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Randomly picked cosmid clones overlap the *pyr*B and *ori*C gap in the physical map of the *E.coli* chromosome

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ABSTRACT

Sets of overlapping cosmid clones generated by random sampling and fingerprinting methods complement data at pyrB (96.5') and oriC (84') in the published physical map of E.coli [7]. A new cloning strategy using sheared DNA, and a low copy, inducible cosmid vector were used in order to reduce bias in libraries, in conjunction with micro-methods for preparing cosmid DNA from a large number of clones. Our results are relevant to the design of the best approach to the physical mapping of large genomes.

INTRODUCTION

There has been widespread interest in the possibility of physically mapping long regions of DNA, and ultimately the entire human genome, by molecular methods. In principle, there are two contrasting approaches. First, restriction enzyme sites, for enzymes such as NotI and SfiI, can be mapped using Southern blotting methods. Extensive maps of the E.coli genome [1] and of several long regions of the mouse [2] and human genome [3] have been analysed by this approach, in conjunction with pulsed-field gel electrophoresis [4]. The other approach is to isolate clones randomly, and, by mapping those with suitable restriction digests, build up "contigs" of overlapping clones. This approach has the advantage that it generates sets of ordered clones, which are available for subsequent biological studies (e.g. identification of genes, controlling elements, study of RFLPs for linkage to genetic loci). Several cloning and mapping projects on small genomes are in progress, specifically on Caenorhabditis elegans [5], Saccharomyces cerevisiae [6] and E.coli K-12 W3110 [7]. The E.coli project has been particularly successful. By combining a random with a directed "walking" approach using λ clones, a detailed restriction map of the entire genome, with the exception of only 8 small gaps, was deduced.

We have attempted to map the E.coli K-12 803 genome as a model project, using entirely random cloning and mapping procedures. With a view to future applications to longer DNA, we have used cosmids as, in theory, fewer analyses are required than are required for phage λ. Because of non-random distribution of clones

Table 1. Vital statistics of the E.coli libraries and the number of clones analysed in each

Cosmid vector	Cloning site	DNA preparations	DNA fragmentation	Library symbol	E.coli host	Nos. clones analysed
pTM	BamHI	Traditional	Sau3AI	T	ED8767 1046 803	900
pDV	ClaI	"	TaqI	D	1046 803 NM554	1000
pDVcosA2	PvuII	"	Sheared	S	NM554	300
lorist b	BamHI	Beads	Sau3AI	L	NM554	600
pDVcos	"	"	"	C	NM554	200
pOU61cos	"	"	"	U	NM554	600

About 1000 clones (from libraries D and L) were pre-matched with the existing computer data base, and only those clones which extended an existing contig were transferred to the main data base.

in our libraries, we have investigated several different cloning strategies and have constructed a new, low copy, inducible cosmid vector, in an attempt to reduce this bias. Our data base, although not as complete as that of others studying the E.coli genome [7], contains clones which overlap 2 of their 8 gaps.

RESULTS

Our computer data base contains 2512 cosmids derived from 6 different E.coli 803 libraries in various vectors and E.coli hosts (Table 1). This includes (1) a sheared DNA library, constructed by a new procedure, incorporating a phosphatase step, and (2) a library in a new low copy, inducible cosmid vector, pOU61cos (see Methods). The clones are arranged in 58 sets or contigs, ranging in length from 40 to 300 kb (results not shown), and we estimate from their cumulative length that they cover 90% of the genome. Nineteen of our contigs have been assigned to genetic loci using oligonucleotide probes by methods similar to those described below (results not shown).

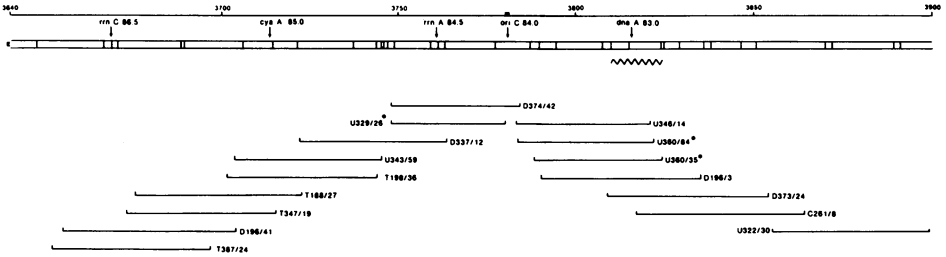

Fig.1 Restriction map of the oriC (84') region. Cosmid clones are represented by horizontal bars and positioned according to their EcoR1 (E) digest pattern; symbols are as in Table 1. Clones with * are contained within a master clone. The scale 3640-3900 kb is from [7]. ~ shows a region of discrepancies in restriction sites between our data and [7]. The solid rectangle shows the gap in [7].

Amongst our contigs, 2 overlap the 84' and 96.4' gaps in the E.coli map [7] and we present these results below.

oriC (84') gap

Figure 1 shows an overlapping set of cosmids covering the oriC region. This contig was positioned by probing individual master clones (see Materials and Methods, Fingerprinting cosmids by HinfI [^{32}P]dCTP labelling, for a definition of "master clone") with cyaA (85.0) and dnaA (83.0) and 5S RNA oligonucleotide probes (see Methods) using both dot blots and Southern blots. Restriction mapping (see Methods) allowed us to align individual clones within this contig to the EcoR1 map [7] and to the restriction map derived from the known sequence of the oriC and unc loci [8]. Some discrepancies (with data in ref.7) in restriction sites were apparent around the dnaA locus

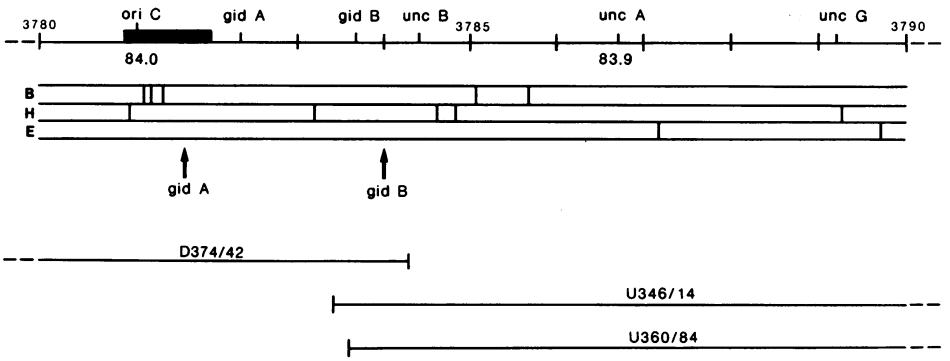

Fig.2 Enlarged map of the oriC (84') region. This shows the restriction map with BamHI (B); HindIII (H) and EcoR1 (E) and the location of the gidA and gidB probes. The solid rectangle represents the gap in [7].

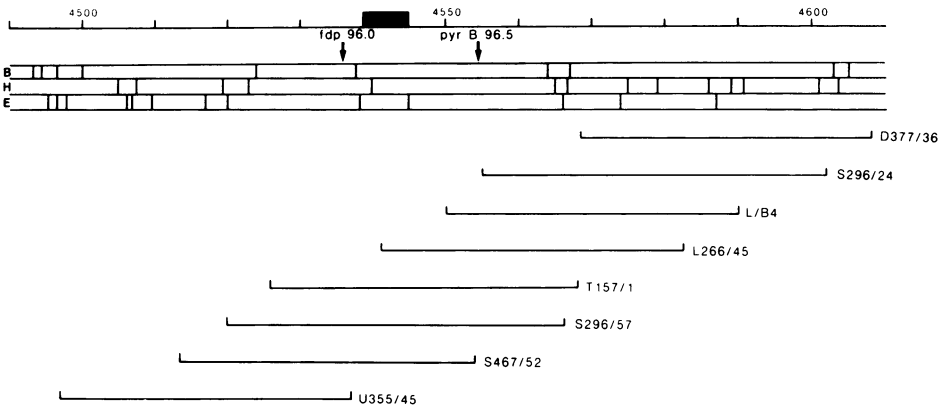

Fig.3 Restriction map of the pyrB (96.5') region. Enzymes as in Fig.2, symbols as in Fig.1. The scale is from [7]. The solid rectangle represents the gap in [7] and is 6 kb long.

and also around the oriC region, implying differences between the E.coli W3110 and 803 strains. Figure 2 is an enlarged view of the central region around oriC showing how hybridization to gidA and gidB oligonucleotide probes was used (see Methods) to determine the 1 Kb overlap of the cosmids as the standard HinfI fingerprint (see Methods) had failed to detect it.

pyrB (96.5') gap

Figure 3 shows another overlapping set of clones covering the gap close to pyrB. This contig was positioned by hybridization to a pyrB oligonucleotide probe and then aligned to the E.coli restriction map [7] by mapping (see Methods). The gap is 6 kb long with the restriction sites as shown.

Frequency of clones around oriC and pyrB

Figures 4 and 5 show histograms of the frequency of clones nearly identical to master clones in the data base around the 2 gaps, showing the distribution for pOUcos61 clones, and all others, separately. In the case of the pyrB gap (Figs.3 and 4) there are 20 independent cosmids covering this gap, 5 of them being derived from pOUcos61. This is the number of clones expected at any locus after sampling 2,500 cosmid clones in an unbiased distribution. In the adjacent (97') region however, there are only 4 master clones with no near identities (see Fig.4). This is a clear example of non-random distribution. In the case of the oriC gap (Figs.1 and 5), there are few clones covering it and the adjacent gid and unc region. The 10 kb between 3,780 and 3,790 kb (Fig.2) is covered by only 3 clones, and that between uncB and uncG by only 2 pOU61cos clones, contrasting with the ease of cloning the immediately adjacent region represented by clones related to 377/12 and 196/3 (Fig.5). In addition, there are other regions on

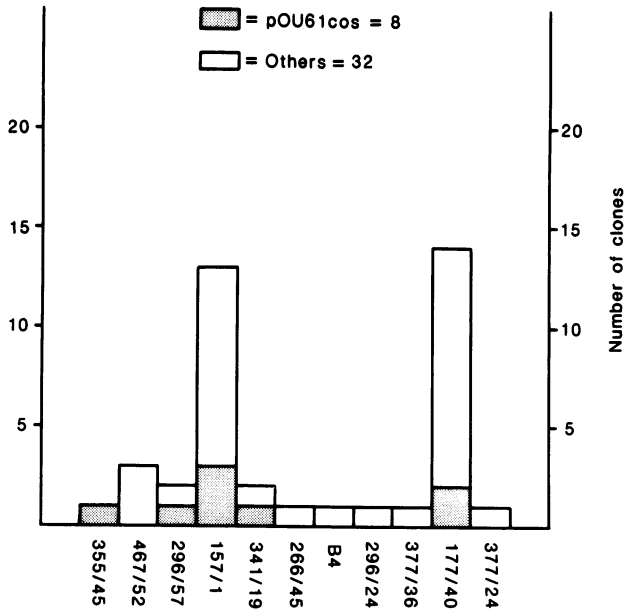

Fig.4 Histogram of distribution of clones in the pyrB region. This shows the number of near identical clones that are contained within each master clone. Shaded area represents pOU61cos clones.

either side where numerous clones were isolated, (e.g. 42 near identities of 176/4, Fig.5).

DISCUSSION

Assuming a total length of the E.coli genome of 4.7 Mbases, the number of cosmid clones one would have to sample to find and overlap a given clone with $p > 99\%$ in a Poisson distribution is 2500. This calculation assumes a $^3/_4$ overlap between clones, which is usually required for our fingerprinting technique. We have examined more than 3,500 randomly picked clones (see Results) and have failed to isolate some regions of the genome. This strongly suggests a biased distribution of clones. Despite this, our database contained clones which overlapped 2 of the 8 gaps in [7] and our studies on the other 6 gaps are in progress.

The gap at 96.4' was represented many times in cosmids and it is surprising that this region failed to clone in phage λ. We estimate that sufficient λ clones were analysed [7] to ensure a $p > 99\%$ of finding a clone for this region, therefore presumably this region was lethal for phage λ in the host strain used [7]. The other gap, at oriC (84')
was overlapped by only 1 cosmid and this was clearly a difficult region to clone both

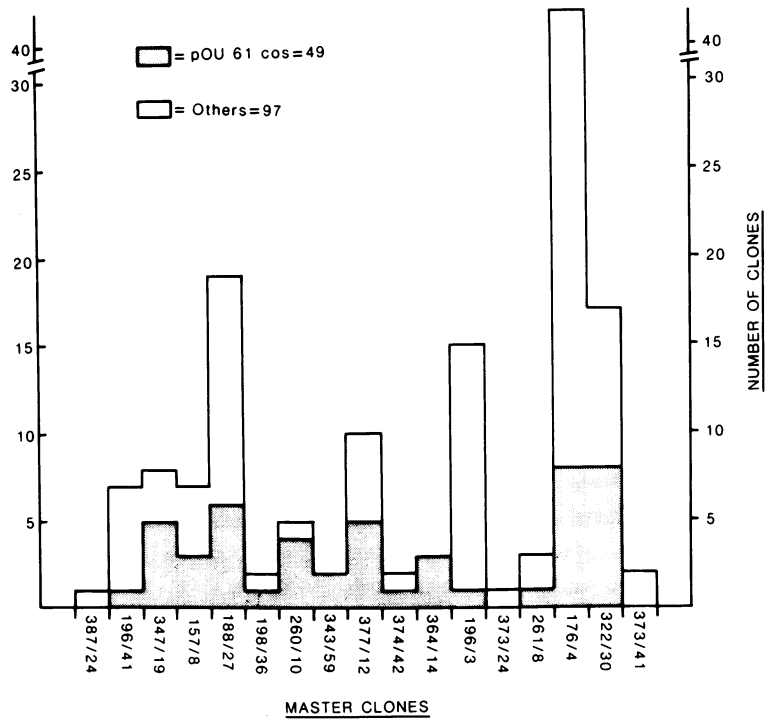

Fig.5 Histogram of distribution of clones in the oriC region. See Fig.4 legend for explanation.

in cosmids and phage λ. Kohara et al [7] suggest this is because of the frequency of Sau3AI sites near oriC and this may be part of the reason as cosmid D374/42 was cloned from a partial TaqI digest of DNA. However, we also failed to find clones overlapping oriC in sheared libraries, so that secondary reasons must be suspected, perhaps related to the high copy vectors. The adjacent unc region is known to be difficult to clone in high copy vectors (F.Gibson, personal communication) and it is interesting that this region (actually to one side of the gap) was only clonable in the low copy vector pOUcos61, based on the R plasmid replicon [9], presumably because the membrane-bound ATP synthetase product of this gene is toxic in high yields.

Our results suggest that a variety of cloning strategies and the use of different cloning vectors, in particular the low copy number, inducible vector pOU61cos, are useful in overcoming difficulties arising from non-random representation in clone libraries. We suggest for mapping longer regions of DNA, that methods should be flexible enough to allow data from phage λ, cosmid, and perhaps even YAC [18] clones to be compared in the same computer database. This will undoubtedly require improved,

Fig.6 Cosmid vector maps. See Methods for construction of cosmid vectors. Restriction enzyme sites are as follows: BamHI (B); ClaI (C); EcoR1 (E); HindIII (H); HpaI (Hp); PvuII (V); SalI (S); XbaI (Xb); XhoI (X). Symbols: —— region derived from pBR322; ▬ region derived from the R1 plasmid; ▨ region derived from λ; ▧ region derived from Tn3; ▧ region derived from Tn5.

automated methods for entering data into the computer, like those recently developed in Cambridge [Sulston, personal communication].

MATERIALS AND METHODS

Cosmid Vectors pTM [10] and lorist b [11] were used for construction of libraries. In addition 3 new vectors were made:

(1) pDV was derived from pLTC (a gift from Dr Grosveld), itself a derivative of pTM, such that an XhoI site replaced the HpaI site in the cos region. The BamHI site was deleted and a BamHI linker cloned into the SalI site. The HindIII site of pLTC was deleted by "filling in" followed by religation. The new plasmid was restricted with EcoR1 and the larger 5.4 kb fragment filled in followed by religation at low concentration, thereby deleting the AGPT region of pTM and forming pDV (5.4 kb) which now lacks an EcoR1 site (Fig.6).

(2) pDVcos and pDVcosA2 The PvuII-XhoI fragment of pDV (containing the cos region, see Fig.6) was cloned into PvuII/XhoI cut pATX (a derivative of pAT153/PvuII/8 [12] which contains the polylinker ClaI, HindIII, PvuII, BamHI, XhoI). This was then cut with XhoI, filled in, cut with ClaI and the resulting fragment (containing cos) cloned back into ClaI/PvuII cut pDV, resulting in a direct repeat of the cos region in the new vector pDVcos. Cloning an XbaI linker into the unique PvuII site of pDVcos gave pDVcos134 (Fig.6). The unique ClaI site of pDVcos134 was deleted by filling in and recircularization and the following polylinker

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 XhoI   EcoRI  BamHI  PvuII   ClaI   HindIII   SalI
TCG AGA ATT CGG ATC CAG CTG TAT CGA TAA GCT TGT CGA
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was inserted between the XhoI and SalI sites to give pDVcosA2.

(3) pOU61cos The amplifiable, low copy, plasmid pOU61 [9] was converted into a cosmid as follows. The 2 kb ClaI-XhoI fragment from pDVcos134 (containing the cos direct repeat) was cloned into the SmaI site of pSP65 [13]. It was then excised with BamHI/EcoR1 and cloned into BamHI/EcoR1 cut pOU61 giving pOU61cos (Fig.6).

Preparation of E.coli DNA High molecular weight DNA was prepared from E.coli 803 (hsdS(rk$^-$, mk$^-$), gal$^-$, met$^-$ [14]) by "traditional" or "agarose bead" methods. In the former, bacteria were grown to late log phase in L-broth at 37°C, chloramphenicol was added to 0.1 mg/ml [15] and growth continued for a further 1 h at 37°C. The cells were centrifuged, cooled, resuspended in lysis buffer (10 mM Tris-Cl, pH 8.0, 10 mM EDTA, 50 mM glucose, 2.5 mg/ml lysozyme) and incubated on ice for 30 min. SDS was added to 1% final concentration, proteinase K to 0.4 mg/ml and the suspension incubated 16 h at 37°C. The resulting solution was gently extracted 3x with phenol

and 3x with phenol/chloroform (1 : 1) and the DNA spooled out after adding 2 vols of ethanol. The DNA was redissolved in TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) at 4°C. This procedure gave DNA fragments of 200-300 kb in length when analysed by pulsed-field gel electrophoresis [4]. In the agarose bead method, after growth of bacteria (as above), cells were washed once in lysis buffer (without lysozyme) and embedded in low melting point agarose [16] at 10^8 cells/ml. The beads were treated with lysozyme (1 mg/ml in lysis buffer as above) for 30 min on ice, washed several times in lysis buffer with 1% SDS at room temperature and incubated 16 hr at 37°C in lysis buffer with 1% SDS containing 0.4 mg/ml proteinase K. Proteins were then extracted by washing 3-4 times with lysis buffer with 1% SDS, followed by washing 6 times in TE and beads were stored in this buffer at 4°C.

Preparation of libraries E.coli 803 DNA was digested partially with Sau3AI or TaqI (Table 1) under conditions which resulted in a wide size range of DNA fragments about a mean of 40 kb [15], followed by size fractionation on a 10-40% sucrose density gradient in 50 mM NaCl, 0.5 mM EDTA, 50 mM Tris-Cl pH 8.0, 0.25% SDS at 26K for 18 hrs at 20°C in an SW28 rotor. After collection and identification of the 35-50 kb fragments [15], the pooled DNA was treated with calf intestinal phosphatase prior to ligation with vector. For partial digestion of agarose bead preparations of DNA, the beads were equilibrated with appropriate buffer and the DNA partially digested with Sau3AI at 6-8 different concentrations of enzyme. The DNA was extracted from the beads by melting the agarose at 65°C, diluting to <0.1% agarose, extracting with phenol/chloroform (1 : 1) and precipitating with ethanol prior to size fractionation as above.

To prepare sheared, blunt ended, DNA for cloning, 0.1-0.2 mg DNA prepared by "traditional" methods in 3 ml TE in a 10 ml B.D. syringe was forced as hard as possible, by hand, through a 25 gauge $^3/_8$ B.D. syringe needle into a 50 ml Falcon centrifuge tube, giving sheared aliquot A. 1.5 ml of this aliquot was further sheared by repeating the above, giving aliquot B. The size of the sheared DNA in each aliquot was assayed by 0.4% agarose gel electrophoresis using λ (48.5 kb) and fragments (33.5 kb, and 15 kb) of λ produced by restriction with XhoI as markers. Usually, DNA ranged from 15 > 50 kb and both aliquots were pooled. Sometimes, the DNA was larger, in which case further shearing was carried out as above; sometimes, the DNA was too small, in which case less pressure was applied on the syringe plunger, when forcing the DNA solution through the fine needle. DNA was ethanol precipitated and digested with calf-intestinal phosphatase in TE in a volume of 800 µl with 40 µg of enzyme for 15 min at 37°C. After heat inactivation (10 min at 70°C) and phenol/chloroform extraction, DNA was precipitated with ethanol and redissolved in 300 µl TE. This solution was treated with 6 units of T4 DNA polymerase (Amersham) using conditions recommended by the manufacturers

in a volume of 1 ml. After 1 hr at 37°C, the work up was exactly the same as above for the calf intestinal phosphatase reaction. DNA was finally dissolved in 0.4 ml TE containing 0.25% SDS for sucrose density gradient centrifugation to purify the 35-50 kb fragments as above.

To prepare arms from pDVcosA2 or pOU61cos, DNA was digested with XbaI to completion, treated with calf intestinal phosphatase and then cut with the desired cloning enzyme (see Table 1). For pDVcos, PvuII replaced XbaI in the arms preparation. After ligation at high DNA concentration, DNA was packaged in vitro using extracts of E.coli 2688 and 2690 [15] and phage stored at 4°C over chloroform. When required, aliquots were used to infect E.coli 1046 (recA$^-$, supE, supF, hsdS, met$^-$ [15]) or E.coli ED8767 (hsdS, supE,supF, recA56, met$^-$ [17]) or E.coli NM554 (sup^o, hsdR, recA13 - a gift from Dr N. Murray, who constructed this as a recA$^-$ derivative of MC1061), or E.coli 803 (see above) and plated at low density on L-plates containing 50 µg/ml ampicillin (or 30 µg/ml kanamycin in the case of lorist b vectors).

Cosmid DNA preparations For mini preparations of DNA from clones, 1-2 ml cultures of individual clones were grown for 16 hr in 2 x TY medium containing 100 µg/ml ampicillin (or 50 µg/ml kanamycin for lorist b clones) at 37°C with vigorous shaking. Miniprep DNA was prepared by the alkaline lysis method. For micropreps, clones were grown in 0.5 ml 2 x TY, 100 µg/ml ampicillin in 1 ml tubes (Micronics, Flow Laboratories) at 37°C for 16 h, usually handling 192 (i.e. 2 x 96) clones per experiment. 50 µl of culture was withdrawn using a multihead pipette with sterile disposable tips, and mixed with an equal volume of 30% glycerol/2 x TY medium in a 96-well sterile plate and immediately frozen at -70°C. The remaining cells were centrifuged in a 96-well plate (U-well, Sterilin bacteriological) and suspended in 30 µl lysis buffer (without lysozyme). 60 µl 0.2 N NaOH, 1% SDS was added, the plate incubated at room temperature for 5 min, 45 µl of 3 M potassium acetate, pH 5.2 added and the plates placed at 4°C for 1 h before pelleting in a MSE 4 L centrifuge at 1 K for 20 min. The supernatant was transferred with a multihead pipette into the wells of a clean 96-well plate and centrifuged again before transferring into wells of another 96-well plate containing an equal volume of isopropanol to precipitate the DNA. The DNA was pelleted by centrifugation, the pellets washed in 70% ethanol and dissolved in 50 µl TE, yielding 0.2-1 µg cosmid DNA. A special procedure was used to amplify the cosmid DNA in pOU61cos clones. They were first grown in 250 µl 2 x TY medium at 30°C for 16 h and glycerol stocks made as above. 10 µl of the overnight growth was used to infect 250 µl of fresh medium and growth continued at 30°C for 5-6 hours (until late log phase). The cultures were then heat shocked at 42°C for 15 mins in a waterbath, a further 250 µl of fresh medium added and growth continued for 16 hr at 37°C. Micropreps were as above.

Fingerprinting cosmids by HinfI (^{32}P) dCTP labelling 96 clones were routinely analysed together and the reactions carried out in a 96 U-well microtiter plate (Sterilin bacteriological). 4 µl reaction mixture containing 10 mM Tris-Cl pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl, 1 unit HinfI, was aliquoted into the pre-cooled wells (plate held on ice) with a Hamilton repetitive dispenser. 1 µl cosmid DNA (4-20 ng) was added to each well, the plate sealed with a self-adhesive plate sealer (Titertech, Flow Laboratories) and incubated at 37°C for 60 min. 2 µl of labelling mixture [10 mM Tris-Cl pH 7.4, 10 mM $MgCl_2$, 6 mM dithiothreitol, 0.1 mM of each of dATP, dGTP and dTTP, 0.2-0.4 µCi ^{32}P-dCTP (Amersham, 3000 Ci/mMole), 0.1 unit Klenow subfragment of DNA polymerase] were added to each well, incubated for 10 min at room temperature and the reaction stopped by adding 7 µl formamide dyes [15]. The DNA was denatured just before electrophoresis by heating the unsealed plate in an 80°C oven for 20 min. The gels were 40 cm x 40 cm x 0.2 mm, 6% denaturing polyacrylamide (38 : 2 of acrylamide : bisacrylamide, 7 M urea, 1 x TBE) and the gel was bonded to one plate with methacryloxypropyl trimethoxysilane (Wacker Chemie, Munich) to permit drying of the gel on the glass plate without distortion. 0.5-1 µl of reaction mixture was loaded into each of 48 wells and electrophoresis carried out at 15 W for 2 hours, or until the bromophenol blue dye reached the bottom of the gel. The gel was fixed in 10% acetic acid for 20 min., washed in tap water for 20 min., dried in an 80°C oven (1 hr) and autoradiographed for 2-3 days at room temperature without intensifying screen on Kodak S X-ray film. The position of restriction fragments between 25-220 bp long was digitised into a computer database using marker tracks (in every 6th lane) as reference. Computer programs [5] were used for matching and for graphic presentation of overlaps. Identical or near identical clones (as judged from their HinfI restriction fragment pattern) were assigned to one arbitrarily chosen member of the set (usually the one with the greatest number of restriction fragments), which was called the "master clone". This strategy avoided unnecessary duplication of information in the computer programs [5] designed to show overlaps graphically.

Restriction mapping of overlapping cosmids 5-20 ng (1 µl) cosmid DNA was digested with BamHI or HindIII or EcoR1 in a 15 µl reaction in the recommended buffer (Amersham) at 37°C for 2 hours. The resulting DNA fragments were end-labelled with ^{32}P dATP by adding 5 µl of the following mixture [10 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 6 mM DTT, 0.1 mM dGTP, dCTP, dTTP, 0.2-0.5 µCi ^{32}P-dATP (3000 Ci/mmole), 0.1 unit Klenow polymerase] and incubating for 10-15 min at room temperature. The reaction was stopped by adding 4 µl 30% glycerol, 10 mM EDTA + bromophenol blue. 10 µl of the reaction mixture was loaded on a 0.4% agarose gel (18 cm x 25 cm x 0.8 cm) and electrophoresis carried out at 28-30 mA in TBE for 16 h. The gel was then washed 2 times in deionized water for 30 min, blotted and dried down in a gel dryer (Pharmacia)

and autoradiographed. λ DNA separately restricted with XhoI and HindIII, and each labelled as above, was used as size markers. The restriction bands were ordered by their appearance and disappearance in the ordered set.

Oligonucleotide probes were synthesized from a knowledge of the sequence at their respective loci (see EMBL database). For 5S rRNA - GTGGTCCCACCTGACCCCAT; for the pyrB locus - TAGCCGTTCGCTTTCACACT; for the cyaA locus -TAACATCCT TGCCAGAGTGA; for the dnaA locus - AAGATCTCTTGCGCAGTTTA; for the gidA locus - CCTGACGGTAGAGCACACGA; for the gidB locus -TCACCACCAGATGACGT TCG. These were labelled with ^{32}P-ATP and T4 polynucleotide kinase and used to probe dot blots and Southern transfers using standard techniques [15].

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