

Methodology article

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Rapid identification and mapping of insertion sequences in *Escherichia coli* genomes using vectorette PCR

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Abstract

Background: Insertion sequences (*IS*) are small DNA segments capable of transposing within and between prokaryotic genomes, often causing insertional mutations and chromosomal rearrangements. Although several methods are available for locating *IS*s in microbial genomes, they are either labor-intensive or inefficient. Here, we use vectorette PCR to identify and map the genomic positions of the eight insertion sequences (*IS1*, 2, 3, 4, 5, 30, 150, and 186) found in *E. coli* strain CGSC6300, a close relative of MG1655 whose genome has been sequenced.

Results: Genomic DNA from strain CGSC6300 was digested with a four-base cutter *Rsa* I and the resulting restriction fragments ligated onto vectorette units. Using *IS*-specific primers directed outward from the extreme ends of each *IS* and a vectorette primer, flanking DNA fragments were amplified from all but one of the 37 *IS* elements identified in the genomic sequence of MG1655. Purification and sequencing of the PCR products confirmed that they are *IS*-associated flanking DNA fragments corresponding to the known *IS* locations in the MG1655 genome. Seven additional insertions were found in strain CGSC6300 indicating that very closely related isolates of the same laboratory strain (the K12 isolate) may differ in their *IS* complement. Two other *E. coli* K12 derivatives, TD2 and TD10, were also analyzed by vectorette PCR. They share 36 of the MG1655 *IS* sites as well as having 16 and 18 additional insertions, respectively.

Conclusion: This study shows that vectorette PCR is a swift, efficient, reliable method for typing microbial strains and identifying and mapping *IS* insertion sites present in microbial genomes. Unlike Southern hybridization and inverse PCR, our approach involves only one genomic digest and one ligation step. Vectorette PCR is then used to simultaneously amplify all *IS* elements of a given type, making it a rapid and sensitive means to survey *IS* elements in genomes. The ability to rapidly identify the *IS* complements of microbial genomes should facilitate subtyping closely related pathogens during disease outbreaks.

Background

Insertion sequences (*IS*) are small DNA segments capable of transposing within and between prokaryotic genomes and episomes, often causing insertional mutations and chromosomal rearrangements [1]. Identifying and map-

ping *IS* elements in microbial genomes is essential to understand their evolutionary significance [2-5]. So rapidly can *IS* elements move that even closely related laboratory strains commonly differ in the positions of their *IS* sequences [6,7]. A swift means to identify *IS* insertions

might therefore allow isolates from specific disease outbreaks to be distinguished from other closely related strains.

Several methods have been used to identify the number and locations of *IS* elements in bacterial genomes, including Southern hybridization [3] and the inverse polymerase chain reaction (iPCR) [4,8,9]. Southern hybridization is rather time-consuming and requires additional procedures for localizing *IS*s. Inverse PCR, a commonly used PCR method for recovering unknown flanking sequences of a known target sequence, uses a library of circularized chromosomal DNA fragments as template and two outward primers located in each end of the known fragment for amplification [8]. However, when a target sequence has multiple genomic locations, the variously sized DNA circles formed are difficult to amplify simultaneously. Also, the length of each restriction DNA fragment containing a target sequence must be determined by Southern hybridization followed by sub-genomic fractioning before intramolecular ligation and PCR amplification [4,8,9]. These difficulties render Southern hybridization and iPCR impractical as techniques for quickly surveying repetitive elements in genomes.

Vectorette PCR (vPCR) [10,11] is another method used to amplify unknown sequences flanking a characterized DNA fragment. It involves cutting genomic DNAs with a restriction enzyme, ligating vectorettes to the ends, and amplifying the flanking sequences of a known sequence using primers derived from the known sequence along with a vectorette primer (Fig. 1). This technique has found many applications, including sequencing cosmid insert termini [10], identifying telomeres [12] and microsatellite sequences [13], mapping deletions, insertions, and translocations [14,15], and determining the 5' and 3' ends of mRNAs [16]. Here, we explore the efficiency of vPCR with regards to identifying and mapping *IS* elements in microbial genomes. We show that multiple copies of an *IS* are readily amplified using an *IS* specific primer in combination with a vectorette primer, and that their genomic locations are readily identified from the flanking DNA sequences.

Results and discussion

The *IS* insertions of CGSC6300

We used *E. coli* strain CGSC6300, a close relative of the sequenced strain MG1655, against which to test the efficiency and reliability of vPCR in detecting *IS* copies. *IS* insertion sites were identified by sequencing flanking DNA fragments amplified using outward *IS*-specific primers in combination with the vectorette primer. Based on the whole genome sequence of strain MG1655 [17], there are 37 *IS* elements, including 7 copies of *IS1*, 6 copies of *IS2*, 5 copies of *IS3*, 1 copy of *IS4*, 11 copies of *IS5*, 3 cop-

ies of *IS30*, 1 copy of *IS150*, and 3 copies of *IS186*. Our results for each *IS* in CGSC6300 are summarized in Table 1 and described as follows:

IS1

Eight and 6 PCR bands, obtained with primers *IS1-A* and *IS1-B* respectively, were observed on ethidium bromide-stained agarose gels (Fig. 2). All 7 *IS1* insertion sites in the sequenced genome of MG1655 [17] were successfully identified by isolating and sequencing these fragments. Sequences obtained from both flanking sequences were used to locate 2 *IS1* elements (*IS1-5* and *IS1-6*). The remaining 5 *IS1* locations were identified from single flanking sequences. Three additional *IS1* elements (*IS1-a* in *b0240*, *IS1-c* in *b1786*, and *IS1-f* in *b2635*) were also found in CGSC6300.

IS2

Primers *IS2-A* and *IS2-B* produced 4 bands and 6 bands, respectively (Fig. 2). Three (*IS2-1*, *IS2-4*, and *IS2-5*) were located from both flanking sequences and the remaining 3 (*IS2-2*, *IS2-3*, and *IS2-6*) were located from one flanking sequence. Gene *b1579*, homologous to the *IS2* transposase [17], was also amplified, sequenced and located in CGSC6300.

IS3

Three *IS3* elements (*IS3-2*, -3, and -4) were each located by sequencing amplified flanking DNAs from both sides, and two (*IS3-1* and *IS3-5*) were each located by sequencing a single flanking sequence (Fig. 2). Three additional *IS3* elements were found at *b0805* (*IS3-b*), *b3604* (*IS3-e*), *b4242* (*IS3-f*).

IS4

One *IS4* was located based on flanking sequences amplified from both sides. No additional *IS4* insertions were found.

IS5

Primers *IS5-A* and *IS5-B* produced 7 and 9 bands, respectively (Fig. 2). Purification and sequencing of these DNA fragments showed that they correspond to flanking sequences of *IS5-2*, -3, -5, -6, -7, -9 for the A-side and *IS5-1*, -2, -5, -6, -7, -8, -9, -10, -11 for the B-side. Fragments flanking either side of *IS5-4* were not identified.

IS30

The three known *IS30* insertions in MG1655/CGSC6300 were identified based on flanking sequences amplified from both sides, and an additional insertion was identified in *b2156*.

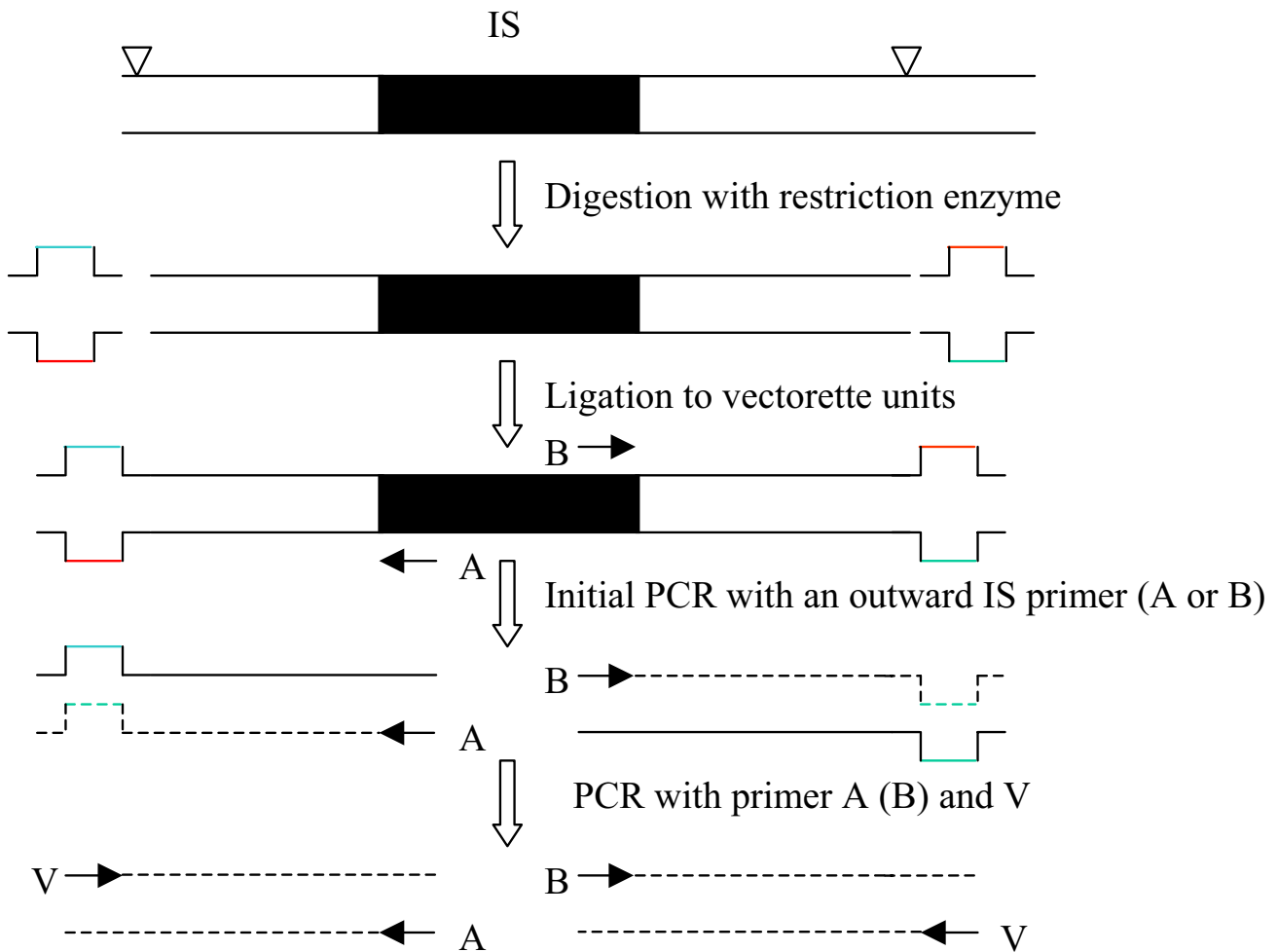


Figure 1
Vectorette PCR for amplification of IS flanking sequences. The shadowed area represents the IS sequence. The solid lines indicate the flanking DNA sequences. ▽ indicates the restriction site. A and B are the outward IS-specific primers located at the ends of the IS. V is a vectorette primer.

IS150

The one known IS150 insertion was identified and no other.

IS186

The three known IS186 insertions were identified based on flanking sequences amplified from both sides (Fig. 2).

Additional IS copies in laboratory strains

Several IS elements located in CGSC6300 are not found in the genomic sequence of MG1655 (Table 1). Lyophilized CGSC6300 was obtained from the *E. coli* Genetic Stock Center, Yale University, and is stored at our laboratory in 15% glycerol at -80°C. It seems likely that the additional

IS transpositions arose after separation from the sequenced MG1655, but prior to arrival in our laboratory, probably during storage on agar slants at room temperature, a condition known to promote IS mobilization [6,7]. These results emphasize that the IS complement of each strain should be characterized prior to experimentation.

Two other *E. coli* K12 derivatives, TD2 and TD10, contain 16 and 18 additional IS insertions (Table 1), respectively. The two additional insertions found in TD10 are: the IS3-a insert between *b0314* and *b0315* and the IS1-b insert associated with a deletion between *b0319* and *b0326*. Originally, TD2 and TD10 were constructed by P1 transduction of different *lac* operons into the *Alac* of K12

Table 1: IS elements in closely related *E. coli* strains MGI655, CGSC6300, TD2 and TD10

Element	start (bp)	end (bp)	Flanking Genes	Direction	A-side		B-side		Strain	
					fragment size ^a	fragment size ^a	MGI655	CGSC6300	TD2	TD10
Known Elements										
IS1-1	19811	20508	b0020-b0023	-	272	199*	P ^b	P	P	P
IS1-2	278402	279099	b0263-b0266	-	174	191*	P	P	P	P
IS1-3	289873	290570	b0273-b0276	-	171*	577	P	P	P	P
IS1-4	1049056	1049753	b0987-b0989	+	281*	139	P	P	P	P
IS1-5	1976542	1977239	b1892-b1895	-	452	226	P	P		
IS1-6	3581114	3581811	b3443-b3446	+	712	1020	P	P	P	P
IS1-7	4516095	4516370	b4293-b4295	+	1354	575*	P	P	P	P
IS2-1	380530	381803	b0359-b0362	+	308	841	P	P	P	P
IS2-2	1465945	1467218	b1401-b1405	-	194	203*	P	P	P	P
b1579 transposase	1649536	1650732	b1578-b1580	+		778	P	P	P	P
IS2-3	2066974	2068247	b1995-b1998	-	287*	444	P	P	P	P
IS2-4	2994394	2995622	b2859-b2862	-	589	219	P	P	P	P
IS2-5	3184203	3185431	b3043-b3046	+	410	672	P	P	P	P
IS2-6	4495795	4497068	b4271-b4274	+	204*	163	P	P	P	P
IS3-1	390963	391829	b0371-b0373	-	489*	142	P	P	P	P
IS3-2	566361	567227	b0540-b0542	+	787	346	P	P	P	P
IS3-3	1093498	1094364	b1025-b1027	-	1009	772	P	P	P	P
IS3-4	2168554	2169420	b2088-b2090	+	502	842	P	P	P	P
IS3-5	314811	315677	b0298-b0300	+	863	158*	P	P	P	P
IS4	4499671	4500999	b4277-b4279	-	629	450	P	P	P	P
IS5-1	273325	274341	b0258-b0260	-	489*	497	P	P	P	P
IS5-2	573960	574976	b0551-b0553	-	590	1605	P	P	P	P
IS5-3	687220	688236	b0655-b0657	-	348	420*	P	P	P	P
IS5-4	1394100	1395116	b1330-b1332	+	569*	471*	(P) ^c	(P)	(P)	(P)
IS5-5	1425770	1426750	b1369-b1371	-	491	1543	P	P	P	P
IS5-6	2064327	2065343	b1993-b1995	-	747	1210	P	P	P	P
IS5-7	2099917	2100933	b2029-b2031	-	306	303	P	P	P	P
IS5-8	2287085	2288101	b2191-b2193	-	314*	411	P	P	P	P
IS5-9	3128193	3129209	b2981-b2983	+	1085	672	P	P	P	P
IS5-10	3363337	3364353	b3217-b3219	-	207*	1126	P	P	P	P
IS5-11	3649812	3650828	b3504-b3506	-	734*	1335	P	P	P	P
IS30-1	269827	270978	b0255-b0257	+	720	314	P	P	P	P
IS30-2	1467382	1468533	b1401-b1405	+	305	297	P	P	P	P
IS30-3	4505034	4506185	b4283-b4285	-	405	766	P	P	P	P
IS150	3718309	3719678	b3556-b3559	+	195	176	P	P	P	P
IS186-1	15445	16557	b0015-b0017	+	503	1501	P	P	P	P
IS186-2	607288	608400	b0581-b0583	+	2204	417	P	P	P	P
IS186-3	2512345	2513463	b2393-b2395	+	764	667	P	P	P	P
Additional Elements										
IS1-a	257908		b0240	-	1488	738		P		
IS1-b	335460		b0319-b0326	-	609	309				P
IS1-c	1871063		b1786	-	1211	484		P		
IS1-d	2037484		b1970-b1971	+	617*	1066			P	P
IS1-e	2623548		b2502	+	282*	368			P	P
IS1-f	2768501		b2635	+	1608	544*		P	P	P
IS1-g	3275070		b3130	+	529	2117			P	P
IS1-h	4539642		b4313	-	175*	1119			P	P
IS2-a	1588558		b1506	+	765	717			P	P
IS2-b	2927499		b2796	+	138	368			P	P
IS3-a	331175		b0314-b0315	+	1002*	563				P
IS3-b	838769		b0805	+	450	870*		P	P	P
IS3-c	2460317		b2351	+	489*	1642			P	P
IS3-d	3427623		b3280	-	953	646			P	P
IS3-e	3776882		b3604	-	1435	935*		P	P	P
IS3-f	4466303		b4242	-	640	935*		P	P	P

Table 1: IS elements in closely related E. coli strains MG1655, CGSC6300, TD2 and TD10 (Continued)

IS5-a	1102866	b1040-b1041	+	365	400		P	P
IS30-a	4115565	b3927	-	692	186		P	P
IS30-b	2246187	b2156	-	303*	445	P	P	P
IS186-a	4541184	b4314	-	606	534		P	P

a * indicates a fragment not recovered. b P, present. c Neither IS5-4 fragment is detected. Conventional PCR of gDNA confirms IS5-4 is present.

derivative strain DD320 [18]. The IS insertion differences between these two strains probably arose when sequences flanking the lac operon were cotransduced during strain construction.

Reliability of technique

Theoretically, the number of flanking DNA fragments amplified with each IS-specific primer should equal the number of copies of each IS element in the genome. Also, the location of each IS copy should be identifiable from the two flanking DNA sequences. However, some copies of IS elements 1, 2, 3 and 5 were initially located by a single flanking sequence only. DNA fragments not recovered may have been masked by fragments of similar size, amplified from other genomic copies of the IS element. This is evidenced by bands in ethidium-stained agarose gels appearing broader and/or staining more intensely (see Fig. 2). While these bands produce clearly readable sequence in the ISs themselves, their flanking sequences are unreadable or show high noisy background, indicating the presence of multiple fragments of similar size (data not shown). In the case where flanking sequences were readable, we located one of the fragments – presumably the one that was amplified most efficiently.

Despite missing fragments, vectorette PCR provides a reliable estimate of the copy number of elements in a genome. Let the number of copies of the *i*th IS element be *n_i*, and the number of unidentified flanking sequences be *u_i*. Then the probability that an IS copy is not identified is simply a product of the probabilities of not obtaining either the A-side or the B-side sequences, *q_i* = (*u_i/n_i*)_{A-side} · (*u_i/n_i*)_{B-side}. The expected number, *x*, of missing copies is determined by summing over all *n_i* copies of each of the *j* = 8 elements in MG1655. Our data provide an estimate of *x* = ∑_{*i*} *n_i* · *q_i* = 2.54 expected missing copies. In fact, only 1 copy was missed entirely. Even when digested by just a single four-cutter restriction enzyme, vectorette methodology is highly reliable with small error rates: 6.8% expected and 2.7% realized.

The actual error rates are even smaller. Our analysis is restricted to the 37 ISs found in the genomic sequence of MG1655; the 7 additional ISs in CGSC6300 were not used

in the calculations even though they may serve to mask fragments and thereby increase the expected and observed error rates.

To determine the reliability of the technique when there are many more than 11 copies of an IS element in a genome requires estimating *m*, the maximum number of amplified fragments likely to be resolved per lane by agarose minigel electrophoresis. Only a small portion of the resolving power of an agarose gel is actually used because approximately 98% (approximately because the calculation $(1/4)^4 \sum_{bp=1}^{1000} (1 - (1/4)^4)^{bp-1}$ assumes equal base frequencies) of amplified fragments produced by a 4-base cutter restriction enzyme are less than 1 kb (excluding the IS and the vectorette). Hence, *m* is less than the maximum number of fragments physically capable of being resolved by agarose minigel electrophoresis.

Consider *m* as the number of discrete positions that an amplified fragment might occupy. The probability that a particular position is not occupied given *n_i* copies of an IS element *i* is $(1 - 1/m)^{n_i}$. The expected number of unoccupied positions is $m(1 - 1/m)^{n_i}$ and the expected number of occupied positions (i.e. bands visualized) is $f_i = m(1 - (1 - 1/m)^{n_i})$. Use *f_i* as an estimate of the number of amplified fragments identified by sequencing. Nonlinear regression of fragments identified, $f_i = m(1 - (1 - 1/m)^{n_i})$, against the number of known genomic copies, *n_i*, yields an estimate of *m* = 11.64 ± 1.79 (Fig. 3A). As a practical matter, no more than a dozen amplified fragments is ever likely to be resolved by agarose minigel electrophoresis when a four-cutter restriction enzyme is used to digest genomic DNA.

Summing the expectations for missing A-side and B-side fragments (i.e. amplified fragments not identified by sequencing) for the *j* = 8 species of IS elements in MG1655 yields

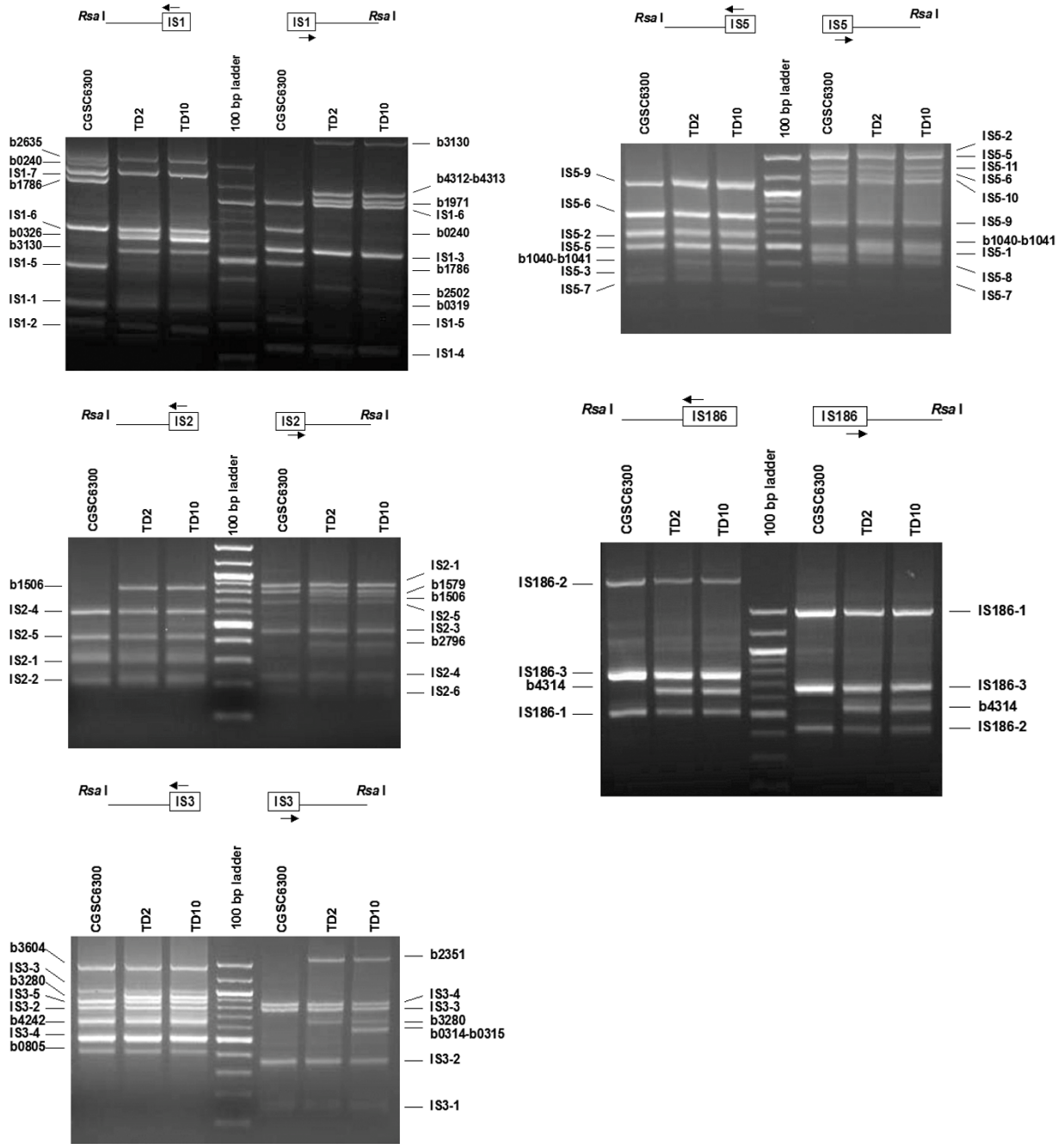


Figure 2
PCR amplification of IS flanking DNA from *E. coli* strains CGSC6300, TD2 and TD10. Results for IS1, 2, 3, and 5 and 186 are shown. Genomic DNA was digested with *Rsa* I, ligated with vectorette units and amplified by vPCR. Each panel shows the PCR products generated by two outward IS-specific primers (arrows) of an IS in combination with the vectorette primer. Flanking DNA fragments from both sides of each IS location were amplified. The PCR products were excised, purified, sequenced and identified from the genome sequence of *E. coli* strain MG1655 [17]. A PCR fragment flanking a known IS site in MG1655 is indicated by the element's name followed by an identifying numeral; for example, IS1-1 is one of 7 IS1 elements in the MG1655 genome. Additional flanking DNAs not found in MG1655 are labeled with the b# of the gene in which the IS is located. PCR products were separated in 1.4% agarose gels and stained with ethidium bromide. Intense bands in the 100 kb ladder correspond to 500 and 1000 bp.

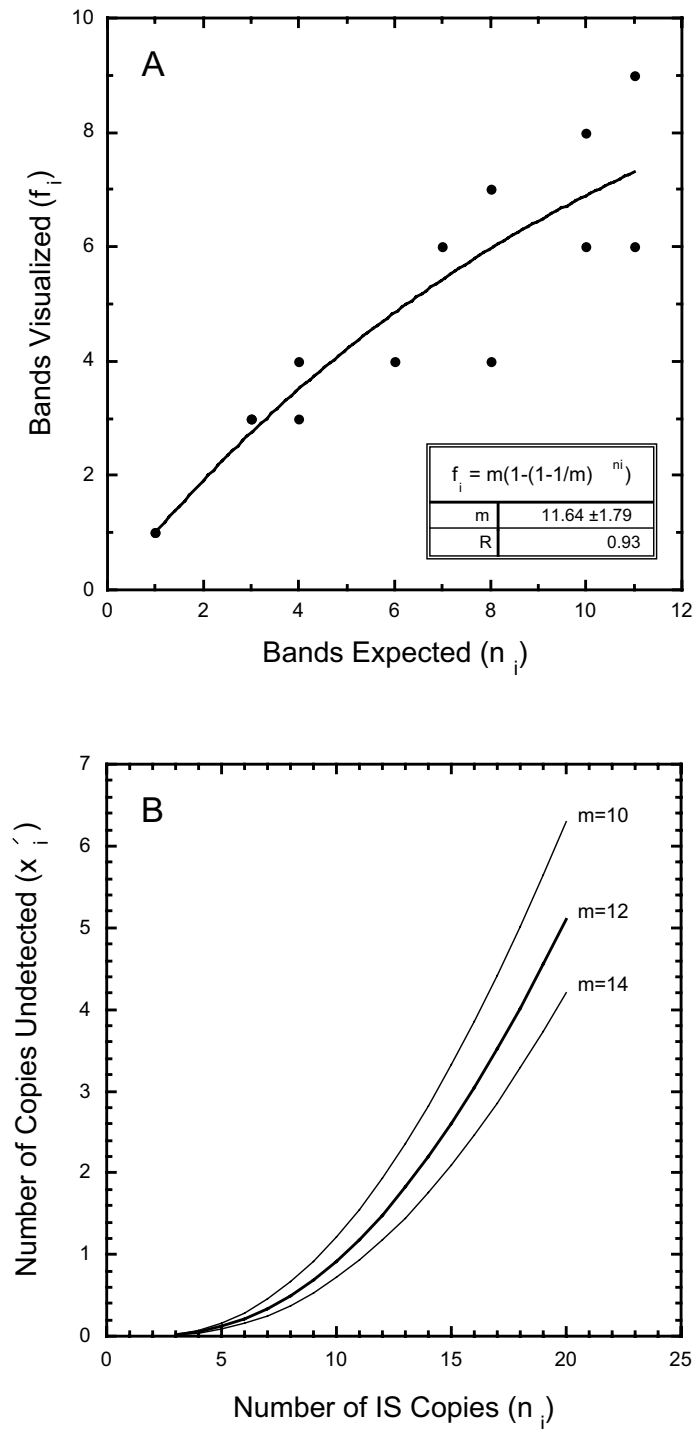


Figure 3

Estimation of IS flanking DNA likely to be resolved and missed. A. The maximum number of fragments likely to be resolved, m , can be estimated by plotting the number of bands observed against the genomic copy number. Only a finite number of bands can be visualized on a gel. Consequently, the likelihood that two amplified fragments comigrate increases with the number of IS copies in the genome. **B.** The number of amplified flanking sequences likely to be missed rapidly increases when 10 or more bands are visualized. Genomic digests with a single restriction enzyme should be restricted to IS elements with fewer than 10 copies per genome. Genomes with more than 10 copies of an IS element should be screened using high resolution agarose gels and/or using a second restriction enzyme to allow all IS copies to be identified.

$\sum_i^j (n_i - f_i)_{A-side} + \sum_i^j (n_i - f_i)_{B-side} = 21.43$ which is slightly larger than the 17 known masked fragments from MG1655 (each marked with an asterisk in Table 1). The probability that an *IS* copy is not identified is $q'_i = (1 - f_i/n_i)_{A-side} \cdot (1 - f_i/n_i)_{B-side}$, where the prime designates that this expectation is based on an ability to resolve a maximum of $m = 12$ fragments per lane. The expected number of missing *IS* copies is $x' = \sum_i^j n_i \cdot q'_i = 3.02$, which is only slightly larger than the direct estimate $x = 2.54$. We conclude that the model provides a robust fit.

A plot of x'_i against n_i (Fig. 3B) reveals that the number of missing fragments increases rapidly with the number of genomic copies. With $n_i = 20$ $x'_i = 5$ copies (25%) remain undetected, and even with $n_i = 10$, $x'_i = 1$ (10%) is expected to be overlooked. To avoid underestimating the number of copies of a highly repeated element, we recommend digesting genomic DNA with a different restriction enzyme and repeating vPCR and sequencing. By using another four-base cutter restriction enzyme *Bst* UI, we identified all flanking sequences not recovered with the enzyme *Rsa* I for *IS1*, *IS2*, *IS3*, and *IS5*, as showed in Fig. 4 for *IS2*. Larger, temperature controlled high resolution agarose gel electrophoresis apparatus available in some laboratories would also improve resolution of the technique.

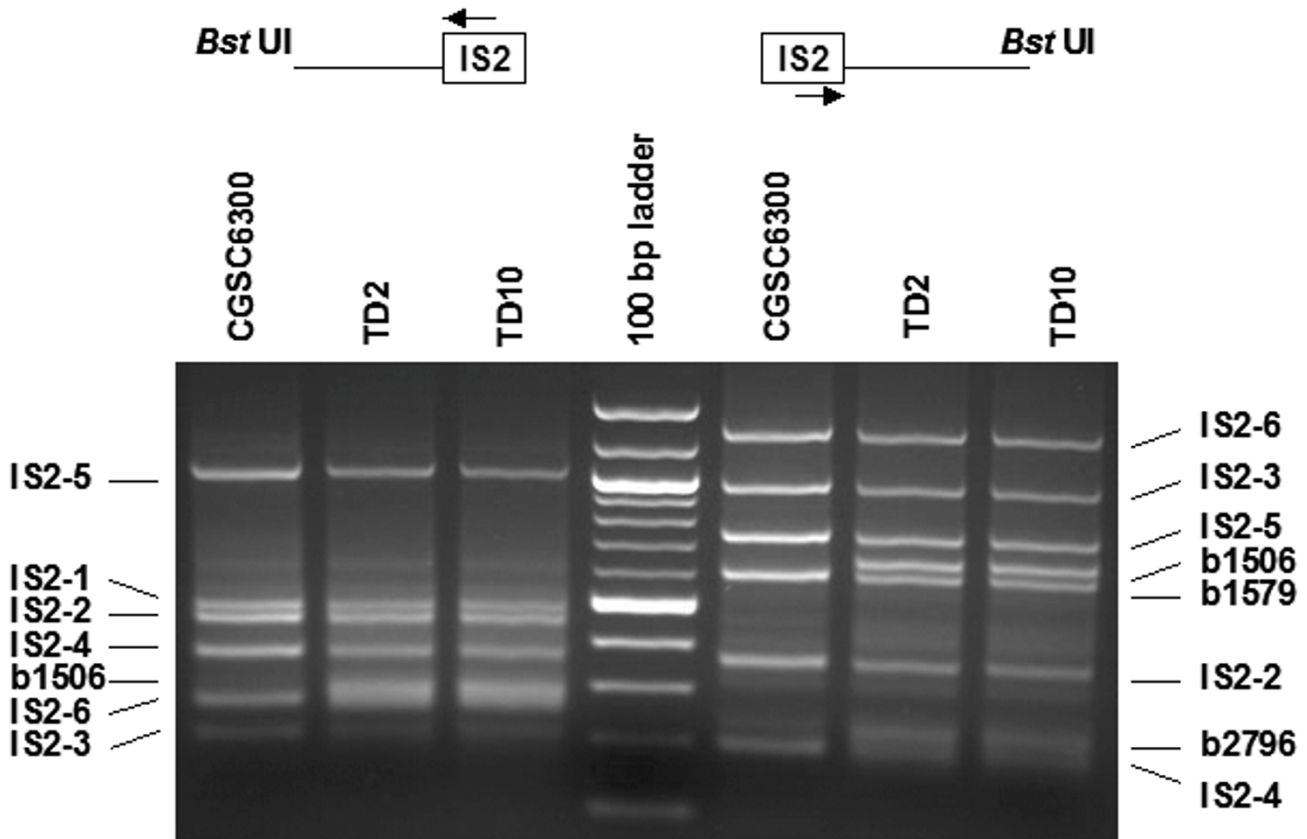


Figure 4
PCR amplification of *IS2* flanking DNA from genomic DNA digested with *Bst* UI. Flanking DNA fragments *IS2*-3A and *IS2*-6A (left hand side) and *IS2*-2B (right hand side), masked by other amplified fragments when genomic DNA was digested with *Rsa* I (see Fig. 2), were recovered with *Bst* UI.

Applications

It is apparent that *IS* complements differ among very closely related laboratory *E. coli* K12 derivatives MG1655, CGSC6300, TD2 and TD10. The rapidity with which these differences have evolved suggests that *IS*s may play important roles in experimental evolution. Indeed, adaptation by *E. coli* to novel laboratory environments is often characterized by *IS* element mobilization [4,19-22]. Using vPCR will provide these workers with a comprehensive view of genomic reorganization during laboratory evolution. Using this method, we characterized *IS* elements in 40 isolates which evolved from TD2 and TD10 during chemostats and found a number of *IS*-mediated gene deletions, duplications and transpositions (unpublished data).

Surveys of natural isolates of *E. coli* reveal that the numbers and locations of *IS* elements differ widely among closely related strains, suggesting a brisk turnover of *IS* elements within and among host lineages [6,23-25]. Comparisons of *E. coli* genomic sequences confirm that *IS* elements are commonly associated with chromosomal rearrangements within lineages [17,26,27]. The ability to rapidly and accurately determine the *IS* complement of the genomes of natural isolates is not only desirable from a population genetic standpoint, but vPCR might also facilitate rapid typing of epidemiological outbreaks of pathogens otherwise indistinguishable from related strains. In this regard it is worth noting that *IS* sequences are highly conserved compared with most *E. coli* house-keeping genes [28]. This will greatly aid using vPCR to type strains because only 1 pair of primers is needed for each type of *IS* element.

Conclusions

This study shows that vPCR is a swift, efficient, reliable method for typing microbial strains and identifying and mapping *IS* insertion sites present in microbial genomes. Flanking DNA sequences from 36 of the 37 *IS* elements in the *E. coli* strain MG1655 were recovered by vPCR and confirmed by DNA sequencing. Unlike Southern hybridization and iPCR, our approach involves only one genomic digest and one ligation step. Vectorette PCR is then used to simultaneously amplify all *IS* elements of a given type, making vPCR a rapid and sensitive means to survey *IS* elements in genomes.

Methods

Strains

Three derivatives of the K12 isolate were used in this study. Strain CGSC6300, obtained from *E. coli* genetic Stock Center, Yale University, was used as a control because it is closely related to MG1655 whose entire genome has been sequenced [17]. TD2 and TD10 (deriv-

atives of DD320, itself a K12 derivative) are routinely used in our experiments in molecular evolution [29].

DNA isolation

Genomic DNA was isolated from overnight culture in LB medium using DNAeasy DNA isolation kit (Qiagen, Valencia, CA, USA).

Vectorette unit

The vectorette unit was made using the protocol of Botstein lab <http://genome-www.stanford.edu/group/botlab/protocols/vectorette.html>[30]. The two anchor bubble primers

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3'-GAGAGGGAAGAGAGCAGGCAAGGAATGGAAGCTGTCTGTCGAGGAGAGGAAG-5'
|||||
5'-GACTCTCCCTTCTCGAATCGTAACCGTTGCTACGAGAATCGTGTCTCTCTTC-3'

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were synthesized by the Advanced Genetic Analysis Center at The University of Minnesota, St. Paul. To anneal bubble primers, 4 μ M of each primer (in ddH₂O) were combined in a total volume of 100 μ l. The mixture was incubated at 65°C for 5 minutes, and then MgCl₂ was added to a final concentration of 1–2 mM before cooling down to room temperature.

DNA digestion and ligation of vectorette units

Genomic DNA from each strain was digested using the restriction enzyme *Rsa* I to produce small, blunt-ended fragments (Fig. 1). The enzyme is a four-base cutter and has 0 to 3 restriction sites within open reading frames (orf) of the eight insertion sequences (*IS1*, *IS2*, *IS3*, *IS4*, *IS5*, *IS30*, *IS150*, *IS186*), but does not cut at the extreme ends of each orf. This allows for the design of outward primers to amplify the *IS* flanking sequence for both sides (see below). Digestion was carried out at 37°C overnight in a 50 μ l reaction containing 1 \times NEBuffer (No. 1), 0.5 μ g DNA and 10 units of *Rsa* I. After digestion, 2 μ l of anchor bubble unit, 1 μ l of 10 mM ATP and 1 unit of T4 DNA ligase (New England Biolabs, Beverly, MA) were added and the reaction was incubated for 5 cycles at 20°C for one hour followed by 37°C for 30 min.

Primers and PCR amplification

Outward primers (Table 2) from each end of the 8 *IS* sequences were designed and used for PCR amplification in combination with the vectorette primer (5' CGAATCG-TAACCGTTCGTACGAGAATCGCT 3') (Fig. 1). The distance between an *IS*-specific primer position and the extreme end of the *IS* orf ranged from 16 to 184 bp, which facilitated identifying *IS*-associated PCR products from DNA sequences. PCR reactions were carried out using Qiagen Multiplex PCR kit (Qiagen, Valencia, CA, USA). Each reaction contained 1 \times Qiagen Multiplex PCR Master Mix, 0.2 μ M of outward *IS* primer and vectorette primer and 2 ng of DNA templates (*Rsa* I-digested DNA ligated

Table 2: Primers used for identification of ISs using vectorette PCR

Primer Name ^a	Sequence (5' to 3') ^b	Length (bp) ^c
IS1-A	gttacgcacc acccctcag ta	22
IS1-B	cggaagtgcg tgccttctc aa	22
IS2-A	ggccc taagacatca atcatctg	23
IS2-B	tcgctcg ccacgggaat atctg	22
IS3-A	agccg ctgcgggcca cccggagcac	25
IS3-B	ggcct cagtcgggaa caatttga	23
IS4-A	cgagagatgag ttcggggtcg agg	24
IS4-B	aaggccttc ccgagagtgg taa	23
IS5-A	gccatggca gaatctgctc catgcggg	27
IS5-B	tgtttcgggc ggacaaaatg ata	23
IS30-A	ccagctcgt atctcctcg gctctg	26
IS30-B	ctagatctgg ttctgctca gc	22
IS150-A	cctgacctgg gttcggggga cac	23
IS150-B	cggaactgaa ggatgctgtt ac	22
IS186-A	ggggcagaat tgctgaccag ttat	24
IS186-B	acctgaac tcgcgaaagc gtggata	25

^aPrimers are named after the insertion sequence with A and B designating each side. ^bSequences obtained from MG1655 [17].

^cLength of primer in base pairs.

with vectorettes). PCR cycling conditions were 95°C for 15 min, 35 cycles of 94°C for 30 s, 60°C for 90 s, 72°C for 2 min, and a final extension step at 72°C for 10 min. The amplified products were separated in 1.4% agarose gel, stained with ethidium bromide and visualized under UV light. DNA bands were excised and purified with Qiagen DNA Gel Extraction Kit (Qiagen, Valencia, CA, USA).

DNA sequencing and analysis

DNA sequencing analysis was carried out on both DNA strands by the AGAC, University of Minnesota, using an IS-specific primer and the vectorette primer. DNA sequences were subjected to BLAST searches against the MG1655 genome sequence.

List of abbreviations

IS: insertion sequences; iPCR: inverse polymerase chain reaction, vPCR: vectorette polymerase chain reaction

Authors' contributions

SZ designed and performed the molecular experiments and prepared the manuscript. AMD provided scientific input and prepared the manuscript. All authors read and approved the final manuscript.

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