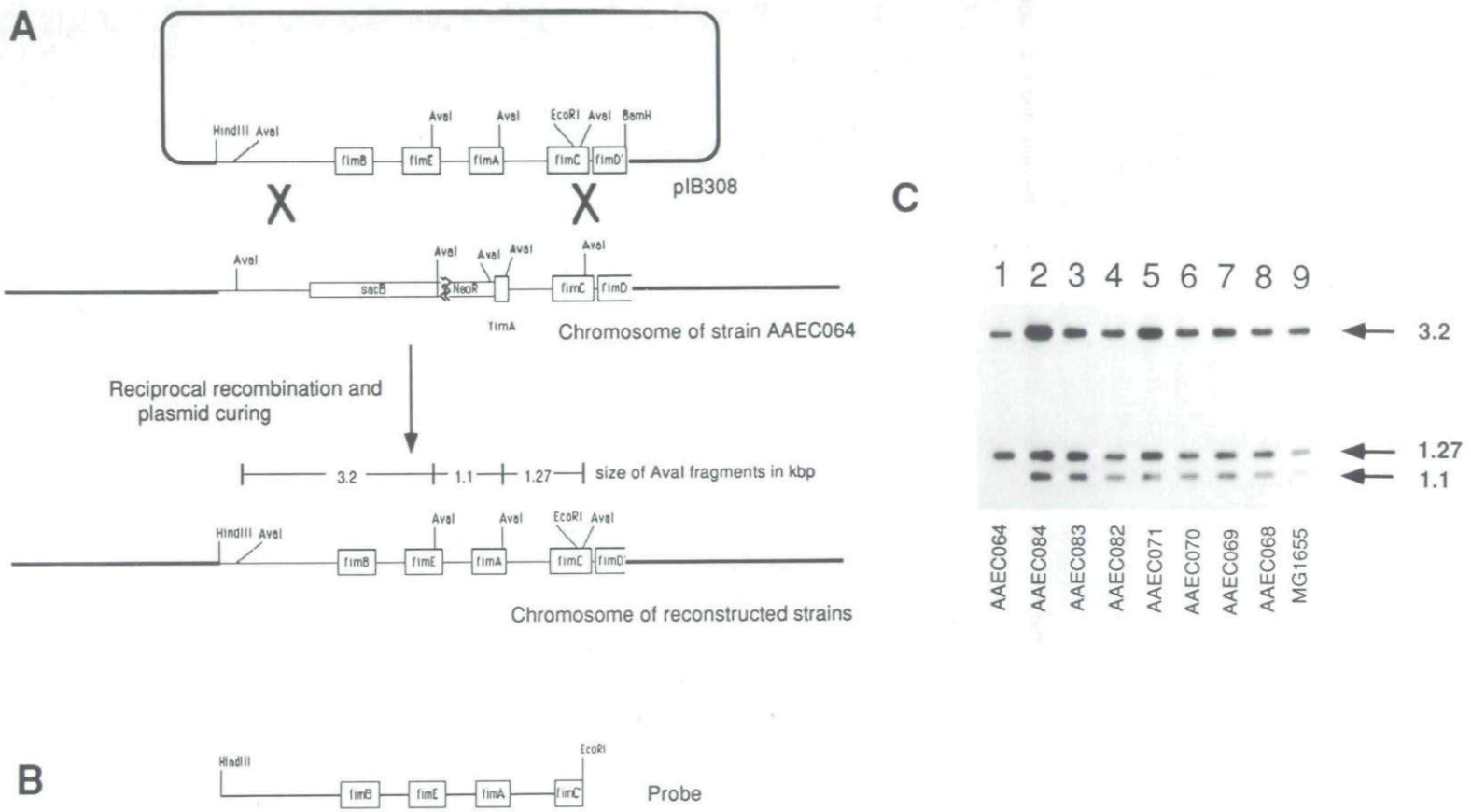

Fig. 3. The recombination between strain AAEC064 and plasmid pIB308 leads to reconstruction of wild-type *fim* gene organization. The recombination reaction is represented schematically (A): plasmid pIB308 is represented at the top of the diagram, the chromosome of strain AAEC064 is shown directly below the two Xs, and the product of the integration-excision (with a fully reconstructed *fim* gene cluster) is shown directly below the arrow. Using the probe indicated (B), the chromosomal structures of seven reconstructed strains (AAEC068–AAEC071 and AAEC082–AAEC084) were examined by Southern hybridization analysis (C). Chromosomal DNA was digested with *Ava*I, and separated on a 1% agarose gel. As expected, the reconstructed strains resemble the wild-type parent, strain MG1655, rather than the intermediate strain, AAEC064. This conclusion was confirmed by a similar analysis using *Hin*cII instead of *Ava*I (data not shown).

1.43 kbp region of *fim* homology on one side of the *sacB*–Neo^R cassette in strain AAEC064. However, since pIB312 lacks *fim* sequences homologous to the other side of the *sacB*-Neo^R cassette, recombination between pIB312 and the chromosome of strain AAEC064 should not produce a *sacB*-containing plasmid. The frequency of mutation to sucrose resistance in two independent pIB312 plasmid integrates is similar to our initial predictions (i.e. 10^{-8}; see Table 4). Moreover, most of these isolates were kanamycin resistant, indicating that the probability of isolating a background mutant during our exchange procedure with plasmid pIB308 is very low (10^{-5}).

The *fim* DNA carried on plasmid pIB308 was cloned from our wild-type strain, MG1655. Accordingly, we expected the sucrose-resistant, drug-sensitive isolates to resemble MG1655. Using Southern hybridization analysis, we found that the organization of the *fim* DNA in seven randomly picked isolates was indistinguishable from that of strain MG1655 (Fig. 3). As expected, these strains produced amounts of fimbriae equivalent to that for strain MG1655, as determined by enzyme-linked immunosorbent assay (ELISA) inhibition (data not shown).

Construction of an unmarked lacZYA *deletion*

Next we attempted to construct an unmarked *lac* deletion strain resulting from recombination between plasmid pRR2 and the chromosome of strain AAEC090. Plasmid integrates were grown and treated exactly as described before (see above). Survivors that were isolated on sucrose agar at 30°C were tested for sensitivity to chloramphenicol and kanamycin. Of the 37 colonies tested, 32 were sensitive to both antibiotics. The five remaining isolates were both chloramphenicol- and kanamycin resistant. Since plasmid integrates containing pRR2 did not show any reduced viability when grown at 30°C, we presume that many of the antibiotic-resistant isolates retained the integrated plasmid. The frequency of sucrose-resistant, antibiotic-sensitive isolates obtained in this experiment (4.8×10^{-5}) was appreciably higher than the spontaneous mutation frequency of strain AAEC090 to this phenotype (8.4×10^{-8}). Two sucrose-resistant, antibiotic-sensitive isolates were examined further by Southern hybridization analysis. The chromosomal structure of these strains confirmed that a successful allelic exchange had taken place (data not shown).

Discussion

We wished to develop an allelic exchange system that would allow the routine transfer of non-selectable markers into the chromosome of our wild-type strain, MG1655. To do this we combined the use of two different approaches: first, the employment of an intermediate strain that contains both a selectable and a counter-selectable marker and, second, the use of a temperature-sensitive plasmid. Used together, these techniques permit positive selection for each step in the allelic exchange process (i.e. plasmid integration, excision, and curing). Moreover, the frequency of plasmid curing, with the accompanying and desired loss of the counter-selectable marker, is increased by the use of a temperature-sensitive vector.

The *B. subtilis sacB* gene was first used as a counter-selectable marker to facilitate the isolation of insertion sequences (Gay *et al.*, 1985). It has been used subsequently in allelic exchanges in *Erwinia* (Reid and Collmer, 1987) and also provides an effective counter-selectable marker in *Legionella* (Cianciotto *et al.*, 1988). We employed the *sacB* gene as a counter-selectable marker primarily because it can be used in wild-type strains. An alternative marker, the wild-type *rpsL* gene, must be used in a merodiploid strain that also contains the recessive, streptomycin-resistance allele (Russel and Dahlquist, 1989).

In constructing the *sacB*-containing intermediate strains, we decided to include a second marker, namely the neomycin-resistance gene of Tn*5*. To do this we first constructed a DNA cassette that contains the two genes cloned in tandem and that can be subcloned on a 3.8 kbp *Bam*HI fragment. The inclusion of the neomycin-resistance gene has a number of advantages. In the first place, the presence of the selectable marker greatly simplifies the construction of intermediate strains. Second, it permits simple transfer of the cassette from one strain to another; this is a useful feature when it is necessary to construct numerous strains that contain an identical mutation. For example, as a first step in constructing an unmarked *lac* deletion mutant, the cassette is transferred from AAEC090 to the strain which the investigator wishes to mutate. Finally, in situations where potential recombinants are likely to include background mutants, kanamycin resistance provides a phenotype that distinguishes many false-positive isolates (i.e. spontaneous sucrose-resistant colonies) from bacteria that have undergone a successful allelic exchange. In this study, we found this to be helpful in identifying *lac* deletion mutants.

In the previously reported studies, the growth of bacteria containing *sacB* was inhibited on LB agar containing 5% sucrose at 37°C. Although these conditions prevented the growth of strains containing *sacB* in either *lac* or *fim* on plasmid DNA (data not shown), the corresponding chromosomal constructs grew well. The sucrose sensitivity of both strains (AAEC064 and AAEC090) was greatly

influenced by temperature and by sodium chloride concentration. Thus, at lower temperatures (30°C), and on media that lacked sodium chloride, the growth of strain AAEC090 (cassette in *lac*) was completely inhibited on agar containing as little as 0.5% sucrose. Strain AAEC064 (*sacB*–Neo^R cassette in *fim*) was fully inhibited on media containing 4% sucrose. In the light of these results, we used LB agar supplemented with 6% sucrose, but lacking sodium chloride, in the allelic exchanges. We interpret the temperature- and sodium chloride-dependent sucrose sensitivity of strains AAEC064 and AAEC090 as suggesting that *sacB* expression may be influenced by DNA supercoiling (Goldstein and Drlica, 1984; Higgins *et al.*, 1988). Transcription of *sacB* in the *sacB*–Neo^R cassette should be initiated at a promoter contained within the cloned *B. subtilis* sequences (Steinmetz *et al.*, 1985). As far as we are aware, neither of our chromosomal constructs results in the transcription of the *sacB* gene from a promoter outside the cassette. Consequently we believe that the variability in sucrose sensitivity between the intermediate strains may reflect differences in local DNA supercoiling.

The use of a temperature-sensitive plasmid pSC101 derivative (pMAK705) in allelic exchange to permit selection for both plasmid integration and excision has been reported before (Hamilton *et al.*, 1989). We have found that the use of these vectors greatly facilitated the isolation of plasmid integrates, but proved unreliable when selecting bacteria that had excised the integrated plasmid. Thus, although plasmid integrates containing insertions in *fim* failed to grow at or below 30°C, as expected, strains with insertions in *lac* grew normally. We also noticed that plasmid integrates containing insertions in *fim* grew normally at temperatures above 30°C (data not shown), and recommend the use of a lower temperature (28°C). Fortunately, the growth inhibition of plasmid integrates is not essential to the success of the allelic exchange procedure described in this communication, and we were still able to isolate unmarked *lac* deletions easily.

Starting with the temperature-sensitive vector pMAK705, we have constructed an additional vector, termed pIB307, that we find more useful than its parent. To reduce the possibility of undesirable recombination between the chromosome and plasmid, we removed most of the *lac* sequences carried by pMAK705. As a result of these and other deletions, the resulting vector is 1.3 kbp smaller than pMAK705 (4.4 kbp rather than 5.7 kbp), but retains the M13mp19 polylinker.

In addition to facilitating the transfer of markers from plasmid DNA to the chromosome, the temperature-sensitive vector has also been used to clone chromosomal alleles (Hamilton *et al.*, 1989). We find that the efficiency of this technique can be improved by employing derivatives that contain the *sacB*–Neo^R cassette. These plasmids contain the *sacB*–Neo^R cassette replacing part of the cloned chromosomal DNA, while preserving some flanking sequences for homologous recombination. The plasmid DNA isolated from plasmid integrates is used to transform cells to chloramphenicol resistance in the presence of sucrose, selecting against the parental, but not the recombinant, plasmid. We have used this technique to clone *fim* genes (data not shown). Finally, the allelic exchange procedure can be used to exchange any DNA segment, in addition to the original or mutant allelic DNA, into the *E. coli* chromosome. Although not formally proven by these studies, we anticipate that virtually any non-essential part of the *E. coli* chromosome could be targeted for these purposes.

Experimental procedures

Bacterial and bacteriophage strains and media

The bacterial strains used in this study are listed in Table 5. P1 transduction was carried out using P1*vir* by standard techniques (Silhavy *et al.*, 1984). Bacteria were grown in Luria Broth (LB) media (Bactotryptone and Bacto-yeast extract were from Difco Laboratories) at 37°C with aeration except where noted. Media containing sucrose (J. T. Baker Co.) were prepared as described in the text. For solid media, agar (Becton Dickinson) was added to 1.5%. Media were supplemented with ampicillin (50 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}) or kanamycin (25 μg ml^{-1}) where appropriate (Sigma Chemical Co.).

Recombinant DNA techniques

Chromosomal DNA was isolated according to published protocols (Ausubel *et al.*, 1987). Restriction endonuclease digestions were carried out according to the specific manufacturers' recommendations. DNA labelling was carried out using the multiprime kit supplied by (Amersham Co). All other molecular genetic procedures and recombinant DNA techniques (plasmid isolation, agarose gel electrophoresis, isolation of restriction fragments, ligation and transformation of plasmid DNA, Southern hybridization) were carried out according to standard published protocols (Ausubel *et al.*, 1987; Maniatis *et al.*, 1982).

Quantification of fimbriation by ELISA

Enzyme-linked immunosorbent assay inhibition determinations were done as previously described (Dodd and Eisenstein, 1982; Eisenstein *et al.*, 1983).

Construction of the sacB–*Neo^R cassette*

The DNA cassette contains the *B. subtilis sacB* gene cloned in tandem with the neomycin-resistance marker from Tn*5*. These markers allow counter-selection (*sacB*) or positive selection (Neo^R) of the cassette and DNA contiguous with it. The cassette can be subcloned on a 3.8 kb *Bam*HI fragment and should contain no significant homology to the *E. coli* chromosome (Berg and Drummond, 1978; Mazodier *et al.*, 1985).

Table 5. Bacterial strains and plasmids.

Strain/Plasmid	Relevant genotype or phenotype	Source/Reference
E. coli		
DH1	*recA1, hsdR17* (r^-m^+), *endA1*	Hanahan, (1983)
MG1655	λ^-, F^-	B. Bachmann; Guyer *et al.* (1981)
AAEC032	*polA12* derivative of MG1655; Tn*10* (Tet^R) near *polA*	Monk and Kinross (1972); Our unpublished data
AAEC064	Δ*fimB–A* replaced by *sacB*–Neo^R; derivative of MG1655	This work
AAEC068-071, AAEC082-084	Fim^+ derivatives of AAEC064 following recombination with pIB308	This work
AAEC090	Δ*lacZ–A* replaced by *sacB*-Neo^R; derivative of MG1655	This work
AAEC100-101	Δ*lacZ–A* derivative of AAEC090 isolated following recombination with plasmid pRR2	This work
Plasmid		
pACYC184	Cm^R, Tet^R	Chang and Cohen (1979)
pUC18	Amp^R	Yanisch-Perron *et al.* (1985)
pMAK705	Cm^R, temperature-sensitive vector	Hamilton *et al.* (1989)
pUCD800	Amp^R, *sacB*	Steinmetz *et al.* (1985)
pRS415	Amp^R, *lacZYA*	Simons *et al.* (1987)
pDS2	Amp^R, 2.8 kbp *Bam*HI–*Pst*I *sacB* fragment from pUCD800 in pUC18	Our unpublished data
pIB215	Amp^R, Kan^R, *Hin*dIII–*Bam*HI Neo^R fragment from Tn*5* in pBR322	This work
pIB248	Amp^R, pBR322 Δ*Aat*II–*Eco*RI, replaced by 8 bp *Eco*RI linker; insertion of 8 bp *Hin*dIII linker in unique *Pvu*II site	Our unpublished data
pIB249	Amp^R, 5.7 kbp *Hin*dIII–*Eco*RI *fimA–D* fragment of MG1655 in pIB248	Our unpublished data
pIB250	Amp^R, Δ*Eco*0109–*Pvu*II *fimB–A* fragment of pIB249; insertion of 10 bp *Bam*HI linker	This work
pIB276	Amp^R, Δ*Nde*I IS*1*-vector fragment of pDS2; insertion of 8 bp *Xho*I linker	This work
pIB279	Amp^R, Kan^R, *sacB*–Neo^R cassette (see text for details)	This work
pIB283	Amp^R, Kan^R, 3.8 kbp *Bam*HI *sacB*–Neo^R cassette from pIB279 in pIB250	This work
pIB298	Cm^R, Δ1.09 kbp *Hin*cII fragment of pACYC184; insertion of 8 bp *Eco*RI linker	This work
pIB300	Cm^R, replacement of 1.4 kbp *Eco*RI fragment of pMAK705 with 1.0 kbp *Eco*RI fragment of pIB298	This work
pIB305	Cm^R, deletion of 0.78 kbp *Eco*0109 fragment of pIB300; *Eco*0109-cut DNA was treated with Klenow enzyme	This work
pIB306	Cm^R, insertion of 8 bp *Hin*dIII linker in *lac Pvu*II site of pIB305	This work
pIB307	Cm^R, deletion of 0.18 kbp *Hin*dIII fragment of pIB306	This work
pIB308	Cm^R, 6.5 kbp *Hin*dIII–*Bam*HI *fimA–D'* fragment of pIB238 into pIB306	This work
pIB310	Cm^R, *fimB–H* deletion in pIB307	Our unpublished data
pIB312	Cm^R, pIB310 Δ 0.5 kbp *Bam*HI fragment; single region of homology to AAEC064	This work
pRR2	Cm^R, 1.0 kbp *Hin*dIII(*Stu*I)–*Sal*I fragment of pRS415 replacing the polylinker of pIB300	This work
pRR5	Cm^R, Kan^R, 3.8 kbp *Bam*HI *sacB*–Neo^R cassette from pIB279 in pRR2	This work

Assembly of the *sacB*–Neo^R cassette required a number of intermediate constructions. The *sacB* gene (on a 2.8 kbp *Bam*HI–*Pst*I fragment) was first subcloned from pUCD800 into pUC18 to generate pDS2 (Gay *et al.*, 1985; Steinmetz *et al.*, 1985; Yanisch-Perron *et al.*, 1985; our unpublished data). Since the subcloned DNA included a 0.6 kbp remnant of the insertion sequence IS*1*, we next deleted a 0.87 kbp *Nde*I fragment from pDS2. To accomplish this, pDS2 was digested with *Nde*I and treated with Klenow enzyme to produce blunt ends. To facilitate subsequent steps, this DNA fragment was recircularized by treatment with T4 DNA ligase in the presence of an 8 bp *Xho*I linker (Pharmacia LKB Biotechnology). The resulting plasmid, which contains a single linker insert, was designated pIB276. Plasmid pIB276 was digested with *Xho*I, and the four-base-pair 5' protruding ends were partly filled in by treating with Klenow enzyme to leave a single-base overhang. Next, the DNA was partially digested with *Eco*RI, and the 1.9 kbp *sacB* fragment was gel-purified. The *sacB*–Neo^R cassette was constructed by ligating the *sacB* fragment from pIB276 with pIB215, a derivative of pBR322 that contains the neomycin-resistance gene from Tn*5* subcloned into the *Hin*dIII–*Bam*HI sites. Plasmid pIB2l5 was digested with *Hin*dIII, treated with Klenow enzyme as described above, and then digested with *Eco*RI before gel purification. The cassette was finally assembled by ligating the purified fragments together to produce pIB279. As expected, pIB279 contains a *Xho*I restriction site between the *sacB* and Neo^R markers.

Construction of pMAK705 derivatives

We wished to use a temperature-sensitive vector for allelic exchange in wild-type *lac* strains. We have constructed a derivative of pMAK705, termed pIB307, that we consider to have two advantages over its parent (Hamilton *et al.*, 1989). By removing the majority of *lac* sequences from pMAK705, we avoided the potential complication of unwanted recombination between the plasmid and chromosome. Moreover, since pIB307 is 1.3 kbp smaller than pMAK705, while still retaining its M13mp19 polylinker cloning site, it is easier to use as a cloning vector (Messing, 1983).

The construction of pIB307 was carried out in four steps. To remove the *lacZ* sequences from pMAK705, we replaced the 1.4 kbp *Eco*RI fragment with a 1.0 kbp *Eco*RI fragment derived from the vector pACYC184. This plasmid was termed pIB300. The *Eco*RI fragment was isolated from pIB298, a derivative of pACYC184 that contains an *Eco*RI linker replacing the 1.09 kbp *Hin*cII fragment (our unpublished data). To remove additional sequences from pIB300, we next deleted a 0.78 kbp *Eco*0109 fragment from the plasmid. Plasmid pIB300 was cut with *Eco*0109, and the overhanging 5' ends were filled in by treatment with Klenow enzyme. The vector and chloramphenicol-resistant fragments were purified and ligated to produce pIB305. Plasmid pIB305, which is refractory to digestion with *Eco*0109 as expected, contains the chloramphenicol-resistance gene in the same orientation as pMAK705. To remove the majority of the remaining *lac* sequences, we next deleted a 180 bp *Pvu*II–*Hin*dIII fragment from pIB305. To retain the *Hin*dIII restriction site in the final construct, we first introduced a *Hin*dIII linker into the *Pvu*II site within the *lacI* region. This plasmid, pIB306, was then digested with *Hin*dIII and the vector fragment was religated to produce plasmid pIB307.

Derivatives of pMAK705 that contain *fim* sequences were constructed from pIB306. All *fim* DNA subcloned into this vector was originally cloned from strain MG1655 (Guyer *et al.*, 1981). Plasmid pIB308 was constructed by subcloning a 6.5 kbp *Hin*dIII–*Bam*HI fragment from pIB238 into pIB306 digested with the same enzymes. The fragment contains the *fimB, fimE, fimA*, and *fimC* genes, as well as part of *fimD* (Klemm *et al.*, 1985; Orndorff and Falkow, 1984; our unpublished data). Plasmid pIB312 was constructed from pIB310 by deleting a 0.5 kbp *Bam*HI fragment comprising the region downstream of the *fimH* gene. Plasmid pIB310 is a derivative of pIB306 that contains a 1.9 kbp *Hin*dIII–*Sal*I fragment including *fim* DNA 5' to the *fimB* gene and 3' to the *fimH* gene (our unpublished data).

The various *lac* derivatives of pMAK705 were constructed from pIB300. The subcloned *lac* DNA was derived from plasmid pRS415, which contains the *lacZYA* genes, as well as 1 kbp of DNA downstream of the *lacA* gene (Simons *et al.*, 1987). To facilitate subsequent manipulation of the *lac* DNA, we constructed a derivative of pRS415 that contains a *Hin*dIII linker inserted into the *Stu*I site downstream of *lacA*. The 1 kbp region 3' to *lacA* was then subcloned into pIB300 on the 1 kbp *Hin*dIII–*Sal*I fragment to produce pRR2. Plasmid pIB300 contains 0.23 kbp of *lac* DNA from the M13mp polylinker region, comprising the 3' end of the *lacI* gene and the *lacZYA* promoter. Since this segment is retained in pRR2, the plasmid contains two sections of *lac* DNA, separated by a *Hin*dIII restriction site. Plasmid pRR5 contains the *sacB*–Neo^R cassette inserted between these two regions of *lac* homology, in the opposite transcriptional orientation to the native *lacZYA* genes. To simplify the construction of pRR5, we made a derivative of pRR2 that contains an *Nhe*I restriction site in place of the *Hin*dIII site. This was accomplished by digesting pRR2 with *Hin*dIII and filling in the recessed ends by treatment with Klenow enzyme, before religating the DNA with T4 DNA ligase. The resulting plasmid, termed pRR4, was then digested with *Nhe*I, and the recessed ends were partly filled in by treatment with Klenow enzyme to leave a single-base overhang. Plasmid pRR5 was constructed by ligating this DNA with the 3.8 kbp *Bam*HI, *sacB*–Neo^R cassette previously treated with Klenow enzyme to leave a complementary base extension.

Construction of strain AAEC064

Strain AAEC064 contains the *sacB*–Neo^R cassette in place of the *fimB–fimA* region of strain MG1655 (Guyer *et al.*, 1981; Klemm *et al.*, 1985; Orndorff and Falkow, 1984). The *sacB*–Neo^R cassette was subcloned into a ColE1-based vector and then transferred into the chromosome of a *polA* derivative of MG1655, as described previously (Cunningham and Weiss, 1985; see below).

Plasmid constructs. Plasmid pIB283 contains the *sacB*–Neo^R cassette flanked on both sides by *fim* DNA (1.4 and 1.3 kbp of homologous *fim* DNA). The transcription of the *sacB* and Neo^R genes in pIB238 is in the same orientation as the native *fim* genes. Plasmid pIB283 was constructed from pIB249 in two steps. (pIB249 contains a 5.7 kbp *Hin*dIII–*Eco*RI *fimB–fimA* fragment cloned from strain MG1655, in pIB248, a vector derived from pBR322 (our unpublished data).) Plasmid pIB249 was digested with *Eco*0109, treated with Klenow enzyme to produce blunt ends, and then digested with *Pvu*II. The plasmid

was then recircularized by treatment with T4 DNA ligase in the presence of a 10 bp *Bam*HI linker (New England Biolabs) to produce pIB250. Plasmid pIB250, which contains a single linker insert, was digested with *Bam*HI and ligated with the 3.8 kbp *sacB*–Neo^R *Bam*HI cassette to produce pIB283.

Transfer of fim–sacB–*Neo^R cassette into the chromosome of MG1655.* Plasmid pIB283, isolated from strain DH1, was transformed into AAEC032, a derivative of MG1655 that contains the temperature-sensitive *polA12* mutation linked to transposon Tn*10* (Guyer *et al.*, 1981; Monk and Kinross, 1972; our unpublished data). The exchange was carried out as described previously, with selection for plasmid integration on ampicillin at 42°C, and selection for the plasmid excision by growth at 30°C (Cunningham and Weiss, 1985). Although growth of the plasmid integrates was reduced at the permissive temperature for plasmid replication (30°C), many survivors retained the integrated plasmid. Because of this problem, it was necessary to screen several hundred colonies to identify a single ampicillin-sensitive, kanamycin-resistant, *fim*$^-$ isolate. Bacteriophage P1*vir*, grown on this strain, was used to transduce strain MG1655 to kanamycin resistance. As expected, the transductants simultaneously became *fim*$^-$ (data not shown). One strain, termed AAEC064, was used in subsequent experiments.

Construction of strain AAEC090

Strain AAEC090 is a descendant of MG1655 in which the *lacZYA* genes are replaced by the *sacB*–Neo^R cassette contained in plasmid pRR5. The transfer of the cassette from pRR5 to the chromosome of MG1655, with the accompanying deletion of the *lacZYA* genes, was accomplished by exploiting the temperature-dependent replication of pMAK705 derivatives (Hamilton *et al.*, 1989). Plasmid integrate derivatives of MG1655 were isolated on chloramphenicol agar at 42°C as described below. Excision of the integrated plasmid, as a result of homologous recombination between the duplicated regions of *lacI* DNA, was expected to produce a strain with the desired chromosomal structure. To select bacteria that had excised the integrated plasmid, we grew plasmid integrates at 30°C as described before (Hamilton *et al.*, 1989). Survivors of this procedure were plated onto MacConkey agar containing kanamycin, and incubated at 30°C. Surprisingly, we found that the majority of colonies were Lac$^+$, and that it was necessary to screen several thousand colonies to identify Lac$^-$ isolates. The correct chromosomal structure of two Lac$^-$ isolates was confirmed by Southern hybridization analysis, and the cassette was transduced into a fresh MG1655 background by P1 transduction employing kanamycin resistance as a selectable phenotype. As expected, all transductants were Lac$^-$ (data not shown). One transductant, strain AAEC90, was employed throughout.

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