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Deoxyribonucleic Acid Homologies Among Some *Pseudomonas* Species

N. J. PALLERONI, R. W. BALLARD, ERICKA RALSTON, AND M. DOUDOROFF

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

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Phylogenetic relationships among a number of strains belonging to the genus *Pseudomonas* were explored by the use of in vitro deoxyribonucleic acid (DNA) hybridization. The fluorescent nomenspecies (*P. fluorescens*, *P. putida*, *P. aeruginosa*, *P. cichorii*, *P. syringae*, and related species), as well as the nonfluorescent species *P. stutzeri*, *P. mendocina*, *P. alcaligenes*, and *P. pseudoalcaligenes*, were shown to belong to a single DNA homology complex which is isolated from other *Pseudomonas* species that have been studied [*P. cepacia* (= *P. multivorans*), *P. caryophylli*, *P. marginata* (= *P. alliicola*), *P. pseudomallei*, *P. acidovorans*, *P. testosteroni*, *P. solanacearum*, *P. diminuta*, *P. facilis*, *P. delafieldii*, *P. saccharophila*, *P. palleronii*]. A limited numerical analysis of the phenotypic properties of the examined strains supported, with some exceptions, their previous allocation to nomenspecies and biotypes. The internal structure and nomenclature of the "*P. fluorescens* homology complex" are discussed.

For the past 7 years, we have been engaged in taxonomic studies of the genus *Pseudomonas*. In the course of this work, we have examined several hundred strains with respect to some 169 phenotypic characters and analyzed the phylogenetic relationships among a number of nomenspecies by deoxyribonucleic acid (DNA) hybridization (1-8, 12). On the basis of phenotypic similarity or DNA homology, or both, we have subdivided the genus into species groups, nomenspecies, and biotypes. Our phenotypic characterization of the subgeneric taxa was based mainly on a subjective analysis of our data, rather than on a numerical or Adansonian approach. In the present studies, some additional experiments with DNA hybridization among members of the genus were performed, and the DNA homologies of the strains examined were related to their previous taxonomic assignments and to their relationships as determined by numerical taxonomy. The data reported also include pertinent information from our previously published studies.

MATERIALS AND METHODS

The "competition" method for determining DNA homologies was the same as that employed previously (2). The 169 phenotypic characters used for numerical taxonomic analyses were also the same as those previously used for distinguishing among different strains and taxa. The pair-wise numerical comparison between strains was made with the aid of a computer to determine the similarity coefficients (S_J), in which positive matches only were taken into account, and the matching coefficients ($S_{S\,M}$), for which both positive and negative matches were considered (11). The cluster analysis based on average similarity coefficients was made for the strains used in the DNA hybridization studies as described by Sokal and Sneath (11).

In the present work, we followed our previous assignment of the various strains to different species and biotypes. Except for those cultures that have been recently added to our collection and are specifically mentioned in the following list, all strains were designated as in our earlier papers, to which references are included for their source of origin and phenotypic characterization:

P. fluorescens, *P. aeruginosa*, *P. putida*, *P. acidovorans*, *P. testosteroni* (12).

P. lemoignei (5).

P. stutzeri, *P. mendocina* (7).

P. cepacia (= *P. multivorans*), *P. marginata* (= *P. alliicola*), *P. caryophylli* (2).

P. diminuta, *P. vesicularis* (1).

P. saccharophila, *P. facilis*, *P. delafieldii*, *P. palleronii* (4).

P. solanacearum (6).

P. pseudomallei (9).

P. alcaligenes strain 142 (12); strains P-37 (K-384) and P-39 (K-477), received from M. J. Pickett, U.C.L.A. Clinical Laboratory (the numbers in parentheses correspond to M. J. Pickett's collection).

P. pseudoalcaligenes strains 63, 65, 66, 197, 299, and 417 (12); strains 440 and 441, received from M. Veron, Pasteur Institute, Paris, France, as strains 63AD (K-311), P-6 (K-379), P-7 (K-401), P-13 (K-504), P-17 (K-532), P-18 (K-546), and P-28 (K-614), received from M. J. Pickett.

P. cichorii strain 758: strain 6 from M. Schroth, Department of Plant Pathology, University of California, Berkeley (NCPPB 1512).

P. glycinea strain 767: strain R-6 from M. Schroth.

P. mori strain 765: strain 1 from M. Schroth (NCPPB 1037).

P. phaseolicola strain 753: strain HB-1-b from M. Schroth; strain 760: strain HB-43 from M. Schroth.

P. savastanoi strain 756: strain OK-21 from M. Schroth; strain 763: strain OK-12 from M. Schroth.

P. syringae strain 754: strain S-7 from M. Schroth; strain 755: strain S-9 from M. Schroth.

RESULTS AND DISCUSSION

The results of the DNA hybridization experiments are presented in Tables 1–9, together with the phenotypic similarity coefficients (S_J) and matching coefficients (S_{SM}) for each pair of strains tested. For the sake of brevity, reciprocal data pertaining to the same pairs of strains are not repeated in the tables, so that all previous tables should be consulted for additional information on the reference strains used in Tables 2–9.

The data reveal that members of the "fluorescent" and "alcaligenes" groups (12), the "stutzeri" group (7), and the fluorescent phytopathogenic species are all interrelated at various levels of DNA homology. None of the strains of the above groups that has been tested shows any significant relationship to any species belonging to other phenotypic or DNA homology groups that have been studied, i.e., *P. acidovorans*, *P. testosteroni*, *P. cepacia* (= *P. multivorans*), *P. mallei*, *P. pseudomallei*, *P. marginata* (= *P. alliicola*), *P. caryophylli*, *P. solanacearum*, *P. diminuta*, *P. saccharophila*, *P. facilis*, or *P. delafieldii* (2, 4, 6, 7). Although our choice of strains for interspecific hybridizations was very limited, our tentative conclusion is that the fluorescent, alcaligenes, and stutzeri groups all belong to a single isolated DNA homology group within the genus *Pseudomonas*. This homology group, which will be henceforth designated as the "*P. fluorescens* complex," includes both fluorescent and nonfluorescent pseudomonads belonging to the nomenspecies *P. fluorescens*, *P. putida*, *P. aeruginosa*, *P. alcaligenes*, *P. pseudoalcaligenes*, *P. mendocina*, *P. stutzeri*, *P. syringae*, *P. tomato*, *P. phaseolicola*, *P. cichorii*, and related species which we have previously reduced to the status of biotypes within the above categories.

In Fig. 1, the species and biotypes belonging to the *P. fluorescens* complex are arranged as well as possible in matrix form according to their relative DNA homologies as determined at 25 C below the T_M (T_M – 25 C). The data used in this figure include those obtained by Palleroni et al. (7) and by E. Ralston (Ph.D. Thesis, Univ. of California, Berkeley, 1971).

TABLE 1. *DNA homologies and phenotypic similarities of P. aeruginosa strain 131*[a]

Species and biotype	Strain	C	C_{80}	S_J	S_{SM}	Species and biotype	Strain	C	C_{80}	S_J	S_{SM}
aeruginosa	45	90	88	94	97	*syringae*	754	*0*		38	59
	52	95	93	93	96	*tomato*	759	*0*		33	56
	132	100	98	94	96	*alcaligenes*	142	**28**	0	31	63
	277	99	98	92	96	*pseudoalcaligenes*	P18	43		41	67
fluorescens A	192	**23**	0	54	69		63	33	22	46	70
fluorescens B	2	22	15	50	68	*mendocina*	CH20	**14**	0	57	74
	413	9	0	58	73	*stutzeri*	220	0		51	71
fluorescens C	18	**24**	**0**	66	79		222	10		50	70
	50	21	10	64	78		223	5		49	70
fluorescens D	31	**28**	**0**	62	75		319	5	0	54	72
fluorescens E	38	17	0	61	74	*solanacearum*	769		*0*	32	60
fluorescens F	83	14		52	68		776	*14*	*0*	39	61
putida A	7	21	0	56	70	*cepacia*	382	**4**		59	68
	49	16		57	72	*marginata*	704	**3**		50	62
	76	18		56	71	*caryophylli*	721	**0**		40	58
	90	**13**	**0**	57	71	*acidovorans*	14	0		50	67
	118	26	0	56	71		105	0	0	50	67
putida B	107	**16**	0	54	67	*diminuta*	501	*0*	0	14	51
phaseolicola	753	*14*	*0*	26	54	*delafieldii*	134	*0*		32	56

[a] *C*: DNA-DNA competition values at T_M – 25 C. C_{80}: DNA-DNA competition values at 80 C. S_J: "similarity coefficients" for positive matches only. S_{SM}: "matching coefficients" for positive and negative matches. Bold-face DNA competition values are average values obtained with both the homologous DNA and heterologous DNA as unsheared immobilized reference DNA. Italicized competition values are those obtained with only the heterologous DNA used as reference.

TABLE 2. *DNA homologies and phenotypic similarities of P. fluorescens biotype A strain 192*[a]

Species and biotype	Strain	C	C_{80}	S_J	S_{SM}	Species and biotype	Strain	C	C_{80}	S_J	S_{SM}
fluorescens A	12	73	58	84	92	*putida* B	53	44	23	60	73
	184	60	49	77	88		98	37	0	59	74
fluorescens B	2	67	49	74	86		107	*32*	*27*	63	76
	93	52	39	71	83	*cichorii*	758	26	18	60	77
	400	**70**	**67**	67	81	*phaseolicola*	753	**37**	**0**	38	66
fluorescens C	18	**52**	**25**	66	80	*savastanoi*	763	30	0	38	66
	50	69	33	64	78	*syringae*	754	**31**	**4**	58	77
	191	64		71	82	*tomato*	759	**30**	**0**	53	75
fluorescens D	31	**53**	**20**	74	84	*alcaligenes*	142	*16*	*3*	31	64
	390	59	30	66	80	*pseudoalcaligenes*	63	*18*	*17*	42	68
fluorescens E	37	62	27	74	85	*mendocina*	CH20	*27*	**0**	53	72
	38	57	18	74	85	*solanacearum*	769	*0*	*0*	34	63
	86	58		74	85		776	*0*	*0*	47	69
fluorescens F	83	58	27	76	87	*marginata*	704	*6*		63	75
putida A	7	38		54	70	*caryophylli*	721	0		49	67
	49	40	8	55	72	*acidovorans*	14	*8*		46	64
	90	**33**	**14**	55	70	*testosteroni*	78	0		36	60
	118	50	9	64	78	*diminuta*	501	**1**		12	52

[a] See footnote *a* of Table 1. Consult also Table 1 for *P. aeruginosa*.

TABLE 3. *DNA homologies and phenotypic similarities of P. fluorescens biotype B strain 400*[a]

Species and biotype	Strain	C	C_{80}	S_J	S_{SM}	Species and biotype	Strain	C	C_{80}	S_J	S_{SM}
aeruginosa	132	21		51	69	*cichorii*	758	9		51	73
fluorescens A	12	81	59	66	82	*phaseolicola*	753	**16**	5	42	72
	184	61	42	66	82		760	16		37	70
fluorescens B	2	55		71	85	*mori*	765	12		44	71
	413	21	28	69	83	*glycinea*	767	13		39	70
fluorescens C	18	**55**	*17*	59	76	*savastanoi*	763	8		36	69
	50	62		64	80	*syringae*	754	**11**	*0*	56	77
	191	56		60	75		755	14		56	77
fluorescens D	390	60	32	63	79	*tomato*	759	*27*	*0*		75
	394	54		62	79	*alcaligenes*	142	33		28	66
fluorescens E	37	63		65	80	*pseudoalcaligenes*	65	**9**		40	69
	38	46	22	62	78	*mendocina*	CH139	14		54	74
putida A	90	**33**	**4**	53	71	*stutzeri*	222	0		46	70
	118	24		57	75	*cepacia*	382	0		53	66
putida B	53	44	39	56	72	*marginata*	704	0		53	67
	107	**40**	41	51	69	*acidovorans*	14	0		42	63

[a] See footnote *a* of Table 1. Consult also previous tables for pertinent data.

Two species unrelated to the complex (*P. acidovorans* and *P. caryophylli*) are also included in the matrix for comparison. In the figures, the areas of different shading represent very approximately the fractions of strains tested that show different levels of interstrain DNA homology. To simplify the figure, *P. fluorescens* biotypes A, B, and F have been combined because of overlapping homology values between strains of these groups. The closely related *P. fluorescens* biotypes D and E are also treated as a unit. The oxidase-negative phytopathogenic pseudomonads are combined under the designation "syringae group," without any implications of the nomenclatural status of the component nomenspecies, because too few representative strains were used in our studies. The single strain of the oxidase-positive species *P. cichorii* is not included in the figure. Three general comments should be made about the construction and significance of the matrix presented in Fig. 1: (i) as in all such simplified diagrams, the selection of shading intensity is purely arbitrary and, in our case, was somewhat influenced by the previous assignment of the strains to various species and biotypes; (ii) the number of strains tested for intergroup homology was generally very small

TABLE 4. *DNA homologies and phenotypic similarities of P. fluorescens biotype C strain 18*[a]

Species and biotype	Strain	C	C_{80}	S_J	S_{SM}	Species and biotype	Strain	C	C_{80}	S_J	S_{SM}
aeruginosa	52	26	15	62	77	*phaseolicola*	753	**18**	**0**	30	59
fluorescens A	12	44	17	67	82	*savastanoi*	756	24	0	38	65
	184	52	27	60	77	*syringae*	754	**25**	**10**	44	66
fluorescens B	2	40	18	64	80		755	29	6	45	66
	93	40	22	67	80	*tomato*	759	**13**	**2**	40	65
fluorescens C	50	78	64	88	94	*alcaligenes*	142	13		30	64
	191	62	52	84	91	*pseudoalcaligenes*	63	*12*		39	67
fluorescens D	31	**46**	**26**	65	78	*mendocina*	CH20	18	0		68
	390	38	0	58	73	*stutzeri*	221	0		44	67
	394	26	1	62	77		222	3		43	66
fluorescens E	37	36	10	59	75	*solanacearum*	769		*0*	32	62
	38	36	0	60	75		776	*0*	*0*	39	63
	86	39	0	62	76	*cepacia*	382	0		58	69
fluorescens F	83	46	26	66	80	*marginata*	704	0		57	70
putida A	7	23	5	52	68	*caryophylli*	721	0		41	61
	90	25	19	56	72	*acidovorans*	14	*8*	*0*	43	62
putida B	53	45	28	58	72	*testosteroni*	78	0		37	61
	107	*41*	**37**	59	74	*diminuta*	501	**6**		14	54
cichorii	758	14	8	45	66						

[a] See footnote *a* of Table 1. Consult also previous tables for pertinent data.

TABLE 5. *DNA homologies and phenotypic similarities of P. fluorescens biotype D strain 31*[a]

Species and biotype	Strain	C	C_{80}	S_J	$S_{S\,M}$	Species and biotype	Strain	C	C_{80}	S_J	$S_{S\,M}$
fluorescens A	12	47	21	72	84	*phaseolicola*	753	**41**	*0*	37	63
	184	60		71	84		760	41		32	61
fluorescens B	2	42	23	67	80	*savastanoi*	763	7		45	68
	93	62	28	71	81	*syringae*	754	**27**	**0**	47	67
	400	26	3	64	78		755	4		49	68
fluorescens C	50	46	20	66	79	*tomato*	759	30	0	45	66
	191	50	21	70	80	*pseudoalcaligenes*	63	23		43	67
fluorescens D	390	87	78	88	93	*mendocina*	CH20	*21*	*0*	55	72
	394	98	86	87	92	*stutzeri*	220		14	43	64
fluorescens E	37	87	77	81	89		221	57	24	44	66
	38	85	71	88	93	*solanacearum*	776	*7*		48	69
	86	86	82	90	94	*cepacia*	382	0		63	72
fluorescens F	83	56	27	76	86	*marginata*	704	*18*		58	69
putida A	7	36	11	62	75	*caryophylli*	721	*0*		47	64
	90	**43**	**9**	63	76	*acidovorans*	14	0		43	60
putida B	53	43	27	62	74		105	0		42	60
	107	*49*	**44**	66	77	*diminuta*	501	0		13	48
cichorii	758	19		56	73						

[a] See footnote *a* of Table 1. Consult also previous tables for pertinent data.

as compared with that tested for intragroup relationship. In some cases, a single pair of strains belonging to different species was analyzed. This may lead to an erroneous impression of intra- and intergroup relationships, especially if an aberrant strain of any given group is used in the DNA hybridization experiments; (iii) the two-dimensional form of the matrix cannot be expected to give a clear picture of the multidimensional branching course of species evolution. Many different graphic orders of presentation of the species and biotypes are possible and have been tried, but none of these appeared to be preferable to the one illustrated.

A comparison of DNA homology values obtained at the more restrictive hybridization temperature of 80 C with those obtained at $T_M - 25$ C (Tables 1-9) shows that, in general, the 80 C values remain high for closely related strains but decrease, often to zero, for more distantly related ones. For example, *P. aerugi-*

TABLE 6. *DNA homologies and phenotypic similarities of P. putida biotype A strain 90*[a]

Species and biotype	Strain	C	C_{80}	S_J	S_{SM}	Species and biotype	Strain	C	C_{80}	S_J	S_{SM}
fluorescens B	2	21		55	70	*cichorii*	758	28	0	50	69
	413	45		69	82	*phaseolicola*	753	**22**	**0**	34	61
fluorescens C	50	31	0	52	69	*savastanoi*	756	26	0	39	65
fluorescens E	38	34		59	73	*syringae*	754	**18**	**2**	46	67
fluorescens F	83	35	0	58	73		755	27	0	47	67
putida A	7	52	27	87	93	*tomato*	759	**24**	**3**	41	64
	42	50	47	89	94	*pseudoalcaligenes*	63	*21*	*24*	43	69
	49	86	92	90	95	*mendocina*	CH50	11		58	75
	76	68	40	88	94		CH139	14		57	74
	77	63	39	87	93	*stutzeri*	222	*0*		45	67
	81	78	66	90	95		319	16		48	68
	100	54	34	71	84	*solanacearum*	776	*13*	*0*	41	64
	118	56	56	74	85	*cepacia*	382	*0*		56	66
	145	55	54	83	91	*marginata*	704	*0*		53	66
	154	60	36	85	92	*caryophylli*	721	*0*		44	63
	160	63	47	84	91	*acidovorans*	14	**3**	*0*	49	66
putida B	53	18		77	85	*diminuta*	501	*0*		14	52
	98	22	0	70	82						
	107	**36**	**17**	65	77						
	110	28	16	74	84						

[a] See footnote *a* of Table 1. Consult also previous tables for pertinent data.

TABLE 7. *DNA homologies and phenotypic similarities of P. putida biotype B strain 107*[a]

Species and biotype	Strain	C	C_{80}	S_J	S_{SM}	Species and biotype	Strain	C	C_{80}	S_J	S_{SM}
fluorescens B	2	24		60	75	*syringae*	754	18		47	67
	413	58	60	60	75	*glycinea*	765	25		31	58
fluorescens E	38	51	46		77	*mori*	765	32		40	63
putida A	7	42		65	78	*mendocina*	CH20	17		51	69
	49	44		66	78	*stutzeri*	221	12		39	61
putida B	53	76	74	81	88		224	30		41	62
	98	51	45	73	84		319	0		42	61
	110	71	64	75	84	*solanacearum*	776	*8*		41	63
cichorii	758	30		54	72	*cepacia*	382	0		64	74
phaseolicola	753	32		37	63	*marginata*	704	*8*		58	69
	760	29		31	59	*diminuta*	501	9		10	47
savastanoi	763	19		36	63						

[a] See footnote *a* of Table 1. Consult also previous tables for pertinent data.

TABLE 8. *DNA homologies and phenotypic similarities of P. phaseolicola strain 753*[a]

Species and biotype	Strain	C	C_{80}	S_J	S_{SM}	Species and biotype	Strain	C	C_{80}	S_J	S_{SM}
cichorii	758	45	14	55	81	*mendocina*	CH120	23	0	32	66
phaseolicola	760	99	99	83	96	*stutzeri*	226	11	0	34	70
mori	765	83	75	59	85	*solanacearum*	730	0		35	72
glycinea	767	87	81	55	85	*cepacia*	725	7	0	31	50
savastanoi	763	80	75	60	88	*marginata*	704	0		34	56
syringae	754	**64**	**44**	55	82	*caryophylli*	720	0		33	66
	755	69	52	58	83	*acidovorans*	14	0		21	53
tomato	759	**61**	**36**	61	86	*testosteroni*	78	0		23	61
alcaligenes	142	12		26	75						

[a] See footnote *a* of Table 1. Consult also previous tables for pertinent data.

TABLE 9. *DNA homologies and phenotypic similarities of P. syringae strain 754 and P. tomato strain 759*[a]

Reference		*P. syringae* 754				*P. tomato* 759			
Species	Strain	C	C_{80}	S_J	S_{SM}	C	C_{80}	S_J	S_{SM}
aeruginosa	132			39	61	10	0	34	58
fluorescens D	394			49	71	34	0	41	66
cichorii	758	39	12	78	92	36	13	68	82
phaseolicola	760	60	25	52	81	63	24	58	85
mori	765	60	43	71	89	70	56	71	89
glycinea	767	55	27	61	85	61	28	69	82
savastanoi	756	59	29	70	89	66	58	67	88
	763	53	19	57	84	55	36	59	85
syringae	755	100	100	85	94	66	53	74	89
tomato	759	**60**	**35**	73	89	NA[b]	NA	NA	NA
alcaligenes	142	0		30	70	0		23	68
mendocina	CH51	5	0	45	70			37	66
	CH120			44	70	30	0	35	65
stutzeri	226	5	0	41	69	20	0	34	66
solanacearum	730	0		46	74	0		41	72
cepacia	725	0		47	62	0		41	58
marginata	704	0		53	70	0		47	65
caryophylli	720	0		59	79	0		48	73
pseudomallei	1691	0							
acidovorans	14	0		37	61	0		27	54
testosteroni	78	0		32	62	0		24	58
diminuta	501	**0**		18	66	0		11	64

[a] See footnote *a* of Table 1. Consult also previous tables for pertinent data.
[b] Not applicable.

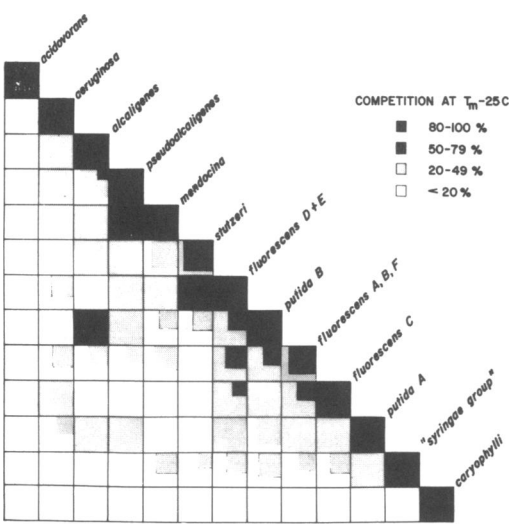

FIG. 1. *Ranges of interstrain DNA homology for nomenspecies and biotypes of the P. fluorescens complex, as well as for P. acidovorans and P. caryophylli.*

nosa strains which have interstrain homologies of 90 to 100% at T_M – 25 C, show homologies of 88 to 98% at 80 C, whereas *P. aeruginosa* and *P. putida* strains, which have homologies of 13 to 21% at the lower temperature, show no homology whatever at 80 C. If the 80 C values are used for constructing a matrix such as that presented in Fig. 1, the *P. fluorescens* complex falls apart into its constituent subgroups or species which, however, can still be arranged in the same graphic order as in Fig. 1. We have not included a figure of such a matrix because it does not show the more distant relationships among the species and because fewer interstrain homologies were determined at 80 C. Furthermore, the values obtained at 80 C are probably less reliable than those at T_M – 25 C because of a greater and less controllable loss of immobilized DNA during hybridization at the higher temperature.

In Fig. 2, the intra- and intergroup phenotypic relationships, as indicated by matching coefficients ($S_{S\ M}$), are shown in simplified matrix form, with the species and biotypes arranged in the same order as in Fig. 1. In this figure all strains used in our DNA hybridization experiments are included.

A different and more detailed analysis of the phenotypic resemblances among the strains of the *P. fluorescens* complex (with the exception of the syringae group) is shown in the dendrogram based on "average similarity coefficients" (S_J) presented in Fig. 3.

It was satisfying to see that, with few exceptions, the numerical taxonomic analysis of

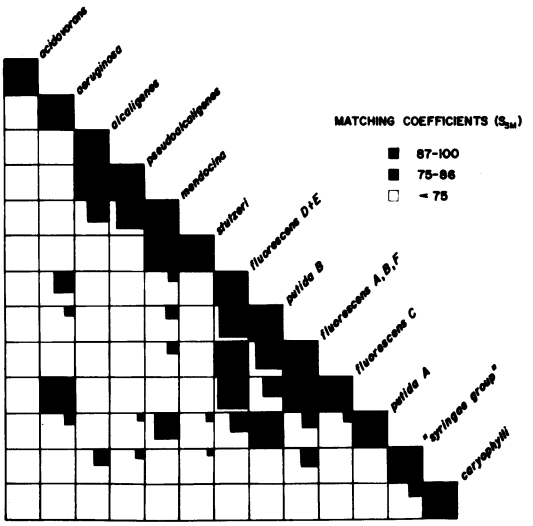

FIG. 2. *Ranges of interstrain phenotypic matching coefficients (S_{SM}) for the species and biotypes arranged as in Fig. 1.*

phenotypic characters (Fig. 2) and the DNA hybridization data (Fig. 1) agree quite well with the assignment of the various strains studied by us to different species and biotypes that had been made previously on the basis of a more or less subjective analysis of phenotypic characters. This subjective analysis was, of course, based on the same data as those used for the computer study, but was partly influenced by the conscious or unconscious differential weighting of certain characters.

Among the more interesting conclusions that can be drawn from the tables and figures are (i) that *P. mendocina*, which had been previously assigned by us to the stutzeri group (7), can, in fact, be regarded as a link between this group and the alcaligenes group (12) on the basis of both matching coefficients and DNA homology with *P. pseudoalcaligenes*, and (ii) that *P. putida* biotype B, although phenotypically most closely related to *P. putida* biotype A, appears to be, in fact, more closely related by DNA homology to *P. fluorescens* and to certain other members of the complex. The nomenclatural problem presented by this group of strains will be discussed later, but it should be obvious that discrepancies between DNA homologies and phenotypic data can be expected *a priori*. Such discrepancies may be due to divergent or convergent evolution, or both, within different phylogenetic lines, or simply to the choice of phenotypic traits used to differentiate among strain clusters. For example, the phytopathogenic *P. syringae*

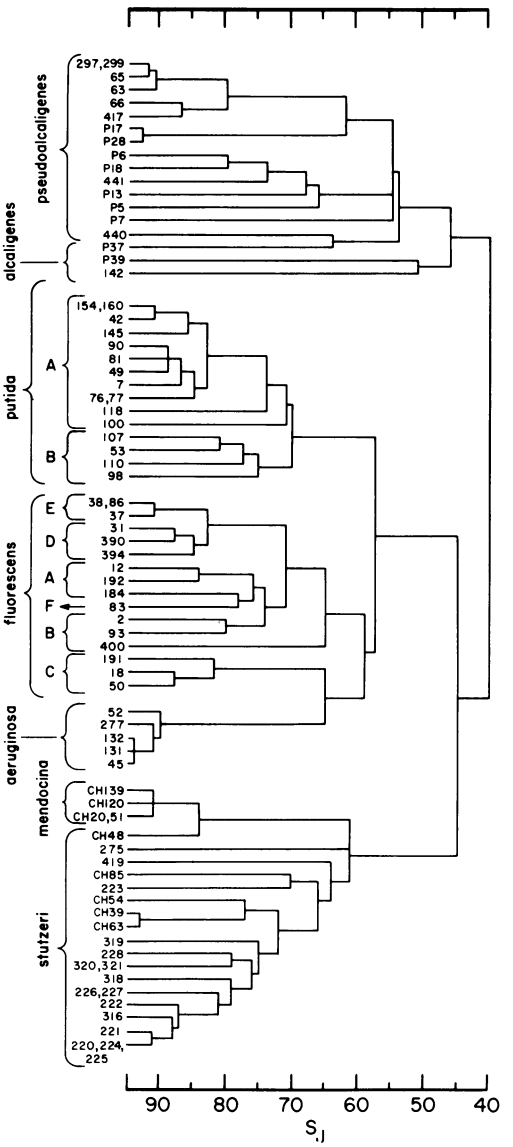

FIG. 3. *Cluster analysis of some strains of the P. fluorescens complex based on average similarity coefficients (S_J).*

shows less phenotypic similarity to some other members of the *P. fluorescens* complex than to certain other phytopathogenic species (e.g., *P. caryophylli*, *P. solanacearum*) which appear to be phylogenetically very distant from *P. syringae* (Table 9). Although it is tempting to ascribe this to convergent evolution, it should be remembered that, without any knowledge of the ancestral forms of the groups in question, it is impossible to draw any conclusions regarding the origin of the apparent similarities.

With some notable exceptions, one taxonomically important phenotypic character distinguishes the *P. fluorescens* complex from all other species that have been included in the DNA homology studies, although not from all other known species of the genus. This character is the inability to accumulate poly-β-hydroxybutyrate as an intracellular carbon reserve, which is characteristic of nearly all members of the complex. However, poly-β-hydroxybutyrate is accumulated by approximately half of the strains of *P. pseudoalcaligenes* (including the type strain). Nevertheless, such polymer-accumulating strains show a high DNA homology with other strains of this nomenspecies and no homology with any of the selected polymer-accumulating species outside the complex. More convincing phenotypic evidence of the phylogenetic relatedness among the fluorescent and nonfluorescent members of the *P. fluorescens* complex has been adduced from the biochemistry of the degradation of aromatic compounds and from immunological studies of the induced isofunctional enzymes involved (12; H. Kita, *personal communication*).

The data presented here and in our previous studies reveal very few clusters consisting of more than two strains that show a very high interstrain DNA homology (C) and phenotypic similarity ($S_{S\,M}$). The notable exceptions are the clusters corresponding to the nomenspecies *P. aeruginosa* (C, 90 to 100%; $S_{S\,M}$, 96 to 97%) and *P. mendocina* (C, 98 to 100%; $S_{S\,M}$, 96 to 98%). These two groups represent what might be considered to be "ideal" bacterial species. Clusters that show somewhat lower, but nevertheless great internal homogeneity are to be found within the nomenspecies *P. pseudoalcaligenes*, *P. stutzeri*, and *P. fluorescens* (biotypes D and E). It is, of course, virtually certain that more such homogeneous clusters will be discovered when more strains are examined. In Fig. 4, some $S_{S\,M}$ values are plotted against DNA competition values for the reference strain of *P. aeruginosa* used in Table 1.

P. alcaligenes is clearly a heterogeneous group, so far consisting of only three strains in our collection (Fig. 3). As mentioned earlier, *P. pseudoalcaligenes* is the only nomenspecies in the "*P. fluorescens* complex" that contains some strains which accumulate poly-β-hydroxybutyrate. This group is also heterogeneous with respect to the arginine dihydrolase reaction, another important diagnostic character in the identification of *Pseudomonas* species. These two properties do not show any correlation with each other in different strains, nor with the DNA homologies between the strains.

P. stutzeri is a heterogeneous group with respect to phenotypic properties, DNA composition [ca. 61 to 66% guanine plus cytosine (GC)], and DNA homology. Members of this group are, however, readily recognizable, and we have previously proposed to retain them all in a single nomenspecies because we could find no suitable phenotypic characters to distinguish among representatives of the four DNA homology groups within this complex (7). Numerical taxonomy supports our previous subjective analysis. In Fig. 5, the DNA competition values reported earlier for one reference strain of *P. stutzeri* are plotted against $S_{S\,M}$ values, and the approximate DNA composition of the various strains is indicated. As can be seen from Fig. 5, the strains most closely related to each other in their DNA composition tend to cluster together, but there is an overlap in the phenotypes of strains that have different DNA compositions and which have been previously assigned to different intraspecific DNA homology groups I–IV by Palleroni et al. (7). A very similar picture is obtained when the S_J rather than $S_{S\,M}$ values are plotted, or when other strains of *P. stutzeri* are used for reference. The discrepancies between phenotypic characters and DNA homologies in

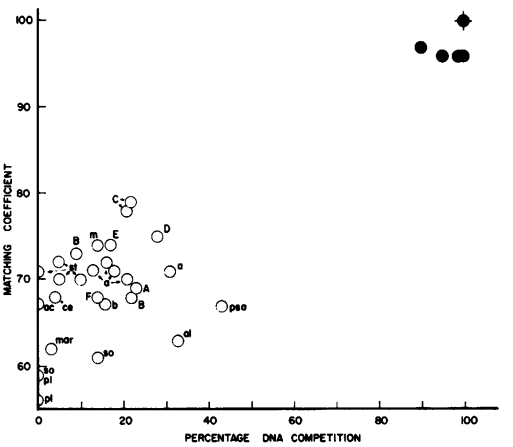

FIG. 4. *Matching coefficients (S_{SM}) versus DNA competition values (at T_M–25 C) with P. aeruginosa strain 131 as reference. Symbols: ✦, reference strain; ●, other P. aeruginosa strains; a, P. putida A; b, P. putida B; A, P. fluorescens A; B, P. fluorescens B; C, P. fluorescens C; D, P. fluorescens D; E, P. fluorescens E; F, P. fluorescens F; pl, fluorescent plant pathogens; m, P. mendocina; st, P. stutzeri; al, P. alcaligenes; psa, P. pseudoalcaligenes; ac, P. acidovorans; ce, P. capacia; mar, P. marginata; so, P. solanacearum.*

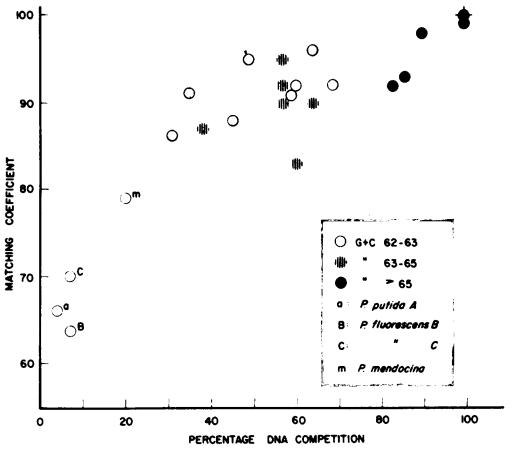

FIG. 5. *Matching coefficients (S_{SM}) versus DNA competition values (at $T_M - 25$ C) with P. stutzeri strain 221 as reference. ✦, reference strain; other symbols are shown in the figure.*

this nomenspecies is also illustrated in Fig. 3 in the light of DNA homology. The almost identical strains 320 and 321 show relatively little similarity to the fairly homogeneous cluster consisting of strains 220, 224, 221, and 316. Yet, strains 320, 220, and 224 belong to the intraspecific DNA homology group I, as defined by Palleroni et al. (7), strains 321 and 221 to their group II, and strain 316 to group IV.

The group of strains representing *P. fluorescens* biotype A (including typical *P. fluorescens*), biotype B (including the nomenspecies *P. marginalis*), and biotype F (nomenspecies *P. lemonnieri*) is clearly heterogeneous in phenotypic properties and shows only moderately high and overlapping interstrain DNA homology values. These biotypes will have to be studied in far greater detail before any significant conclusions can be drawn with respect to their phylogenetic and nomenclatural relationships. Biotype C appears to represent a separate evolutionary line within the species, which may, on further study, be found to merit specific rank. *P. fluorescens* biotype D and biotype E (nomenspecies *P. chlororaphis* and *P. aureofaciens*, respectively) are very closely related to each other as evidenced by numerical taxonomy and DNA homology. They have identical DNA composition with a GC content intermediate between that of *P. fluorescens* and of *P. aeruginosa*, and are characterized by the production of closely related green or yellow phenazine pigments.

E. putida biotype A (including typical *P. putida*) shows a relatively low level of DNA homology with several members of the *P. fluorescens* complex and appears to represent a separate phylogenetic line, deserving specific rank. This conclusion is strongly supported by the immunological studies of isofunctional enzymes involved in benzoate metabolism (13). As mentioned earlier, *P. putida* biotype B, although similar to *P. putida* A in overall phenotype, appears to be more closely related to *P. fluorescens* with respect to DNA homology. Convincing evidence of the phylogenetic relationship of several strains of this biotype has been adduced from the studies of Stanier et al. (13) and the unpublished results of H. Kita, who have demonstrated that the muconate lactonizing enzyme of selected strains of *P. putida* B showed closer immunological relationship to the homologous enzymes of *P. fluorescens* B than of *P. putida* A. Clearly, the location of *P. putida* B in the species *P. putida* is not warranted on the basis of phylogenetic considerations, although it might be defended for pragmatic purposes.

The arginine dihydrolase-negative, oxidase-negative, phytopathogenic fluorescent pseudomonads, lumped together as the syringae group, represent a separate evolutionary branch of the *P. fluorescens* complex. We have studied too few strains belonging to the innumerable nomenspecies or "pathotypes" to draw any unequivocal conclusions about the speciation with this group. Although experienced plant pathologists can recognize some of these nomenspecies, and a numerical analysis of the nutritional and physiological characters of a number of strains has revealed some phenotypic clusters corresponding to the pathotypes (10), there are still considerable problems of identifying the nomenspecies. Our findings that two strains labeled *P. syringae* show virtually complete DNA homology, as do also two strains received as *P. phaseolicola*, suggest that there may also be very homogeneous DNA clusters within the group. A similar conclusion has been reached by Paul Pecknold of the Plant Pathology Department at the University of California at Davis, who has examined the DNA homologies of a larger collection of *P. syringae* and some related nomenspecies (*personal communication*).

P. cichorii, of which only one strain was studied by us, and which is not included in the figures, is a fluorescent plant pathogen that resembles phenotypically the members of the syringae group; it differs from the syringae group mainly in possessing cytochrome *c* (positive oxidase reaction), which is absent in this group but present in all other species of the *P.*

fluorescens complex. As can be seen from the tables, the DNA hybridization experiments show that this nomenspecies is, indeed, more closely related to the syringae group than to other fluorescent pseudomonads, but less closely than the members of the group are related to each other.

On the basis of our very limited examination of the fluorescent phytopathogens, we concur with the proposal of Sands et al. (10) to retain the nomenspecies *P. cichorii* and to provisionally combine the oxidase-negative nomenspecies in a single species, *P. syringae*. It should be noted that such a species, which would encompass several pathotypes or biotypes, would be no less homogeneous than *P. putida* (biotype A), *P. stutzeri*, *P. fluorescens*, or *P. alcaligenes*. It seems to us that the syringae group offers particularly interesting and useful material for more detailed studies on bacterial speciation.

Although our studies represent the collaborative effort of several members of our group extending over a period of some 3 years, we do not pretend to have done more than scratch the surface of the problem of speciation in the genus *Pseudomonas*. We hope, however, that we have at least indicated some areas in which further studies might be most fruitfully pursued. We do not think that the data presented in our tables are sufficient to attempt any meaningful statistical analysis of phenotypic resemblance and DNA homology of different species and biotypes, because too few reference strains were used and too few interspecific hybridizations were attempted. We do, however, believe that the information provided may be useful to other workers interested in bacterial phylogeny, taxonomy, and genetics. For example, amino acid sequence analyses and immunological studies of homologous enzymes of different *Pseudomonas* species are now under way. Obviously, the selection of the bacterial strains used for such studies and the interpretation of the results can be facilitated by the knowledge of the DNA homologies and the overall phenotypic similarities among the available cultures.

With our present methodology, a complete analysis of the DNA homology relationships among all strains included in our study could easily occupy a lifetime. The reference strains that we used were selected with a certain amount of prejudice (e.g., because they were type strains or were received by us as typical representatives of various nomenspecies). The choice of reference strains is crucial to the circumscription of DNA homology clusters as well as of phenotypic taxa. In future work, it is hoped that reference strains will be chosen because they are "centrally" located in any given group. Ideally, such strains will be found by preliminary DNA homology studies with a few different reference strains within each group. Alternatively, such central strains may be selected on the basis of numerical taxonomic analysis of phenotypic properties, although the pitfalls inherent in this approach have been clearly demonstrated in the case of *P. stutzeri* and *P. putida*.

Our demonstration of a large and isolated DNA homology complex within the genus *Pseudomonas* does not, unfortunately, give any immediate promise of being helpful in subdivision of this uncomfortably large genus into taxonomically useful generic groups, because no clear-cut phenotypic characters differentiate between members of this complex and most other *Pseudomonas* species. Obviously, DNA homology determination is not a useful tool for the primary assignment of an unidentified strain to a given genus. An arbitrary splitting of the genus into several genera would simply shift the burden of using complicated phenotypic determinative keys from recognizing species to recognizing genera. It should also be emphasized that even the isolation of the *P. fluorescens* complex from other DNA homology groups within the genus may disappear when more species are examined. Further studies using DNA hybridization, as well as hybridization between DNA and ribosomal ribonucleic acid, should, however, give us a greater insight into the phylogeny of this group of bacteria and, hopefully, into broader problems of bacterial speciation. Such studies are now being pursued in our laboratory.

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