Nucleic Acid Homologies in the Genus *Pseudomonas*

N. J. PALLERONI,¹ R. KUNISAWA, R. CONTOPOULOU, and M. DOUDOROFF

Department of Bacteriology and Immunology, University of California, Berkeley, California 94720

On the basis of ribosomal ribonucleic acid homologies, the genus *Pseudomonas* can be divided into at least five distinct groups, some of which are as distantly related to each other as they are to *Escherichia coli*. One of these groups contains members of the genus *Xanthomonas*. The data presented support and extend the previous grouping based on deoxyribonucleic acid homologies and support the current view that the portion of the genome coding for ribosomal ribonucleic acid is more conserved in the course of evolution than the bulk of the genome.

For about nine years our laboratory has been largely involved in taxonomic studies of the genus *Pseudomonas*. Our first approach was the phenotypic characterization of a large number of strains using mainly nutritional characters. On the basis of phenotypic resemblance, we recognized several "species groups," comprised of what we regarded as recognizable species. Below the specific rank, we further recognized a number of "biotypes," some of which had been previously described as species, and many of which may deserve specific rank (14). Deoxyribonucleic acid (DNA)-DNA hybridization experiments generally supported our conclusions based on phenotypic data but showed that some of the species and biotypes were quite heterogeneous with respect to DNA homology. It was also found that some species groups were sufficiently closely related to each other to be united in a larger DNA homology complex. For example, the "*P. fluorescens* complex" was found to contain not only the "fluorescent group" but also the nonfluorescent pseudomonads that had been assigned to the "*alcaligenes*" and "*stutzeri*" groups (10, 14). Our studies have been reviewed recently (9).

To pursue further our studies on bacterial phylogeny and speciation, we have resorted to ribosomal ribonucleic acid (rRNA)-DNA hybridizations among selected members of the various DNA homology groups, and included three species of *Xanthomonas* in the studies. The results are reported in the present paper.

¹Present address: Research Division, Hoffmann-La Roche, Inc., Nutley, N.J. 07110.

(This paper was presented at The Annual Meeting of the American Society for Microbiology, 1973.)

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in the presently described experiments are numbered as in our previous publications (1, 2, 4, 7, 8, 10-12, 14). Where applicable, the American Type Culture Collection (ATCC) number has been included. *Pseudomonas viridiflava* VIRID 1 (NCCPB 1249) and *Xanthomonas campestris* XC-1 were received from D. C. Hildebrand, Department of Plant Pathology, University of California, Berkeley, and *Xanthomonas pruni* XP-218 and *Xanthomonas translucens* XT-122 were sent by M. P. Starr, Department of Bacteriology, University of California, Davis.

Preparation of rRNA. For the preparation of unlabeled rRNA, most strains were grown in a medium containing 0.033 M K-Na phosphate buffer, pH 6.8; $(\text{NH}_4)_2\text{SO}_4$, 0.1%; asparagine, 0.2%; yeast extract, 0.5%; Hutner mineral base (3), 10 ml per liter. For the growth of *P. maltophilia*, DL-sodium lactate was substituted for asparagine. *P. saccharophila* was grown in a medium containing 0.033 M K-Na phosphate buffer, pH 6.8; NH_4Cl , 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05%; ferric ammonium citrate, 0.005%; CaCl_2 , 0.0005%; and sodium succinate, 0.2%. For the growth of *P. diminuta*, the same basal medium was used as for *P. saccharophila* except that succinate was replaced by asparagine and the essential growth factors (1) were added. The cultures were grown at 30°C on a rotary shaker. The cells were suspended in 0.02 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.4, containing 0.01 M MgCl_2 . Cell concentrations ranging from 10 to 20% (wet weight/vol) were used. DNase was added to the suspension to a final concentration of 10 µg/ml. The suspension was

passed through a French pressure cell at 12,000 psi. All the above operations were carried out in the cold. For most rRNA extractions the procedure was as follows. The extract was centrifuged for 15 min at $27,000 \times g$, the pellet was discarded, and the supernatant fluid was centrifuged for 45 min at $127,000 \times g$ or 75 min at $88,700 \times g$. The ribosome pellet was suspended in about 0.25 of the original volume of 0.05 M phosphate buffer, pH 6.8, containing 0.1 M NaCl, and the suspension was extracted three times using each time one volume of liquefied phenol saturated with 0.5% sodium dodecyl sulfate in 0.02 M phosphate buffer, pH 6.8, containing 0.1 M NaCl. The extractions were performed at room temperature, and every time the phases were separated by centrifugation in a refrigerated centrifuge. The final aqueous phase was treated 5 to 10 times with several volumes of anhydrous ethyl ether to remove the dissolved phenol, and the RNA was precipitated with two volumes of 95% ethanol.

Some variations of the above method were used for the extraction of the rRNA from bacteria that gave unsatisfactory preparations. For the extraction of *P. maltophilia* and of *P. aeruginosa*, bentonite was added to the ribosome suspension in an amount equivalent to half the estimated RNA content (wt/wt). In addition, fresh bentonite was added to the aqueous phase with each phenol extraction. In the extraction of *P. delafieldii*, the French-press extract was allowed to stand for 10 min at room temperature (to promote DNase reaction) before bentonite was added. For the preparation of *Escherichia coli* rRNA, the French-press extract was received directly into buffer-saturated phenol containing bentonite.

All RNA preparations were stored in 70% (vol/vol) ethanol at -10°C . The RNA was centrifuged and resuspended in $0.1 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl and 0.015 M trisodium citrate in water adjusted to pH 7.0 with HCl) before use in competition experiments. Samples of all RNA preparations were checked by sucrose-gradient centrifugation (2 to 20% sucrose in $1 \times \text{SSC}$) for 16 h at $53,000 \times g$. The bulk RNA was used as competitor RNA without further fractionation.

Labeled RNA was prepared from bacteria grown either in ^3H -uridine or ^{32}P -ortho-phosphate-containing media. ^3H -labeled uridine (9×10^{-7} M, 28 Ci per mmol) was added to media containing phosphate buffer (0.033 M, pH 6.8); $(\text{NH}_4)_2\text{SO}_4$, 0.1%; Hutner mineral base (3), 10 ml per liter; (Difco) vitamin-free Casamino Acids, 0.2%; and DL-asparagine, 0.2% for *P. fluorescens*, or DL-lactate, 0.2% for *P. aeruginosa* and *P. maltophilia*. For the labeling of *X. campestris* the composition of the medium was: phosphate buffer (0.033 M, pH 6.8); NH_4Cl , 0.1%; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.05%; ferric-ammonium citrate, 0.005%; CaCl_2 , 0.0005%; and Casamino Acids, 0.5%.

Those strains that did not take up uridine were labeled with ^{32}P (2.5 mCi of carrier-free phosphate per 100 ml of medium). The medium used for *P. cepacia*, *P. acidovorans*, and *P. delafieldii* contained Tris-hydrochloride buffer (0.01 M, pH 7.4); KH_2PO_4 , 0.0025%; NH_4Cl , 0.1%; ferric-ammonium citrate,

0.005%; CaCl_2 , 0.0005%; Casamino Acids, 0.2%; and DL-lactate, 0.2%. To this medium, growth factors (1) were added for the growth of *P. diminuta*.

The cells were harvested when the culture reached a density equivalent to 150 to 175 Klett units (red filter no. 66). The rRNA was extracted by the methods described above, and after sucrose-gradient fractionation, the peaks corresponding to the 23S and 16S RNA were pooled and used as reference RNA. For the hybridization experiments, the DNA was immobilized on nitrocellulose membranes 10 mm in diameter (Schleicher and Schuell, type B-6); each filter had an average of $20 \mu\text{g}$ of DNA. Hybridizations were carried out at 65°C for 8 h in vials containing $1 \mu\text{g}$ of labeled reference RNA and $100 \mu\text{g}$ of bulk competitor RNA in a final volume of 0.25 ml of $2 \times \text{SSC}$. Control vials with labeled RNA and no competitor RNA were also included. After hybridization, the filters were washed once with $2 \times \text{SSC}$ at 65°C and placed individually in vials with 0.2 ml of $2 \times \text{SSC}$ containing 25 μg of pancreatic RNase and 1,500 U (about 4 μg) of T_1 RNase per ml. The vials were incubated for 1 h at 37°C , and the filters were washed twice with $2 \times \text{SSC}$ at 37°C , dried, and counted in a scintillation counter. The percentage of competition was calculated as described by Ballard et al. for DNA-DNA competition experiments (2).

DNA homologies. DNA-DNA homologies were taken from our previously published data and were based on DNA-DNA hybridizations performed at $T_m - 25^\circ\text{C}$ by the competition technique (2).

Phenotypic similarities. Phenotypic matching coefficients (S_{SM}) for 169 characters were calculated as described by Sokal and Sneath (13).

DNA base composition (G plus C mol percent) was determined by using the buoyant density method of

FIG. 1. Ranges of intra- and intergroup rRNA homologies, expressed as percentage of competition in rRNA-DNA hybridizations. The groups are designated by Roman numerals.

TABLE 1. Nucleic acid homologies of group I

Strain	GC in DNA (%)	Reference: <i>P. aeruginosa</i>			Reference: <i>P. fluorescens</i> D31		
		Phenotypic matching coefficient S_{SM}	Competition (%)		Phenotypic matching coefficient S_{SM}	Competition (%)	
			DNA-DNA	rRNA-DNA		DNA-DNA	rRNA-DNA
<i>P. aeruginosa</i> 131, 17503 ^a	68				75	28	87
<i>P. fluorescens</i> D31, 9447 ^a	63	75	28	87			
<i>P. fluorescens</i> A 192, 13525 ^a	60	69	23	93	85	53	102
<i>P. fluorescens</i> B 400, 17815 ^a	62	69	21 ^b	ND ^c	80	26	105
<i>P. fluorescens</i> C 18, 17400 ^a	61	79	24	91	87	46	101
<i>P. fluorescens</i> E 38, 13985 ^a	63	74	17	ND	93	85	101
<i>P. fluorescens</i> F 83, 12983 ^a	59	68	14	ND	79	56	95
<i>P. putida</i> A 90, 12633 ^a	63	71	13	80	75	43	94
<i>P. putida</i> B, 53, 17430 ^a	60	67	16 ^b	85	77	43	97
<i>P. cichorii</i> 758	58	64	ND	88	74	19	99
<i>P. syringae</i> 755	59 ^b	59	0 ^b	92	69	4	97
<i>P. savastanoi</i> 756	58 ^b	60	ND	ND	69	7 ^b	102
<i>P. phaseolicola</i> 753	58 ^b	54	14	88	64	41	97
<i>P. mori</i> 765	ND	56	ND	ND	64	ND	96
<i>P. glycinea</i> 767	58 ^b	51	ND	ND	61	ND	101
<i>P. tomato</i> 759	ND	56	0	89	67	32	98
<i>P. viridiflava</i> VIRID 1	ND	54	ND	ND	62	ND	97
<i>P. stutzeri</i> 221, 17588 ^a	65	72	10 ^b	96	66	57	94
<i>P. stanieri</i> 224, 17591 ^a	62	72	0 ^b	ND	66	ND	93
<i>P. mendocina</i> CH 20, 25411 ^a	62	74	14	95	72	21	88
<i>P. pseudoalcaligenes</i> 63, 17440 ^a	63	70	33	92	67	23	87

^a ATCC number.^b Value obtained with a related strain.^c ND, not done.

TABLE 2. Nucleic acid homologies of groups II and IV

Group	GC in DNA (%)	Phenotypic matching coefficient S_{SM}	Competition (%)	
			DNA-DNA	rRNA-DNA
Group II Reference: <i>P. cepacia</i> 382, 17759 ^a				
<i>P. cepacia</i> 717, 727	68, 66	89, 91	82 ^b	101
<i>P. marginata</i> 704	69	79	35	98
<i>P. caryophylli</i> 721	66	71	24	97
<i>P. pickettii</i> K-288, 27511 ^a	64	69	26	87
<i>P. solanacearum</i> 779	66	56	0 ^c	88
Group IV Reference: <i>P. diminuta</i> 501, 11568 ^a				
<i>P. vesicularis</i> 500, 11426 ^a	66	92	27	96

^a ATCC number.^b Value obtained with strain 727.^c Value obtained with a related strain.

TABLE 3. Nucleic acid homologies of group III

Group III	G + C in DNA (%)	Reference: <i>P. acidovorans</i> 14			Reference: <i>P. delafieldii</i> 134		
		Phenotypic matching coefficient S _{SM}	Competition (%)		Phenotypic matching coefficient S _{SM}	Competition (%)	
			DNA- DNA	rRNA- DNA		DNA- DNA	rRNA- DNA
<i>P. acidovorans</i> 14, 15668 ^a	67				68	0	83
<i>P. testosteroni</i> 78, 11996 ^a	61	85	33	92	70	0	85
<i>P. delafieldii</i> 134, 17506 ^a	65	68	0	89			
<i>P. facilis</i> 332, 17695 ^a	64	63	0	87	89	100	92
<i>P. saccharophila</i> 15946 ^a	69	66	0	79	76	0	95

^a ATCC number.

TABLE 4. RNA homologies of group V

Group V	G + C in DNA (%)	RNA competition (%)	
		Reference	
		<i>P. maltophil</i> 67	<i>X. campestris</i> XC-1
<i>P. maltophilia</i> 67, 13637 ^a	67		95
<i>X. campestris</i> XC-1	68 ^b	92	
<i>X. pruni</i> XP-218	67 ^b	96	97
<i>X. translucens</i> XT-122	68 ^b	93	101

^a ATCC number.^b Value reported for a related strain.

M. Mandel. In a few cases, values were taken from the literature.

RESULTS

Under the conditions of our experiments, the binding of homologous RNA in the absence of competitor ranged from 1.1 to 1.9 µg of immobilized DNA per mg for different species. We do not consider these differences to be significant, particularly in competition experiments, because of good agreement between reciprocal hybridizations. In addition, for technical reasons we have not yet solved the problem of maximal binding of homologous RNA, and therefore no conclusions as to the

fraction of the genome coding for rRNA can be drawn from our data.

The RNA hybridization experiments demonstrate that the pseudomonads that we have studied fall into five RNA homology groups. The internal values for each group are considerably higher than those obtained in hybridizations using strains selected from different groups. In fact, members of different groups are as distantly related to each other as they are to *E. coli* (Fig. 1). The figure also shows the overlap in the ranges of G plus C content of the DNA of the different groups.

Group I corresponds to the *P. fluorescens* DNA homology complex, and group II to the *P. pseudomallei*-*P. cepacia* DNA homology complex (9). Group III comprises two DNA homology groups (the *P. acidovorans* and the *P. facilis*-*P. delafieldii* groups) as well as *P. saccharophila*. Group IV corresponds to the *P. diminuta* DNA homology group. Group V contains *P. maltophilia* and members of the genus *Xanthomonas*.

The intragroup relationships with respect to DNA-DNA and rRNA-DNA homologies using one or two reference strains of each group are shown in Tables 1, 2, 3, and 4. Also included in Tables 1, 2, and 3 are the DNA base compositions and matching coefficients for all strains tested. Because the *Xanthomonas* species have not been analyzed by our methods, no matching coefficients and DNA-DNA homologies are available. That *P. maltophilia* was related by DNA-DNA homology to *Xanthomonas* to a larger extent than other species was suggested by M. P. Starr and Rita Colwell (personal communications).

Were it not for the rRNA-DNA hybridization experiments, we could not have established any relationship among the members contained in this group.

The five groups that we have established on the basis of rRNA homologies seem to be very distantly related to each other phylogenetically. In our opinion, the five RNA homology groups deserve at least independent genus (and possibly family or order) assignment. Although there are certain phenotypic characters studied by us that can be used to differentiate among species within the genus *Pseudomonas*, we have not been able to find enough useful characters (including DNA base composition) to distinguish all species of any one group from those belonging to other groups, with the possible exception of group IV, which has some special

[illegible]

FIG. 2. *Pseudomonas* species and biotypes arranged according to rRNA and DNA homologies. The shaded circles represent rRNA homology groups.

morphological and physiological properties. Hopefully, better characters will be found in the future to distinguish clearly among the groups.

On the basis of many DNA-DNA hybridization experiments previously reported by us and the rRNA homologies reported here, we may at present visualize the relationships among *Pseudomonas* species that we have studied as shown in Fig. 2. The relative distances of the five homology groups from each other are shown in the figure as well as we could achieve in a simple two-dimensional graph.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Grant AI-1808 from the National Institute of Allergy and Infectious Diseases.

REPRINT REQUESTS

Address requests for reprints to: N. J. Palleroni, Research Division, Hoffmann-La Roche, Inc., Nutley, N. J. 07110.

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