A combined physical and genetic map of *Pseudomonas aeruginosa* PAO

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(Received 21 June 1990; revised 28 August 1990; accepted 10 September 1990)

A combined physical and genetic map of *Pseudomonas aeruginosa* PAO was constructed by pulsed-field gel electrophoresis and Southern hybridization using cosmid clones from a genomic library carrying known genes. A total of 37 *SpeI* restriction fragments have been mapped on the 5862 kb genome, and fragment contiguity demonstrated by hybridization with clones from a *SpeI* junction fragment library and fragments obtained by partial *SpeI* digestion, both derived from the *P. aeruginosa* PAO chromosome.

Introduction

The genetic map of *Pseudomonas aeruginosa* now has over 300 markers located (Holloway & Zhang, 1990) and a comparison of gene arrangement with the related species *P. putida* has led to proposals regarding the evolution of the chromosome of these species (Morgan & Dean, 1985; Holloway & Morgan, 1986). The development of pulsed-field gel electrophoresis (PFGE), which allows the resolution of DNA fragments at least a megabase in size, has enabled restriction mapping to be extended to entire chromosomes. This permits the accurate determination of genome size and together with cloning techniques provides a means of generating combined genetic and physical maps with the accuracy required for interspecific genome comparisons.

Physical-genetic maps of varying complexity have been constructed for *Escherichia coli* (Smith *et al.*, 1987), *Caulobacter crescentus* (Ely *et al.*, 1990), *Anabaena* sp. strain PCC 7120 (Bancroft *et al.*, 1989) and *Rhodobacter sphaeroides* (Suwanto & Kaplan, 1990). Römmling *et al.* (1989) have presented a physical map for *P. aeruginosa* PAO, but with limited genetic data.

In this paper we report the construction of a combined physical and genetic map of *P. aeruginosa* PAO based on the existing genetic map (Holloway & Zhang, 1990). A preliminary account of this work was given at a meeting on *Pseudomonas*: Biotransformations, Pathogenesis and Evolving Biotechnology (Holloway *et al.*, 1990).

Methods

Bacterial strains and plasmids. These are listed in Table 1.

Media and cultural conditions. Nutrient agar, nutrient yeast broth and solid and liquid minimal medium have been described elsewhere (Leisinger *et al.*, 1972; Stanisich & Holloway, 1972).

DNA isolation and manipulation. Chromosomal DNA was prepared by the method of Scott *et al.* (1981). Purified plasmid DNA was prepared by the method of Nayudu & Holloway (1981) and rapid small-scale plasmid preparation performed by the method described in Maniatis *et al.* (1982). Restriction endonucleases (Biolabs) were used according to the conditions of Maniatis *et al.* (1982). T4 DNA ligase (BRESA) and calf alkaline phosphatase (Boehringer Mannheim) were used according to the manufacturer's instructions. Bacteriophage λ packaging kit was supplied by Promega; *in vitro* packaging was performed according to Maniatis *et al.* (1982) and the manufacturer's instructions. Electrophoretic analysis and DNA-DNA hybridization were done essentially as described by Lyon *et al.* (1988). Dot blotting to Hybond-N (Amersham) was performed according to the manufacturer's instructions.

Construction and storage of a genomic library of P. aeruginosa PAO. The vector used was the broad host range cosmid pLA2917. Cosmid constructs were transfected into *E. coli* S17-1 and individual clones stored in microtitre trays as described by Lyon *et al.* (1988).

Complementation analysis. The ability of recombinant cosmids to complement known mutants of *P. aeruginosa* PAO was tested using spot matings. Donor strains grown in microtitre trays were transferred using a 50-point multi-inoculator onto minimal agar plates containing tetracycline and appropriate growth factors and which had been prespread with 0.1 ml of an overnight culture of the recipient grown at 43 °C to minimize host restriction (Holloway, 1965).

Pulsed-field gel electrophoresis (PFGE). *P. aeruginosa* PAO1293 was used for this study. The materials and methods were as described by Smith *et al.* (1986) with the following alterations. The chloramphenicol concentration used to align the chromosomal replication origins was

Abbreviation: PFGE, pulsed-field gel electrophoresis.

Table 1. *Bacterial strains and plasmids used in this study*

All *P. aeruginosa* strains are derived from PAO1 (ATCC 15692). Genotype symbols are the same as those used by Holloway & Zhang (1990). Genotypes of the following *P. aeruginosa* PAO strains are given in full elsewhere: PAO2, PAO129, PAO303, PAO307, PAO472, PAO473, PAO890, PAO894, PAO898, PAO900, PAO903 (Morgan, 1982); PAO12, PAO697, PAO682, PAO684, PAO685, PAO686, PAO687, PAO889, PAO891, PAO892, PAO895, PAO899, PAO913, PAO917, PAO918, PAO919, PAO921, PAO922, PAO926, PAO928 (Moore *et al.*, 1983); PAO765, PAO767, PAO1219, PAO1480 (O'Hoy & Krishnapillai, 1985); PAO1490 (O'Hoy & Krishnapillai, 1987); PAO483 (Isaac & Holloway, 1968); PAO909, PAO1818 (Bray *et al.*, 1987); PAO979 (Früh *et al.*, 1985); PAO965, PAO6020 (Meile & Leisinger, 1982); *P. aeruginosa* PRP900 (Roehl & Phibbs, 1982); *P. aeruginosa* PFB2 (Phibbs *et al.*, 1974). The *E. coli* cosmid clone mobilizing strain S17-1 is described by Simon *et al.* (1983); *E. coli* JM109 by Yanisch-Perron *et al.* (1985), the wide host range cosmid vector pLA2197 by Allen & Hanson (1985), and the IncP1 plasmid R68 by Chandler & Krishnapillai (1974).

<i>P. aeruginosa</i> strain	Genotype	Source/derivation*
PAO660	<i>phe-2</i>	1
PAO667	<i>met-9020 eda-9001 rif-155</i>	2
PAO705	<i>aro-1</i>	1
PAO716	<i>cys-54 rif-965</i>	1
PAO1153	<i>argH32 rif-104</i>	1
PAO1278	<i>thr-9001 rif-125</i>	2
PAO1293	Prototroph	3
PAO2196	<i>met-9020 trp-9029 lys-9015</i>	2
	<i>nar-9011 catA1 chu-9002</i>	
PAO4044	<i>met-9020 trp-9029 lys-9015</i>	2
	<i>nar-9011 catA1 dcu-9041</i>	
PAO4299	<i>met-9020 catA1 vtu-9001</i>	2
	<i>chu-9183 hluH9051 hisIV9122</i>	
	<i>pur-9054</i>	

* 1, B. W. Holloway collection; 2, H. Matsumoto collection; 3, constructed from PAO2 using the transducing phage E79 *tw-2* (Morgan, 1979) with selection for prototrophy. This strain was chosen for genome analysis by PFGE as PAO1, the progenitor of all PAO strains, now carries a mutation for chloramphenicol resistance.

300 µg ml⁻¹. Bio-Rad ultra-pure agarose (1%, (w/v)) was used in the preparation of DNA blocks. The concentration of proteinase K in ESP was 0.4 µg ml⁻¹. After digestion, the sample was washed twice in electrophoresis running buffer at 45 °C for 30 min.

The PFGE apparatus with its hexagonal electrode (CHEF) system was obtained from LKB Produkter. The electrophoresis was performed in 0.1 × TBE buffer, 12 °C, 170 V. The agarose concentration was 1% for 48 h electrophoresis and 1.3–1.5% for 65 h. The pulse time was varied between 2 s and 70 s depending upon the size range of the fragments to be separated.

The DNA from agarose was transferred to Hybond-N membrane according to the standard method (Maniatis, *et al.*, 1982). In some cases, part of the DNA still remained in the gel after blotting, which could be seen by restaining the gel in ethidium bromide solution. In this case, the blotting was repeated using the alkali transfer method as described by Amersham. Both membranes give a satisfactory signal on hybridization, which was performed by the method as described in Maniatis *et al.* (1982).

Preparation of λ-concatemer as a standard for PFGE. The method used was that described by Waterbury & Lane (1987). In order to break the

large concatemer formed into smaller fragments, the insert was incubated at 65 °C for 2 min prior to loading.

Construction of SpeI junction clones. About 10 µg of chromosomal DNA of *P. aeruginosa* PAO1293 was digested to completion with one of a number of different restriction endonucleases. The digested DNA was purified by ethanol precipitation, circularized by self-ligation, cut with *SpeI*, cloned into the *SpeI* site of pGEM5Zf(+) vector (Promega) and then transformed into *E. coli* JM109. The restriction patterns of the clones were checked by gel electrophoresis. Putative *SpeI* junction clones gave two fragments when cut with *SpeI*, and three when cut with *SpeI* and the chromosome cutting enzyme.

Results

Measurement of genome size – choice of restriction enzyme

Accurate measurements of genome size rely on the ability to resolve all fragments upon electrophoresis, and for this to be feasible restriction endonucleases that cut infrequently are required. McClelland *et al.* (1987) predicted from an examination of prokaryotic sequence data that enzymes that included the tetranucleotide CTAG in their recognition sequence should cut infrequently. Fig. 1 shows 30 *SpeI* (ACTAGT) generated fragments from genomic *P. aeruginosa* PAO DNA, separated by means of PFGE using different pulse regimes. Together with a fourth gel (details in legend) 34 *SpeI* fragments ranging in size from 10 to 525 kb were detected, totalling 5844 kb. The addition of three other small *SpeI* fragments, detected in our search for linking clones (see below) brings the genome size to 5862 kb. The enzymes *XbaI*, *DraI*, *AsnI*, *NotI* and *SfiI* all yielded too many fragments less than 50 kb to be useful in this study, but will be valuable in mapping of additional enzyme sites within *SpeI* fragments (see Discussion).

Strategy for construction of combined physical and genetic map

The primary strategy employed to order the *SpeI* fragments and simultaneously to correlate the physical map thus generated with the established genetic map was to probe Southern blots of PFGE-separated genomic *SpeI* fragments with clones from a *P. aeruginosa* PAO genomic cosmid library which have been shown to carry known chromosomal genes. These are listed in Table 2. This ordered unambiguously the majority of large (>100 kb) *SpeI* fragments, accounting for more than 80% of the genome. Of particular use were the six cosmid clones which fortuitously contained a *SpeI* site. This proved contiguity of the two *SpeI* fragments identified by probing, and also provided precise points of alignment for the physical and genetic maps. However, except for

Fig. 1. PFGE of PAO1293 DNA pre-digested with *SpeI* to demonstrate the separation of fragments. Panels (a), (b) and (c) are gels showing the readily identifiable separation of *SpeI* fragments, numbered 1 to 30 in descending size, by comparison with the phage λ concatameric ladder ranging in size from 582.0 to 48.5 kb shown on tracks A and C of each panel. Electrophoresis was in $0.1 \times$ TBE buffer at 170 V at 12 °C. The agarose gel concentration in (a) was 1.5%; in (b), 1.3% and in (c), 1.1%, and the pulse times were: (a) 15 s for 16 h; 30 s for 16 h; 45 s for 16 h; 60 s for 17 h; (b) 15 s for 12 h; 25 s for 52 h; (c) 5 s for 15 h; 10 s for 10 h; 15 s for 15 h. Fragments 30–34 were visualized on a 1.2% agarose gel run at a pulse of 2 s for 17 h, 3 s for 6 h and 4 s for 19 h. Phage λ digested with *XhoI* was used as a size standard. The fragments were measured as 15 kb, 11.5 kb and three of about 10 kb.

the five junctions thus identified, this approach could not detect small *SpeI* fragments lying between located fragments, nor could it identify all fragments from the 55–70 min region of the genetic map in which genetic markers and hence useful cosmid clones are scarce. The remaining *SpeI* fragments were mapped using a variety of approaches, as described below.

SpeI junction clones. Cloned fragments of genomic DNA that contain an internal *SpeI* site were constructed with a view to demonstrating contiguity of *SpeI* fragments mapped to adjacent locations by probing with cosmid clones (above), and identifying small fragments contiguous with the mapped fragments. If clones representing every *SpeI* site had been isolated it would also have been

Table 2. Cosmids identified as carrying known chromosomal markers and used in Southern hybridization of PFGE-separated *SpeI* fragments, listed in map order

Cosmid	Marker(s) carried/ strain used for complementation	Location (min)*	<i>SpeI</i> fragment†
pMO010602	<i>ilvB</i> , <i>C</i> (PAO900)	0	1, 2, 27, 29, 30‡
pMO010749	<i>car-9</i> (PAO889)	1	2
pMO011105	<i>pur-8001</i> (PAO4299)	2.5	2
pMO012243	<i>hisIV</i> (PAO891)	4	2
pMO010113	<i>ser-33</i> (PAO765)	4	2
pMO010725	<i>hisIIA</i> (PAO892)	7	7
pMO011318	<i>hisIIB</i> (PAO913)	7	7
pMO012844	<i>argA</i> (PAO894)	8	7
pMO010303	<i>argH</i> (PAO1153), <i>lysA</i> (PAO895)	10	7
pMO010128	<i>argB</i> (PAO303), <i>pyrE70</i> (PAO483)	11	7
pMO010229	<i>pur-136</i> (PAO917)	12.5	11
pMO011919	<i>trpA</i> (PAO918), <i>trpB</i> (PAO890)	14	11 + 28§
pMO010817	<i>hisV</i> (PAO919), <i>ser-3</i> (PAO2)	19	8
pMO013624	<i>hisV</i> (PAO919), <i>ilvD</i> (PAO898)	19	8
pMO010739	<i>met-28</i> (PAO899), <i>proC</i> (PAO697), <i>pyrB</i> (PAO129), <i>pyr-81</i> (PAO767)	20	8
pMO010705	<i>trpE</i> (PAO472)	22.5	22
pMO011925	<i>trpC</i> (PAO921)	23	27
pMO012425	<i>trpC</i> (PAO921), <i>trpD</i> (PAO922), <i>argC</i> (PAO307)	23.5	27
pMO011243	<i>argC</i> (PAO307)	23.5	27 + 1§
pMO010638	<i>proA</i> (PAO682)	26	1
pMO011722	<i>leu-8</i> (PAO12)	30	1 + 19§
pMO011145	<i>pur-66</i> (PAO684)	30.5	19
pMO010920	<i>thr-48</i> (PAO1278), <i>thr-59</i> (PAO1480), <i>thr-60</i> (PAO1490)	31	19
pMO011234	<i>lys-9015</i> (PAO2196)	33.5	19 + 20§
pMO012329	<i>argF</i> (PAO686), <i>argG</i> (PAO685)	34	3
pMO010337	<i>phe-2</i> (PAO660), <i>aro-1</i> (PAO705)	38	3
pMO012437	<i>zwf-1</i> (PRP900), <i>eda-9001</i> (PAO667), <i>edd-1</i> (PFB2)	39	3
pMO012140	<i>leu-10</i> (PAO687), <i>trpF</i> (PAO473)	40	9 + 21‡
pMO011618	<i>leu-10</i> (PAO687), <i>trpF</i> (PAO473), <i>pur-9013</i> (PAO1818), <i>met-9011</i> (PAO926)	40	9
pMO012223	<i>pyrD</i> (PAO928)	41.5	9
pMO012509	<i>dcu-9041</i> (PAO4044)	43.5	16
pMO011137	<i>cys-50</i> (PAO1219)	45	16
pMO1967	<i>ben-4,2,1</i> , <i>ant-1,3</i> , <i>catABC</i>	47–48	17
pMO1981	<i>gcu-2</i>	45.5	17 + 10§
pMO1983	<i>gcu-1</i>	45.5	10
pMO011236	<i>cys-54</i> (PAO716)	56	12
pMO010617	<i>pur-70</i> (PAO909)	66	6
pMO011644	<i>oruI</i> (PAO979)	66.5	6
pMO011302	<i>pruA</i> (PAO965), <i>pruB</i> (PAO6020)	67.5	6
pMO011809	<i>hisI</i> (PAO903)	69	5

* From Holloway & Zhang (1990).

† From Fig. 1.

‡ These cosmid clones contain an unidentified repeated sequence.

§ These cosmid clones contain a *SpeI* site.|| These cosmid clones were generated from pLA2197 and DNA from a *P. aeruginosa* PAO R-prime selected as carrying *catA* (C. Zhang & B. W. Holloway, unpublished data).

possible to 'walk' into the 55–70 min region of the chromosome. However, although *Bam*HI, *Eco*RI, *Kpn*I, *Hind*III and *Xho*I (chosen because they do not cut the cloning vector) and *Sac*I were each used for the cutting of genomic DNA prior to junction clone construction, and a total of 108 junction clones isolated, only 22 different junctions were identified in subsequent probing, with some *SpeI* junctions being cloned many times.

In addition to junction fragments, four families of clones were identified which did not contain a restriction site of the enzyme used in their construction. These clones contained inserts of 11.5 kb, 6.8 kb, 6.4 kb or 5.1 kb and were presumed to represent small *SpeI* fragments which happened not to contain cutting sites for those enzymes used in linking clone construction. The largest clone did in fact hybridize with one of the smallest

bands seen in the PFGE gels, but the remaining three were presumed to be too small to be seen on the gel. That this was the case was shown by using members of these families as probes against dot-blot of the cosmid library. This yielded four classes of cosmid clones, each carrying 2 *SpeI* sites, 11.5, 6.8, 6.4 or 5.1 kb apart. These cosmid clones were in turn used as probes against Southern blots of PFGE-separated *SpeI* fragments, revealing in each case two large *SpeI* fragments flanking the small cloned fragment (data not shown).

Chromosomal *TnI* inserts. Krishnapillai *et al.* (1981) determined the genetic location of 12 *TnI* inserts in the *P. aeruginosa* PAO chromosome, 11 of which mapped between 46 and 58 min. *SpeI*-digested genomic DNA from these derivatives was separated by PFGE, blotted, and probed with the IncP1 plasmid R68, which carries *TnI*. This allowed us to order the seven *SpeI* fragments 17 (150 kb) through 18 (150 kb) shown in Fig. 1, several of which had not previously been located by any of the above methods (data not shown).

Probing of partial *SpeI* digests. Despite the assertion of Smith *et al.* (1987) that with hindsight they would have used probing of partial enzyme digests in order to identify adjacent fragments, we did not find this approach particularly useful in the initial stages of mapping. Once the majority of fragments had been located, however, it was used to confirm or deny that particular fragments were adjacent. In particular, that fragment 8 (300 kb) was flanked by fragments 22 (90 kb) and 14 (175 kb) was confirmed by probing a partial *SpeI* digest with cosmid clone pMO10817, which carries DNA from fragment 8 (see Table 2). In addition to hybridization with fragment 8, the two bands with the next-highest intensity were of sizes 390 kb and 475 kb (data not shown). These two junctions had not been identified by any of the procedures described above.

Discussion

With the recent advent of electrophoretic techniques for the separation of large DNA fragments in the megabase range such as PFGE and variations of it, it has become possible to construct restriction maps of bacterial chromosomes. Such a map accurately measures genome size and reveals aspects of genome complexity such as the two chromosomes in *Rhodobacter* (Suwanto & Kaplan, 1989) or the presence of any megaplasmids. Such a map is of greater genetic use if the location of known genes can be identified in relation to the restriction enzyme sites. However, it is not yet clear which is the most appropriate strategy for physical mapping of prokaryotic genomes, either from the point

of view of ease and accuracy of mapping, or from that of the genetic usefulness of the results. This problem has been raised elsewhere (Smith & Condemine, 1990), and is highlighted by a comparison of the strategies and findings that we present here and those of Römling *et al.* (1989) in the construction of a physical and genetic map for the genome of *P. aeruginosa* PAO. Our primary approach of using cosmid clones carrying known genes, together with *SpeI* junction clones to prove fragment contiguity, is technically simpler while more time consuming than the use of two-dimensional gels together with *SpeI* partial digests and complete digests (Römling *et al.*, 1989), but is more accurate in regard to the placing of small fragments. Thus while we agree with Römling *et al.* (1989) on the size and position of the 27 largest *P. aeruginosa* PAO *SpeI* fragments (accounting for more than 95% of the genome), we differ from them in the number and sizes of small *SpeI* fragments and also in the position of some small *SpeI* fragments.

The construction of *SpeI* junction clones also provides the means for increasing the resolution of the physical map. Preliminary experiments have shown that these clones (cut with *SpeI* and the chromosome cutting enzyme to separate the two halves of the junction) can be used as probes against PFGE separated and Southern-blotted genomic DNA digested to completion with *SpeI* and partially digested with either *XbaI* or *DraI*. The resulting ladder of partial *XbaI* (*DraI*) fragments allows mapping of *XbaI* (*DraI*) sites within a particular *SpeI* fragment.

Our finding that *P. aeruginosa* has a genome size of 5862 kb makes it one of the largest among prokaryotes, with only *Anabaena* sp. strain PCC 7120 at 7110 kb being larger amongst those genome sizes measured by means of PFGE (Bancroft *et al.*, 1989). The genomes of a large number of human and animal pathogenic bacteria are in the smaller size range of 900–2650 kb, including those for *Chlamydia*, *Rickettsiella*, *Porochlamydia*, *Haemophilus influenzae* and many *Mycoplasma* species (Frutos *et al.*, 1989; Lee & Smith, 1988; Pyle *et al.*, 1988). *E. coli* K12 and *Bacillus subtilis* 168 both have chromosome sizes of 4700 kb (Smith *et al.*, 1987; Ventra & Weiss, 1989). One reason for the larger genome size of *P. aeruginosa* PAO is that unlike human and animal pathogens that are obligate intracellular parasites, e.g. *Mycoplasma*, *Rickettsiella* and *Chlamydia*, which rely on their cellular hosts for the provision of many of their nutritional requirements, *P. aeruginosa* being a free-living bacterium and an opportunistic pathogen has had to be nutritionally independent. While *E. coli* with its smaller genome of 4700 kb (Smith *et al.*, 1987) is also capable of independent existence, the size difference is probably due to the extensive repertoire of catabolic genes found on the *P. aeruginosa* PAO chromosome (Holloway & Zhang,

Fig. 2. The partial physical and genetic map of *P. aeruginosa* strain PAO. The physical map of 5862 kb shows the alignment of the numbered *SpeI* fragments, with their sizes in parenthesis, on the left and the genetic map of 75 min showing the location of genes identified by probing complementing cosmids against *SpeI* fragments on the right. The proven contiguity of *SpeI* fragments either by the hybridization with junction fragments or cosmids is shown with a closed dot on the horizontal stalk. Those with contiguity yet to be demonstrated are shown without the dot. The thicker lines joining the genetic and physical maps indicate complementing cosmids which have a *SpeI* site linking contiguous *SpeI* fragments.

1990). *P. putida* PPN has a similar genetic organization to *P. aeruginosa* PAO (Holloway & Morgan, 1986) and also a similar genome size (R. Saffery & A. Morgan, unpublished data), but lacks virulence determinants,

suggesting that *P. aeruginosa* PAO has only recently become an opportunistic pathogen.

The combined physical and genetic map presented here has sufficient resolution to reveal inaccuracies in the distances between markers in some regions of the genetic map (Holloway & Zhang, 1990). This is shown by the change in slope of the lines connecting the physical and genetic maps in Fig. 2, in particular in the 50–65 min and 70–10 min regions of the map. While the inaccuracies are not large, they are sufficient to require a reassessment of conclusions drawn from marker-for-marker comparison with the genetic map of *P. putida* PPN (Morgan & Dean, 1985; Holloway & Morgan, 1986). We are at present improving the resolution of the map presented here by mapping additional restriction enzyme sites and increasing the number of genetic markers located by probing PFGE Southern blots with cloned genes, from both *P. aeruginosa* PAO and other bacterial species. Essential genes of common ancestry from divergent species often show sufficient sequence conservation to allow a signal upon hybridization. Together with parallel genome studies on *P. putida* PPN and *P. solanacearum* which are being carried out in this laboratory, it will be possible to gain a more accurate picture of the comparative organization of the genomes of *Pseudomonas* spp.

This work was supported by the Australian Research Council. E.R. was supported by a scholarship from IUC-World Bank, S.D. by an AIDAB scholarship, and M.S. by a Commonwealth Postgraduate Research Award.

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