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A physical genome map of *Pseudomonas aeruginosa* PAO

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A complete macrorestriction map of the 5.9 Mb genome of *Pseudomonas aeruginosa* PAO (DSM 1707) was constructed by the combination of various one- and two-dimensional pulsed field gel electrophoresis techniques. A total of 51 restriction sites (36 *Spe*I sites, 15 *Dpn*I sites) were placed on the physical map yielding an average resolution of 110 kb. Several genes encoding virulence factors and enzymes of metabolic pathways were located on the anonymous map by Southern hybridization. Distances between the gene loci were similar on the genetic and physical maps, suggesting an even distribution of genome mobility throughout the bacterial chromosome. The four rRNA operons were organized in pairs of inverted repeats. The two-dimensional macrorestriction techniques described herein are generally applicable for the genome mapping of any prokaryote and lower eukaryote which yields resolvable fragment patterns on two-dimensional pulsed field gels.

Key words: genome map/*Pseudomonas aeruginosa*/pulsed field gel electrophoresis/rRNA operons

Introduction

Bacteria of the genus *Pseudomonas* occupy a dominant position in the biosphere in terms of the variety of habitats from which they can be isolated; they are found in large numbers in soil, freshwater and marine environments and some are found in association with plants and animals. The species *P.aeruginosa* is a metabolically versatile organism that is of increasing biotechnological and medical interest (Sokatch, 1986). This saprophytic microorganism has the genetic potential to dissimilate a wide range of organic molecules left in the biosphere by natural and synthetic routes (Clarke and Ornston, 1975). For example, bacteria are able to degrade aliphatic and aromatic hydrocarbons from industrial waste.

Although *P.aeruginosa* is ordinarily a soil and water organism, it is in addition an important opportunistic pathogen for animals, plants and man (Sokatch, 1986). Due to its high antibiotic resistance it has become an important etiological agent for severe nosocomial infections and sepsis. In cystic fibrosis, the most common fatal genetic disease in Caucasian populations, the chronic lung infection with mucoid, alginate-producing strains of *P.aeruginosa* is a major cause of morbidity and reduced life expectancy (Devault *et al.*, 1989).

For the genetic and molecular analysis of the metabolic versatility and pathogenicity of *P.aeruginosa*, a knowledge of the genome organization is essential. A chromosome map of the *P.aeruginosa* reference strain PAO has been generated by mapping of auxotrophic markers using the established mechanisms of gene exchange in bacteria, i.e. transduction, transformation and conjugation, and, more recently, transposon mutagenesis and gene cloning (Holloway and Matsumoto, 1984; O'Hoy and Krishnapillai, 1987).

In order to facilitate the genetic analysis of the organism at the molecular level, the existence of a physical map based on long-range restriction sites would be advantageous. To date, complete macrorestriction maps from bacteria are available for *Escherichia coli* K12 (Smith *et al.*, 1987) and the smaller genomes of *Haemophilus influenzae* (Lee *et al.*, 1989) and two *Mycoplasma* species (Bautsch, 1988; Pyle and Finch, 1988). Here we present a complete physical genome map of *P.aeruginosa* PAO (DMS 1707), constructed mainly by various two-dimensional pulsed field gel electrophoresis (PFGE) techniques.

Results

Rare restriction sites in the P.aeruginosa chromosome

Long-range restriction mapping of bacterial genomes requires restriction endonucleases that cut only rarely in the chromosome. Criteria for the selection of appropriate enzymes are GC content, oligonucleotide frequency and the degree of methylation of the organism to be mapped. In the GC-rich *P.aeruginosa* genome (67 mol% GC) the tetranucleotide CTAG occurs more than an order of magnitude less frequently than calculated from mononucleotide frequencies (McClelland *et al.*, 1987; Bautsch *et al.*, 1988). Hence, any restriction enzyme containing only A,T or A,T plus CTAG in its recognition sequence is a promising candidate for an appropriate rare-cutter. In our PFGE analysis on the *P.aeruginosa* PAO (DSM 1707) chromosome, the number of restriction sites increased from ~40 to >100 sites in the order *Spe*I (5'-ACTAGT), *Asn*I (5'-ATTAAT), *Dra*I (5'-AAATTT), *Xba*I (5'-TCTAGA), *Ssp*I (5'-AATATT). Moreover, we found that *Dpn*I (5'-G^{m}ATC) cuts the strain PAO DNA at only very few recognition sites: separation of a *Dpn*I single digest by PFGE (Figure 1B) yielded 15 fragments in the range from 806 to 86 kb (Table I).

One-dimensional PFGE of a single *Spe*I digest resolved 33 fragments SpA to SpZ from 517 to 23 kb in size when 0.25 μg DNA was applied per lane (Figure 1A). Under these conditions resolution was optimal in the mol. wt range above 50 kb, and in particular the seven double bands SpD, -I, -L, -N, -R, -W and -X could be clearly discriminated from single bands by ethidium bromide staining (Figure 1A). However, fragments <20 kb in size contained <1 ng of DNA and could not be reliably detected. For visualization of fragments smaller than SpZ, 10–30 μg of *Spe*I-digested

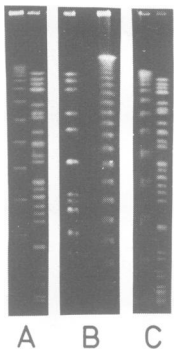

Fig. 1. Separation of *Spe*I (**A**), *Dpn*I (**B**) and *Spe*I/*Dpn*I(**C**) digests of the genome of *P.aeruginosa* PAO by CHEF. Pulse times were linearly increased in two ramps: 3−15 s for 8 h, 1−33 s for 30 h (**A**); 5−40 s for 14 h, 1−80 s for 29 h (**B**); 3−15 s for 15 h, 1−27 s for 23 h (**C**). The size markers are oligomers of λ cI857 Sam7, the lowest band in (A) and (C) being the monomer, and in (B) the dimer.

Table I. Sizes of restriction fragments of *P.aeruginosa* PAO (DSM 1707) DNA

Fragment	Fragment size (kb)	
	*Spe*I	*Dpn*I
A	517	806
B	451	757
C	412	696
D	377[a]	580
E	326	506
F	304	394[c]
G	293	302
H	258	290
I	234[b]	269[c]
J	211	215[c]
K	201	169
L	169[c]	86
M	160	
N	147[a]	
O	133	
P	126	
Q	114	
R	82[d]	
S	57	
T	50	
U	45	
V	40	
W	34[e]	
X	30[e]	
Y	25	
Z	23	
AA	19	
AB	15	
AC	7	
Total	5933	5948

The average absolute error of the size determination is ~ ±6 kb for fragments <600 kb and ~ ±10 kb for fragments >600 kb. The systematic error of size calibration by comparison with yeast chromosomes and λ oligomers as markers is maximal 10 kb. This uncertainty reflects the influence of DNA concentration on mobility in PFGE (Grothues *et al.*, 1989).
[a]Double band of nearly identical size; [b]doublet, fragments differ by 3.4 kb; [c]doublet, fragments differ by 2.4 kb; [d]doublet, fragments differ by 2.0 kb; [e]doublet of identical size.

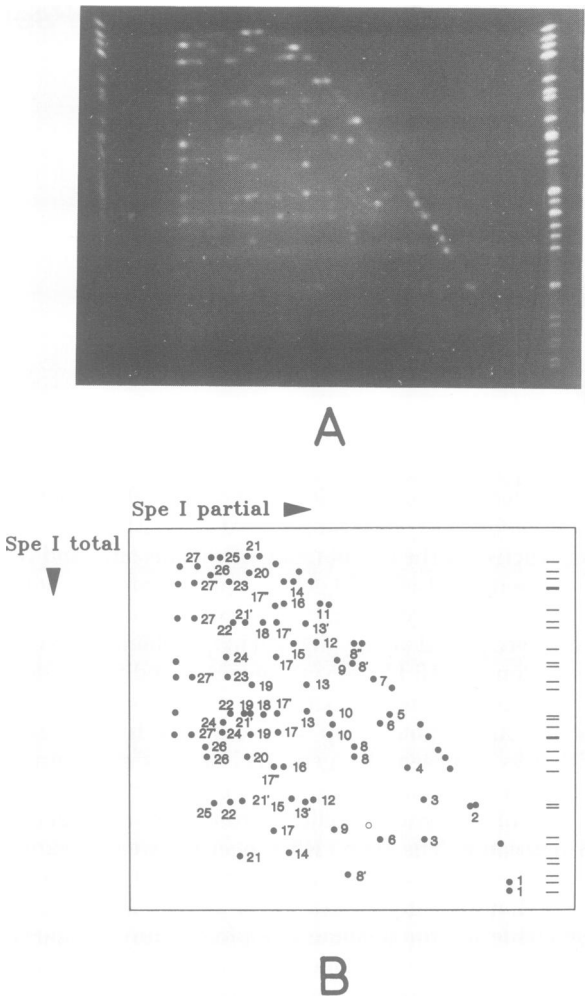

Fig. 2. Two-dimensional gel of a partial−complete *Spe*I restriction digest. After cleavage of agarose-embedded DNA with 0.5 U *Spe*I for 4 h,the fragments were separated by CHEF in two linear ramps (1−20 s, 8 h; 1−70 s, 30 h), redigested to completion, and separated in the second dimension under conditions as in Figure 1(A). Complete *Spe*I digests were applied as size markers to the outermost lanes. In the diagram (**B**) of the gel (**A**) the corresponding spots of a lane are identified by the same number.

DNA was run in separate PFGE experiments with shorter pulse times. The fragments listed in Table I add up to a comparable genome size of 5933 kb for the *Spe*I digest and 5948 kb for the *Dpn*I digest; for mapping, we have assumed a genome size of 5940 kb.

Mapping strategy

We attempted to construct the genome map entirely by physical methods. The following strategies were employed.

Two-dimensional restriction fragment analysis of partial and complete single digests. A partial digest of the genomic DNA with one enzyme was separated by PFGE followed by redigestion to completion with the same enzyme and separation by PFGE in the second dimension (Figure 2). The fragments that were linked to each other were directly read off from the two-dimensional gel. Fragments were identified by comparison with a complete single digest in the outermost lane. The mol. wt of a partial digestion fragment was

determined by extrapolation of the straight line of linked fragments on the first dimension. Completely cleaved fragments located on the upper contour line served as mol. wt markers. Any difference in size between the fragments of the partial digest and the sum of visible fragments in the respective lane provided indirect evidence for linkage with additional tiny fragments which do not contain enough DNA to be visualized. In case of overlaps between partial digest lanes, the corresponding spots were identified by mass law and fluorescence intensity as further criteria.

Reciprocal pairs of complete double digests (Bautsch, 1988). Two complementary PFGE gels with complete double restriction digests and reverse order of the two digestions were run. Identical spots on both two-dimensional gels were identified by mol. wt and assigned to the respective fragments of the first dimension which must therefore overlap on the map. Linking fragments were only present in the one-dimensional separation of double, but not of single digests. Fragments that do not contain the restriction sequence of the second enzyme were located on the contour line and were identified by comparison with the respective single digest. If more than two fragments are linked together which were not cleaved by the other enzyme, their order is inherently indeterminate. This ambiguity could be resolved by partial digest analysis.

Two-dimensional restriction fragment fingerprinting. After separation of two single restriction digests of the genomic DNA with enzymes A and B in independent runs in the first dimension, each digest was redigested with approximately one order of magnitude more frequently cutting enzyme C and subsequently run in parallel in the second dimension. Overlaps were identified by the similarity of the fragment pattern. Only those C fragments within the pattern which were cleaved neither by A nor B were informative. For smaller fragments, the intensity of an informative fingerprint was too low. Hence, comparative fingerprinting is primarily useful for the detection of overlaps between large A and B fragments with many C subfragments.

Southern hybridization analysis. In this case links and overlaps were established by hybridization of DNA probes with partial as well as complete single and double restriction digests separated by one-dimensional PFGE.

Construction of the genome map

The *Spe*I and *Dpn*I macrorestriction map of *P.aeruginosa* PAO was constructed as follows: first of all, several partial – complete *Spe*I digests were analyzed on two-dimensional gels. An example is shown in Figure 2. This single gel was sufficient to identify 23 links of *Spe*I fragments. The majority of links were independently found in several lanes of partially digested fragments which allowed 21 *Spe*I fragments to be ordered. Figure 2(B) assigns 27 clearly visible lanes of partially cleaved fragments. Faint DNA spots were not analyzed. The reader may identify the individual fragments of a partial digest by comparison with the complete *Spe*I digest in the outermost lane. The pattern of spots displayed in Figure 2 turned out to be the optimum compromise of maximum linkage information consistent with interpretable signal to noise ratios. In this case, contour clamped

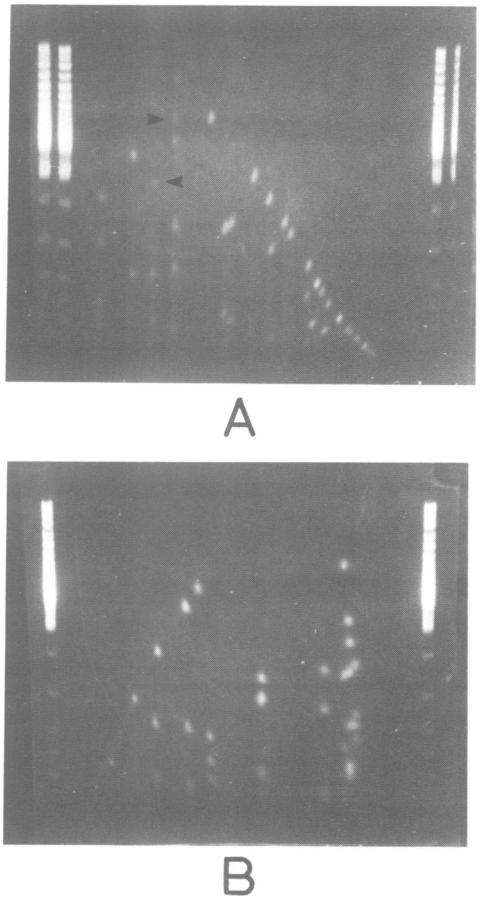

Fig. 3. Matched restriction enzyme analysis of *Spe*I/*Dpn*I (**A**) and *Dpn*I/*Spe*I (**B**) double digestions. FIGE was performed on 1% (w/v) agarose gels at 5.6 V/cm with a linear ramp from 1 s to 25 s over 21 h in both dimensions. The ratio of the forward to the reverse direction was chosen as 3:1. The arrowheads in (A) indicate partially cleaved SpC and SpD fragments (see text).

homogeneous electric field electrophoresis (CHEF) was optimized for a resolution between 20 and 600 kb. In further experiments, the conditions of partial cleavage were varied, and different pulse times were selected for the preferential resolution of larger or smaller fragments respectively. Another three certain and several probable links were detected. By the end of the first round, linkage had been repetitively established for 25 *Spe*I restriction fragments.

As a second step, the method of reciprocal pairs of complete double digests (see above) was employed. Figure 3 displays two complementary two-dimensional gels of complete double digests with *Spe*I and *Dpn*I separated by field inversion gel electrophoresis (FIGE). Redigestion of a *Spe*I digest with *Dpn*I revealed two hard to cut sites in the SpC and one of the SpD fragments between DpD and DpK and between DpA and DpH respectively (see arrows in Figure 3A: SpC = DpD/SpC + DpK + DpJ/SpC, [86 + 169] + 156 kbp; SpD = DpA/SpD + DpH + DpI/SpD, [30 + 290] + 57 kb). As visualized in Figure 3(A), the majority of *Dpn*I sites are located in the large *Spe*I fragments. An important feature to prove the consistency of the map was the fact that only one out of the two fragments of the SpI and SpL doublets had a *Dpn*I cleavage site, whereas the other one remained uncut. In the *Dpn*I/*Spe*I gel shown in

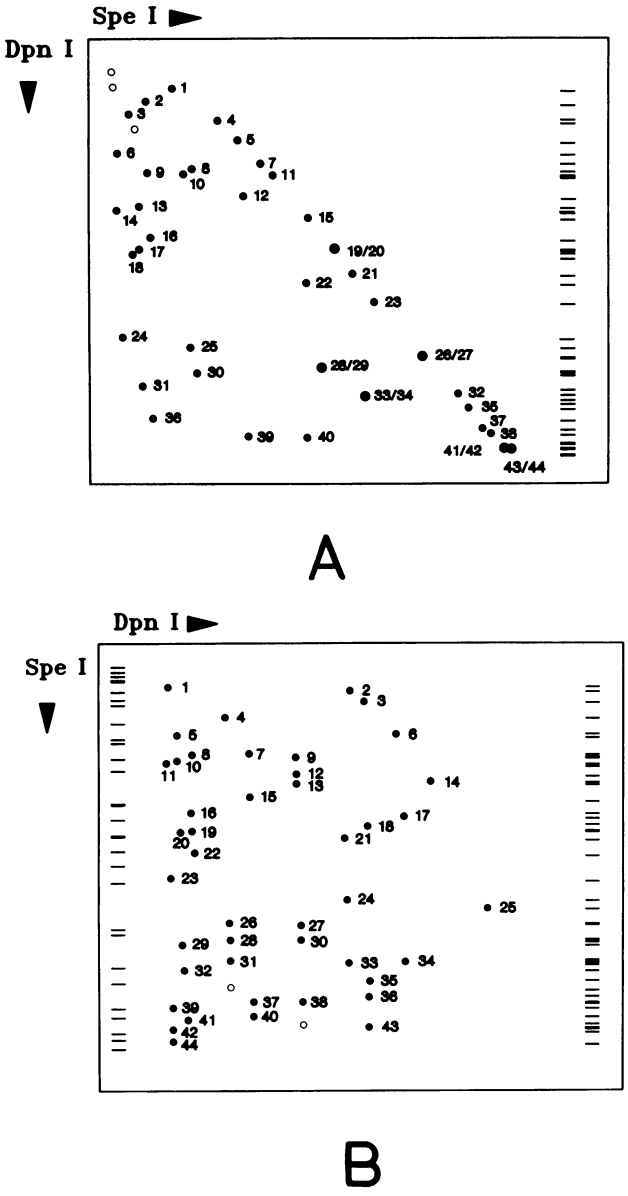

Fig. 4. Diagrams of the separation of reciprocal double restriction digests on two-dimensional CHEF gels. The *Spe*I (**A**) and the *Dpn*I (**B**) cleavages in the first dimension and the double digest in the second dimension were separated under the same conditions as shown in Figure 1A–C respectively. The DNA spots are ordered by decreasing mol. wt (assignment see Table II). Partial digests are indicated by open circles. The rightmost lanes display the location of fragments of a complete double digest, the left lane in (B) that of a complete *Spe*I cleavage. The diagram only shows the position of the 44 largest fragments which could be reliably visualized under optimized CHEF conditions both by ethidium bromide stain and autoradiography.

Figure 3(B) the small *Dpn* fragments from DpE to DpL were preferentially resolved in the first dimension. Figure 4 displays the location of all identified DNA spots of double restriction analysis on high-resolution two-dimensional CHEF gels. Fragments <25 kb are not shown. Radioactive end-labeling verified that *Spe*I fragments smaller than SpP did not contain any *Dpn*I sites (Figure 5). The diagram in Figure 4 was obtained by data accumulation from sets of gel pairs that had been performed under identical conditions. Spots are numbered from top to bottom by decreasing mol. wt. Unambiguous assignment of the corresponding restriction

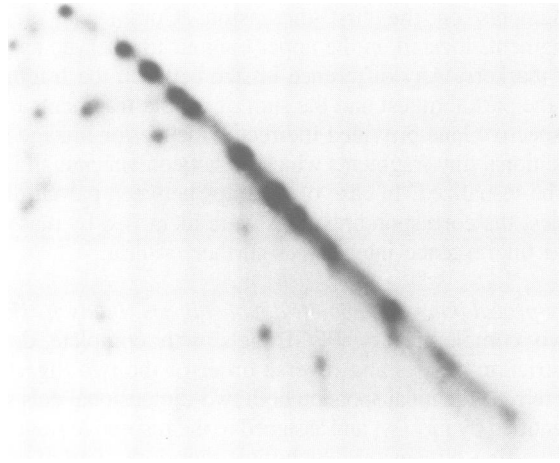

Fig. 5. Autoradiogram of a reciprocal *Spe*I/*Dpn*I two-dimensional gel. After radioactive end-labeling with terminal transferase, restriction fragments were separated by CHEF as in Figure 1A and C.

fragments on both gels was possible for the DNA spots 1–6, 11, 15–17, 21–27 and 31. Clusters of indistinguishable mol. wt differences occurred for the spots at positions 7–8, 9–10, 12–14, 18–20, 28–30, 32–34, 35–44. The assignment of fragments is given in Table II.

The information on partial–complete single digests and reciprocal double digests that was obtained from a series of two-dimensional gels was sufficient for the construction of the complete *Spe*I/*Dpn*I map. The smallest *Spe*I fragment with a *Dpn*I site was chosen as the starting point, because *Dpn*I cleavage of the 126 kb SpP fragment yielded two pieces of identical size (see Figure 3). Correspondingly, the DpG/DpJ cleavage site was placed in the middle of SpP. The size of the linking fragments was used as an independent criterion for the consistency of the assignment. The physical genome map of *P.aeruginosa* PAO derived from these experiments is shown in Figure 6.

After construction of the map, fragment order could be confirmed by the analysis of previously unassigned lanes on two-dimensional gels of partial *Spe*I digests. In addition, the accuracy of mapping was verified independently. Two-dimensional fingerprinting of *Spe*I and *Dpn*I digests with the restriction endonuclease *Xba*I (Figure 7) confirmed 15 overlaps of *Spe*I and *Dpn*I fragments. Inconsistencies with the map were not observed. Moreover, the fragments located by Southern hybridization with gene probes (Table III) were in complete agreement with the physical map.

A total of 51 restriction sites (36 *Spe*I sites, 15 *Dpn*I sites) were placed on the map. The resolution of the physical map varies between 7 and 330 kb with an average of ~115 kb.

Mapping of genes

Cloned genes were placed on the anonymous map by Southern hybridization analysis (Table III). The physical distance between the operons of tryptophan biosynthesis, *trpIBA* and *trpGDC*, was determined to be 580 ± 40 kb (Figures 6 and 9). The unassigned genes *phnAB* which encode another anthranilate synthetase not involved in tryptophan biosynthesis (Crawford *et al.*, 1986) was located in close vicinity to the structural gene *toxA1* of the major virulence factor of *P.aeruginosa*, the ADP-ribosyltransferase

Table II. Assignment of the restriction fragments of identical mol. wt on the reciprocal gels of complete double digests

Fragment no.[a]	Gel *SpeI/DpnI*	Gel *DpnI/SpeI*
1	SpE	on DpA
2	on SpD	DpH
3	on SpB	on DpI
4	SpH	on DpD
5	SpI_u	on DpB
6	on SpA	DpJ
(7, 8)	SpJ, on SpG	on DpE, on DpC
(9, 10)	on SpD, on SpF	on DpF, on DpB
11	SpK	on DpA
(12, 13, 14)	on SpI_l, on SpC, on SpA	on DpF, on DpF, DpK
15	SpL	on DpE
16	on SpD	on DpC
17	on SpC	on DpJ
(18, 19, 20)	on SpB, SpN, SpN	on DpI, on DpC, on DpB
21	SpO	on DpG
22	on SpL	on DpC
23	SpQ	on DpA
24	on SpA	on DpG
25	on SpF	DpL
26	SpR	on DpD
27	SpR	on DpF
(28, 29, 30)	on SpM, on SpM, on SpG	on DpD, on DpB, on DpF
31	on SpC	on DpD
(32, 33, 34)	SpS, on SpP, on SpP	on DpB, on DpG, on DpJ
(35, 36, 37,	SpT, on SpD, SpU	on DpI, on DpI, on DpF
38, 39, 40,	SpV, on SpI, on SpL	on DpE, on DpA, on DpE
41, 42, 43,	SpW, SpW, SpX	on DpB, on DpA, on DpI,
44)	SpX	on DpA

[a]Fragments are numbered by decreasing mol. wt as in Figure 4. Clusters of spots of indistinguishable mol. wt differences are indicated by parentheses. The prefix 'on' indicates the overlap with the respective fragment.

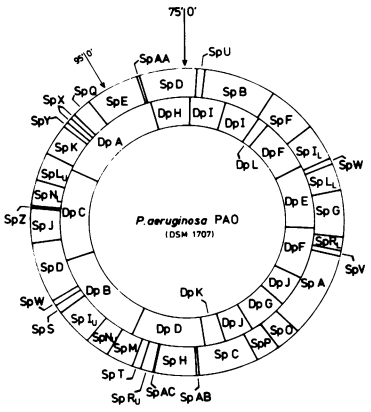

Fig. 6. *SpeI/DpnI* macrorestriction map of *P.aeruginosa* PAO (DSM 1707). The arrows indicate the zero times of the time-of-entry maps of the 95′ map (Holloway and Matsumoto, 1984) and its recalibrated 75′ version (O'Hoy and Krishnapillai, 1987). Within a double band, the fragment of larger mol. wt is designated by the index 'u' and that of lower size by 'l'.

exotoxin A (Figure 8) (Douglas *et al.*, 1987). Exotoxin A and alginate are species-specific products of *P.aeruginosa*. The synthesis of alginate is regulated by *algR1* (Devault *et al.*, 1989) which positively controls the expression of a clustered set of *alg* structural genes like *alg60* (Wang *et al.*, 1987). *alg60* and *algR1* are separated from each other by a remarkable 1670 ± 140 kb. The *recA* gene (Kokjohn and

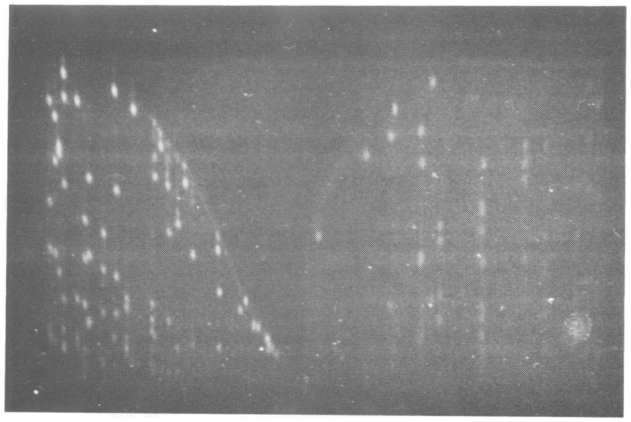

Fig. 7. *XbaI* fingerprints of complete *SpeI* (**left**) and *DpnI* (**right**) digests. After two independent CHEF runs in the first dimension as in Figure 1A and B, FIGE was employed for the second dimension on a 1% (w/v) agarose gel in 0.25 × TPE at 14°C (electric field 5.6 V/cm; two linear ramps: pulse times 1−9 s for 10 h, 1−20 s for 9 h; pulse ratio 3:1).

Miller, 1987) was aligned in close proximity to the *alg* structural genes on the same *SpeI* fragment (Table III).

Comparison of genetic and physical maps

For the comparison of the genetic maps (Holloway and Matsumoto, 1984; O'Hoy and Krishnapillai, 1987) with the anonymous physical map, the zero times of the respective genetic maps of the FP2 transfer origin (Holloway and Matsumoto, 1984) and of the *ilvB,C* locus (O'Hoy and Krishnapillai, 1987) were allocated to the physical map by using the *tripI* gene as the calibration marker (Figure 6). *trpIBA* was chosen as the reference locus because it was found to be located on the small 30 kb *SpeI* fragment SpW, and it had independently been mapped on both the detailed chromosome map from 1984 (Holloway and Matsumoto, 1984) and its recalibrated version using high-frequency-of-recombination donors (O'Hoy and Krishnapillai, 1987). No gross differences were apparent between the genetic and physical maps (Figure 9): the gene order is the same in all three maps. For the four marker loci whose location on the recalibrated 75′ map is known, the genetic distance is compatible with the physical length within 2 min or less, i.e. within 150 kb or less. Hence, physical and genetic distance correspond with each other at a resolution of 10^2 kb, i.e. there is no evidence for large regions of unusual high or low recombination frequency within the *P.aeruginosa* genome. In contrast, a small but significant distortion is required for superimposing the physical map on the 1984 version of the genetic map (Holloway and Matsumoto, 1984) (Figure 9): if compared with the physical size, the marker region between 20 and 50 min is compressed by 10%, and the region between 85 and 10 min is accordingly expanded on the genetic map.

Genomic organization of rDNA operons

Pseudomonas aeruginosa contains four transcriptional units coding for rRNA genes. The rDNAs are organized as operons in the typical eubacterial fashion 5′-P1 promoter−P2−16S rDNA−~500 bp intergenic spacer with tRNA genes−23S rDNA−5S rDNA−rho-independent terminator (Hartmann *et al.*, 1986). Restriction analysis gave identical fragment patterns for all four operons, indicating close

Table III. Assignment of genes to the physical map

Locus	Gene	Probe	Reference	Physical map		Genetic maps[a]	
				*Spe*I	*Dpn*I	95′ map	75′ map
algD	*alg60* GDP-mannose dehydrogenase	pAD5053	Wang *et al.* (1987)	SpP	DpJ	45′	34′
algR	*algR1*	pAD1029	Devault *et al.* (1989)	SpF	DpF	19′	10′
phnAB	anthranilate synthetase (not used in tryptophan synthesis)	p1514	Crawford *et al.* (1986)	SpQ	DpA	*93′–95′*	*68′–70′*
rec-102	*recA*	pKML2003	Kokjohn and Miller (1987)	SpP	DpG	42′–45′	*33′–34′*
rrnA	23S rRNA	pHF360	Festl *et al.* (1986)	SpF	DpI	*14′–17′*	*6′–8′*
rrnB				SpF	DpF	*19′–22′*	*10′–12′*
rrnC				SpA	DpF	*34′–35′*	*23′–24′*
rrnD				SpE	DpA	*93′*	*72′–73′*
toxA1	exotoxin A	pCDPT2	Douglas *et al.* (1987)	SpY	DpA	85′	67′–68′
tripIBA	*trpI*, activator gene of tryptophan synthase	pMC602	Chang *et al.* (1989)	SpW	DpE	28′	14′
trpGDC	anthranilate synthetase, small subunit	p1395	Essar *et al.* (1989) (unpublished)	SpV	DpF	34′	23′

[a]Assignment in italics indicates extrapolation from the physical map.

homology. We determined the location and orientation of the *rrn* operons by Southern hybridization with a 354 bp fragment of the 23S rDNA (Festl *et al.*, 1986) that according to sequence data (Toschka *et al.*, 1987a,b; H.Y.Toschka, personal communication) starts 120 bp downstream from the unique *Spe*I site in the 23S rDNA. The four operons are located from 72 to 21 min prior to the auxotrophic marker region (Holloway and Morgan, 1986). The operons are organized in pairs of inverted repeats with *rrnA* and *rrnB* each being 152 ± 4 kb, *rrnC* 1004 ± 36 kb and *rrnD* 1033 ± 28 kb apart from the symmetry center (Figure 9). A chromosomal region bounded by inverted repeats is topologically equivalent to a transposon (Kleckner, 1977). Hence, we would like to propose that the current organization of the *rrn* operons in *P.aeruginosa* evolved from an ancestor operon by gene duplication and inversion followed by another duplication.

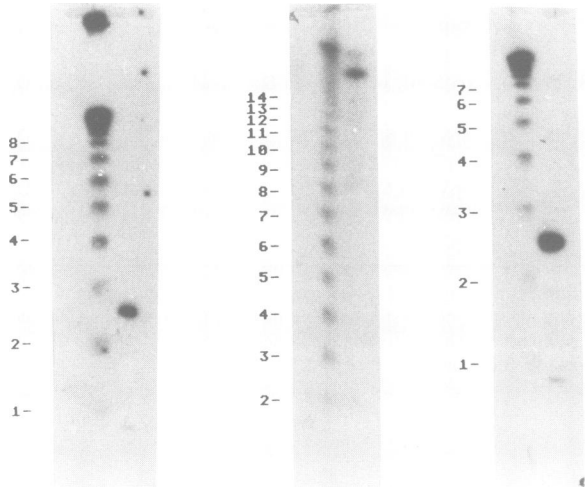

Fig. 8. Hybridization of probe *phnAB* to *Spe*I (**left**), *Dpn*I (**middle**) and *Spe*I/*Dpn*I digests of *P.aeruginosa* DNA separated by CHEF.

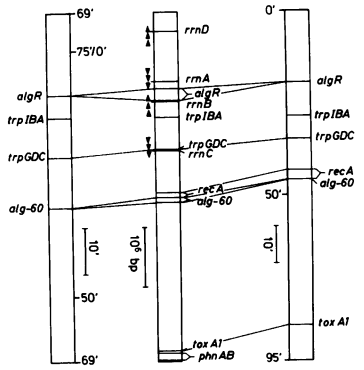

Fig. 9 .Comparison of the physical genome map of *P.aeruginosa* PAO (**middle**) with the genetic maps. **Right**: 95′ map (Holloway and Matsumoto, 1984); **left**: recalibrated 75′ map (O'Hoy and Krishnapillai, 1987). The arrowheads indicate the orientation of the *rrn* operons from 5′-16S rDNA to 3′-5S rDNA.

Discussion

The macrorestriction map of *P.aeruginosa* PAO (DSM 1707) has been entirely constructed by physical methods without any need for hybridization or preparative gel electrophoresis and cloning. The restriction fragments were ordered according to the results of two-dimensional pulsed field gels. The success of such an approach critically depends on the colinearity of separation in both dimensions in order to allow the unambiguous identification of fragments. In our experience CHEF (Chu *et al.*, 1986), CFGE (Southern *et al.*, 1987) and FIGE (Carle *et al.*, 1986) gave straight lanes in both dimensions. FIGE was hampered by a somewhat lower resolution which became critical when >100 spots had to be analyzed on a single two-dimensional gel (cf. Figures 2 and 3 for two-dimensional CHEF and FIGE gels respectively). The high resolution of two-dimensional CHEF gels is illustrated by the fact that after separation of partial–complete *Spe*I digests the fragments of a doublet could be clearly assigned to either the upper or lower fragment which differ in size by 2 kb or less (see Figure 2 as an example).

Another method to construct long-range restriction maps of bacterial genomes utilizes Southern blot analysis of fragments separated by pulsed field gels. Restriction sites are located by probing Southern blots of digests with individual fragments isolated from other digests and by correlating the products of double and triple digestions.

Although maps of the *H.influenzae* (Lee *et al.*, 1989) and *Mycoplasma mycoides* genomes (Pyle and Finch, 1988) have been successfully constructed by this method, several caveats need to be mentioned as already outlined by Lee *et al.* First of all, gel-purified genomic fragment probes are usually contaminated by randomly broken DNA from the rest of the chromosome which produce a faint background hybridization to all fragments that will prevent the unambiguous detection of very short overlaps. This happens in *P.aeruginosa* PAO for a number of small *Spe*I fragments which neighbor considerably larger fragments and overlap with a large *Dpn*I fragment (see Figure 6). Secondly, the assignment of fragments becomes ambiguous if a restriction site is bounded by repetitive DNA such as the four *Spe*I sites in each of the four *rrn* operons of *P.aeruginosa*. In this case the relationship between large genomic segments cannot be established. In addition, the analysis of doublets such as SpD, -I, -L, -N, -R, -W and -X becomes a major obstacle if long-range restriction mapping is based on the evaluation of Southern hybridization with gel-purified fragments. These problems are avoided if mapping is based on the macrorestriction analysis of two-dimensional gels as described in this report.

A third approach for physical genome mapping has been the combination of available genetic information together with PFGE data. These methods, however, are only applicable to organisms where extensive genetic mapping data already exist. The 22 *Not*I fragments of *E.coli* K12 were ordered by compiling the information obtained from Southern blotting with cloned genes and linkage probes, shifts in fragment size due to known genetic rearrangements and analysis of partial digest patterns (Smith *et al.*, 1987). The second example in the literature is the construction of a partial physical map of the *Caulobacter crescentus* chromosome (Ely and Gerardot, 1988). Genetic loci were assigned to specific large *Dra*I restriction fragments by PFGE analysis of DNA from a number of mutants containing Tn*5* insertions at known map positions. Without any other mapping strategy, it is difficult to close the gaps between the large fragments due to the lack of appropriate Tn*5* insertion mutants of small fragments.

Although our mapping strategy avoids the ambiguities of Southern analysis with gel-purified fragments and allows us to verify the physical map by independently obtained genetic data, several technical problems of two-dimensional PFGE need to be addressed that require adequate precautionary measures. One general intrinsic problem with PFGE is the reliable detection of tiny restriction fragments. We are confident that under our routine conditions all *Dpn*I and *Spe*I fragments >20 kb in size were reliably visualized. However, smaller fragments may have escaped notice due to unfavorably low fluorescence signals of the ethidium bromide stain. Moreover, tiny fragments may diffuse out of the agarose blocks during cleavage with restriction enzymes (Fan *et al.*, 1989; Grothues *et al.*, 1989). We applied the following precautions in order to ensure the completeness of the map: to detect small fragments, gels were overloaded with 10–100 times more DNA than usual, immediately after restriction enzyme cleavage had been terminated, and pulsed field or conventional agarose gel electrophoresis was conducted to resolve preferentially the range between 0.5 and 50 kb. In addition, restriction digests were end-labeled with radioactive nucleotides in order to visualize small and large fragments at comparable intensity. However, as exemplified by the non-equal labeling of DNA segments shown in Figure 5, terminal deoxynucleotidyl transferase did not only incorporate radioactive label at the terminal 3′ end, but also at free internal 3′-OH groups which were probably generated by single strand breaks during processing of the sample (Winnacker, 1984). Accordingly, the larger fragments were more heavily labeled than the smaller ones.

After the existence of smaller fragments had been demonstrated on one-dimensional gels, the subsequent two-dimensional PFGE of partial digests proved to be the most helpful in assigning the tiny fragments. The absence of next neighbors of a large fragment on such a two-dimensional gel was taken as evidence for linkage with tiny fragments. The major problem in mapping *P.aeruginosa* PAO was the region of large *Dpn*I fragments stretching from DpD to DpA (Figure 6) where the reciprocal digests were not informative for the ordering of several *Spe*I fragments. Consequently, linkage of *Spe*I fragments was mainly based on the evaluation of partial digests. Fragment order became difficult to assign if small fragments were neighboring considerably larger *Spe*I fragments and no further information could be extracted from fingerprinting or Southern analysis. Hence, both possibilties for the order between fragments SpX and SpQ and between SpE and SpAA remained compatible with the experimental data. The map in Figure 6 displays the most likely configuration which was deduced from fragment size and hybridization analysis of partial digests. In addition, although we have no evidence for it, we cannot definitely exclude the possibility that tiny restriction fragments have escaped our notice.

The combined *Spe*I and *Dpn*I macrorestriction map has an average resolution of 110 kb. Hence, the chromosomal position of any cloned DNA sequence of *P.aeruginosa* PAO can be assigned with an average accuracy of 2% of genome size. The agreement between the long-range restriction map described here and the time-of-entry maps (Holloway and Matsumoto, 1984; O'Hoy and Krishnapillai, 1987) is satisfying. No inconsistency in the order of two genes was found between the physical and either of the two genetic maps. Distances between marker loci vary more between the two genetic maps than between the physical map and the recalibrated genetic map. At the present resolution, the physical distance determined by macrorestriction mapping corresponds well with the relative distance measured by genetic techniques such as the conjugative plasmid systems FP2 (Holloway and Jennings, 1958), IncP-1 RP1 (Haas *et al.*, 1981) and R68 (Holloway and Morgan, 1986). We conclude that there is no evidence for stretches of hot spots of recombination on the chromosome.

The four *rrn* operons of *P.aeruginosa* PAO (Hartmann *et al.*, 1986) were found to be organized as pairs of inverted repeats (Figure 9). Among eubacterial species the number of rRNA operons varies, e.g. *Bacillus subtilis* has ten copies (Jarvis *et al.*, 1988), *E.coli* and *Salmonella typhimurium* each have seven (Ellwood and Nomura, 1982; Lehner *et al.*, 1984), *H.influenzae* has six (Lee *et al.*, 1989) and *C. crescentus* and *M.mycoides* each have two (Morgan *et al.*, 1978; Feingold *et al.*, 1985). Four copies, as in *P. aeruginosa*, is about in the middle of the range seen in bacteria.

The chromosomal location of *rrn* operons has been mapped for the species *E.coli* (Ellwood and Nomura, 1982), *S.typhimurium* (Lehner *et al.*, 1984), *H.influenzae* (Lee *et al.*, 1989) and *B.subtilis* (Jarvis *et al.*, 1988). In these bacteria, all *rrn* operons are clustered. In *E.coli*, the direction

of transcription for the rRNA cistrons is the same as that of DNA replication (Nomura *et al.*, 1977). Moreover, ~90% of the known genes in the *E.coli* chromosome encoding proteins involved in DNA, RNA and protein synthesis have a transcriptional orientation that is co-directional with replication (Brewer, 1988). These results have been interpreted to mean that the selection for the genome organization that minimizes head-on collisions between replication forks and RNA polymerases may be a major force in the shaping of the *E.coli* chromosome (Nomura *et al.*, 1977; Brewer, 1988).

The *rrn* operons in *P.aeruginosa* are organized as pairs of inverted repeats, which does not fit into the scheme found in *E.coli*. This finding confirms the exprience of the earlier genetic mapping studies that the gene arrangement in *P. aeruginosa* is different from that established in *E.coli* and *S.typhimurium* (Holloway and Morgan, 1986). In particular, the biosynthetic genes are not clustered and contiguous as they commonly are in enterobacteria (Bachmann, 1983; Sanderson and Rother, 1983). We suspect that long-range inversion, duplication and transposition played a substantial role during the evolution of the *P.aeruginosa* genome. The large separation of the *trp* genes and the organization of the *rrn* operons as tandem inverted repeats may have evolved by such long-range genome rearrangments.

The two-dimensional macrorestriction analysis described herein allows the rapid and reliable mapping of any bacterial genome of interest provided appropriate rare-cutting restriction endonucleases can be found that give resolvable fragment patterns on two-dimensional gels. However, in contrast to the 'bottom-up' strategy whereby the genome map is established by the sorting of clones from a genomic library (Kohara *et al.*, 1987; Wenzel and Herrmann, 1988), a 'top-down' approach such as long-range restriction mapping has the intrinsic disadvantage that it only yields abstract information about the relative location of fragments. If necessary, however, the DNA fragments of interest can be cloned in separate experiments according to the map information.

Two-dimensional agarose gels for restriction mapping is a well-known approach (Zehetner *et al.*, 1987). Using conventional agarose gel electrophoresis, restriction maps of plasmids (Villems *et al.*, 1978) and chloroplast DNA (Hildebrand *et al.*, 1985) were constructed by the analysis of two-dimensional gels. Other applications were the analysis of repetitive sequences (Boehm and Drahovsky, 1984) and the determination of genome size (Yee and Inouye, 1982). Nevertheless, reports about two-dimensional techniques for the analysis of DNA remained rare in the literature, presumably because of low resolution and unfavorably low signal to noise ratios (Zehetner *et al.*, 1987). This paper shows that the current stage of PFGE technology is sufficient to obtain informative two-dimensional gels of high resolution and reliability for the two-dimensional restriction mapping of any bacterial genome. In addition, it should also be possible to apply the two-dimensional PFGE techniques to the genome analysis of eukaryotic organisms. In the case of lower eukaryotes such as yeast and protozoa, the chromosomes may be separated in the first dimension followed by the separation of partial restriction digests in the second dimension. Subsequent assignment of DNA probes by Southern hybridization yields a physical chromosome map. For more complex eukaryotic genomes which give non-resolvable fragment patterns on stained two-dimensional gels, two-dimensional separation of partial–partial digests and subsquent hybridization may be performed. The gene mapping of gene families will be facilitated. Woolf *et al.* (1988) have already employed a combination of PFGE with conventional electrophoresis for the mapping of the T cell receptor γ gene family. However, in our opinion the major impact of these PFGE techniques will reside in the characterization of large stretches of DNA that have been cloned into 'yeast artificial chromosomes' (YAC) (Burke *et al.*, 1987). The computer-aided two-dimensional fingerprint analysis of YAC clones should allow the alignment of YAC contigs within a reasonable period of time.

Materials and methods

Bacterial strain

The strain *P.aeruginosa* PAO (DSM 1707) (Phibbs *et al.*, 1974) was purchased from the Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG. Subcultures in tryptone broth supplemented with 15% (v/v) glycerol were stored at −70°C until use.

DNA probes

Escherichia coli HB101 was transformed by plasmids containing cloned *P.aeruginosa* sequences (see Table III) using the calcium chloride procedure (Maniatis *et al.*, 1982). Plasmids were isolated from 1.5 ml overnight cultures of bacteria as described by Birnboim and Doly (1979). For isolation of the probe, a restriction digest of the plasmid was separated by agarose gel electrophoresis, and the cloned fragment was eluted by the 'freeze and squeeze' method (Thuring *et al.*, 1975).

Pulsed field gel electrophoresis

Sample preparation (Grothues and Tümmler, 1987). Pseudomonas aeruginosa PAO was grown overnight in tryptone broth to late exponential phase. Bacteria were harvested by centrifugation for 10 min at 1400 *g* and washed twice with SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.4). 1.5×10^8 cells were encapsulated in 60 µl 1% (w/v) low melting point agarose (Sigma type VII, St Louis, MO). Agarose plugs were incubated overnight at 56°C with 0.5 mg/ml proteinase K in 0.5 M EDTA, 1% (w/v) *N*-laurylsarcosine, pH 9.6. Agarose blocks were equilibrated in TE buffer (10 mM Tris–HCl, 10 mM EDTA, pH 7.4) at 4°C until used. Complete restriction digests of a 1/4 agarose plug were performed with 20 U restriction endonuclease, 7 mM dithiothreitol, 20 µg bovine serum albumin (Miles) in 155 µl reaction buffer recommended by the manufacturers. Double digests were performed sequentially. To obtain maximum specificity of cleavage with *DpnI*, the non-specific endonucleolytic activity has to be as low as possible. We observed a considerable batch to batch variation. Best results were obtained with *DpnI*, lot no.14 from New England Biolabs (Beverly, MA). For radioactive end-labeling, a *SpeI* or *DpnI* digestion in a 15 µl agarose block was incubated for 15 min at 37°C with 15 U terminal deoxynucleotidyl transferase (TdT), 2 µCi [α-^{32}P]dCTP (>3000 Ci/mmol) in 20 µl 140 mM potassium cacodylate, 30 mM Tris–HCl, 1 mM $MnCl_2$, 0.1 mM dithiothreitol, pH 7.6. The reader may note that TdT labeled agarose embedded DNA only in the presence of $MnCl_2$, but not in the presence of $CoCl_2$. Mol. wt markers were provided by concatamers of λ cI857 Sam7 (monomer size 48.5 kb) and by the chromosome of *Saccharomyces cerevisiae* strain X2180-IB (10^7 cells/60 µl agarose block).

Electrophoresis. Electrophoresis was conducted in a commercial Biorad CHEF DR^{TM}II cell (Chu *et al.*, 1986) or in a home-made FIGE (Carle *et al.*, 1986; Grothues *et al.*, 1989) or CFGE (Southern *et al.*, 1987) apparatus. Technical details on the construction and the software of the instruments are available upon request (D.Grothues). Blocks were inserted into a 1.5% (w/v) agarose gel in 0.5 × TBE buffer and DNA fragments were resolved by CHEF or CFGE for 24–43 h at 17°C at an electric field of 5.6 V/cm using a linear increase of pulse intervals. In the case of two-dimensional PFGE, the respective lanes were cut out after the first dimension and incubated in a small reaction chamber for 40–48 h with 150–200 U restriction endonuclease in 3 ml reaction buffer. The lane was fixed to a second 1.5% (w/v) agarose gel with the width of the lane of the first dimension becoming the height of the second dimension. This procedure yielded highly resolved DNA spots without loss of sensitivity. DNA spots

were visualized by staining with ethidium bromide or by autoradiography of blotted ^{32}P-labeled fragments.

Southern blot hybridization

Prior to blotting, the PFGE gels were irradiated with 302 nm light for half the time required to take a standard Polaroid positive. DNA was transferred onto nylon membranes by capillary blotting for 48 h. For hybridization, probes were labeled according to the random primer labeling technique developed by Feinberg and Vogelstein (1983) using [α-^{32}P]dCTP (3000 Ci/mmol). PFGE blots were pre-hybridized with 0.5 M sodium phosphate, 7% (w/v) SDS, 1 mM EDTA, pH 7.2 (Church and Gilbert, 1984) for 1 h at 68°C and subsequently hybridized for 24–48 h with 10 μCi of DNA probe (~25 ng) and 7 × 10^4 c.p.m. of λ standard in the same buffer. Washings were performed with 40 mM sodium phosphate, 1% (w/v) SDS, pH 7.2, for 5 min at room temperature and then twice for 40 min at 68°C (Church and Gilbert, 1984).

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