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"Genetic Species" Concept in *Xanthomonas*

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ABSTRACT

FRIEDMAN, S. (State University, Ghent, Belgium), AND J. DE LEY. "Genetic species" concept in *Xanthomonas*. J. Bacteriol. **89**:95–100. 1965.—Deuterated, N^{15}-labeled deoxyribonucleic acid (DNA) from *Xanthomonas pelargonii* forms distinct hybrids with ordinary DNA from *X. hederae*, *X. juglandis*, and *X. carotae*. Hybridization is less pronounced with *X. phaseoli* and *X. begoniae*. There is evidence that some hybridization occurs with *X. vesicatoria*, *X. campestris*, and *X. tamarindi*. These results favor the concept of a "genetic species," rather than a division of the genus into many separate species based almost entirely on phytopathogenic host specificity.

It appears that bacterial classification is now in a transitory state, and that changes may be expected in the near future as the result of the application of new approaches such as Adansonian analysis, molecular biology, and comparative biochemistry. At the subgeneric level, evidence is accumulating in favor of the concept of a "genetic species" or "genospecies" (Ravin, 1963), which is opposed to the current practice of splitting up genera into many species based only on small phenotypic differences. Some aspects of this problem were briefly discussed on a previous occasion (De Ley and Friedman, 1964). With the genus *Acetobacter* as a model system, evidence for this view was presented on the basis of biochemical and enzymatic data (De Ley, 1961), deoxyribonucleic acid (DNA) base composition (De Ley and Schell, 1963), and DNA hybrids (De Ley and Friedman, 1964). It is to be expected that a similar situation may hold in many, if not all, genera.

In this study, the genus *Xanthomonas* was selected as another example. *Bergey's Manual* and Prévot (1961) list over 60 species. Krassilnikov (1959) includes some 70 xanthomonads and related strains in *Pseudomonas*. A critical examination of the differences among these strains makes it doubtful that so many separate species actually exist (Starr, 1959; De Ley, 1964). Species differentiation is almost entirely based on phytopathogenic host specificity. The results of Dye (1958), however, show that considerable cross-infection exists. There is also a high degree of cross-reactivity among antisera to their strains (Soda and Cleverdon, 1960). Physiologically and biochemically, all xanthomonads are nearly indistinguishable, except for small differences in pigmentation, gelatin liquefaction, starch hydrolysis, nitrate reduction, and ammonia production. Colwell and Liston (1961) carried out an Adansonian analysis of 16 species of *Xanthomonas*, and were unable to find significant differences among them. The DNA base composition of several of these strains was determined by De Ley and Van Muylem (1963). The "melting points," T_m, were between 96.4 and 97.3 C, indicating that *Xanthomonas* may comprise a narrow group of closely related strains, possibly representing only one or a few genetic species. One obvious check on this hypothesis, the formation of DNA hybrids between these strains, is reported in the present paper. The positive outcome of these experiments substantiates the prediction by one of us (De Ley, 1964) "that the number of species in *Xanthomonas* will be reduced to very few in the near future." As with *Acetobacter* (De Ley and Friedman, 1964), the present results are in accord with the concept of a "genospecies."

MATERIALS AND METHODS

Organisms. The strains were the same as those used in a previous study (De Ley and Van Muylem, 1963). These strains, and some of the characteristics of their DNA, are listed in Table 1.

Techniques of cultivation. The strains were grown at 22 to 25 C for 2 to 3 days in Roux flasks in a medium containing 2% glucose, 2% $CaCO_3$, 1% yeast extract (Nederlandsche Gist & Spiritusfabriek, Brugge, Belgium), and 2.5% agar (Difco).

Cultivation of deuterated, N^{15}-labeled X. pelargonii P121. A synthetic medium was devised to allow the uptake of $(N^{15}H_4)_2SO_4$ as sole nitrogen source. It contained 0.545% KH_2PO_4, 0.368% Na_2HPO_4, 0.025% $MgSO_4 \cdot 7H_2O$, 0.0005% $FeCl_3$,

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0.1% NaCl, 1% $CaCO_3$, 0.1% $(N^{15}H_4)_2SO_4$, trace elements, traces of amino acids and vitamins (De Ley and Friedman, 1964); the final *p*H was 7.0. This medium was lyophilized overnight. Then 2% (w/v) anhydrous glucose, 2.5% agar, and heavy water were added. The medium was dispensed in 100-ml portions in Roux flasks and sterilized in a pressure cooker containing heavy water at 120 C for 20 min. The bacteria had previously been adapted to the heavy-water medium by serial transfer onto slants of increasing concentrations of D_2O. The Roux flasks were inoculated from a slant with fully adapted organisms, and growth was spread evenly after a few days. On the D_2O medium, 10 to 14 days were required for full growth, compared with 3 to 4 days on H_2O medium. *X. pelargonii* P121 was selected for the preparation of deuterated N^{15}-labeled DNA (DN^{15}-DNA) because it grew fairly well, its guanine plus cytosine (G + C) content was about average, and the compositional distribution of its DNA molecules was broad enough to overlap with the DNA of all the other strains.

Preparation of DNA, heating and annealing of DN^{15}-DNA and ordinary DNA, and CsCl density-gradient centrifugation. We followed exactly the same procedures described in our previous paper (De Ley and Friedman, 1964), except that the heating was done at 110 to 112 C and the *Escherichia coli* phosphodiesterase treatment was carried out for 4 hr with 50 units of enzyme. (The phosphodiesterase preparation was kindly donated by I. R. Lehman, Stanford University, Palo Alto, Calif.) The activity of the preparation on single-stranded DNA was determined by incubating the enzyme with a mixture of single- and double-stranded DNA from *X. pelargonii* P121. DNA from *Cytophaga* sp. 292 was always used as a reference (De Ley and Friedman, 1964).

Estimation of molecular weight of DNA. The procedure was carried out with a Spinco model E ultracentrifuge at 37,020 rpm and 20 C, in ultraviolet light and with a concentration of 40 μg of DNA/ml of buffer (0.15 M NaCl and 0.015 M trisodium citrate, *p*H 7.0). The molecular weight was calculated from the sedimentation coefficient, with the formula $S_0^{20\,C} = 0.063\ M_w^{0.37}$ (Doty, McGill, and Rice, 1958). The high molecular weights (Table 1) show that the DNA was in good condition for hybridization.

RESULTS

DN^{15}-DNA from *X. pelargonii* P121 was found by CsCl density-gradient centrifugation to have a buoyant density of 1.7475 ± 0.0006 g/cc (average of 30 estimations). The buoyant density of ordinary DNA from the same strain was calculated to be 1.7255 g/cc (Table 1). These values agreed very well with the experimentally observed values for native and heated and annealed DNA (Fig. 1c). Density-gradient centrifugation of a mixture of native ordinary DNA of homologous or heterologous strains and DN^{15}-DNA *X. pelargonii* resulted in two completely separate peaks, similar to the bands when the two DNA preparations were heated and annealed separately (Fig. 1c, 4c, and 5a).

As a control, ordinary and DN^{15}-DNA from *X. pelargonii* P121 were heated and annealed together. The resulting band (Fig. 1a) persisted,

TABLE 1. *Properties of purified DNA from various strains of Xanthomonas, and hybridization with DN^{15}-DNA from X. pelargonii P121**

H strain	Strain no.	T_m	G + C	σ	Buoyant density	Molecular wt	Hybridization with DN^{15}-DNA from strain P121	
							Peak	Per cent
		C	%		*g/cc*			
X. phaseoli	P162	96.4	66.0	0	1.7251	11.5×10^6	Weak	35 ± 3
X. juglandis	J107	96.5	66.3	0.5	1.7253	5.2×10^6	Distinct	43 ± 1
X. vesicatoria	V136	96.55	66.4	0.06	1.7253	11.7×10^6	None	26 ± 3
X. pelargonii	P121	96.6	66.5	0.65	1.7255	10.0×10^6	Distinct	(complete)
X. pelargonii (DN^{15}-labeled)		96.5	66.3	0.62	1.7475	13.5×10^6		
X. carotae	C104	96.8	67.0	0.75	1.7260	18.0×10^6	Distinct	49 ± 2
X. begoniae	B3	96.85	67.1	0	1.7261	9.8×10^6	Weak	32 ± 5
X. hederae	H1	96.9	67.2	0.63	1.7265	15.0×10^6	Distinct	52 ± 3
X. tamarindi	T20	97.1	67.6	0.42	1.7268	11.6×10^6	None	22 ± 5
X. campestris	C129	97.3	68.2	0.3	1.7272	6.8×10^6	None	25 ± 3

* Values for T_m, G + C, and σ (variance of the compositional distribution) are taken from De Ley and Van Muylem (1963), except for the values of DN^{15}-DNA and for the σ value of strain C129 which, upon redetermination with our present preparation, was found to be 0.3 instead of 0.04. The buoyant density = 0.098 (molecular fraction of G + C) + 1.660 g/cc (Schildkraut, Marmur, and Doty, 1962). The buoyant density of DN^{15}-DNA was determined experimentally.

after hydrolysis with phosphodiesterase, as a major hybrid peak with both types of renatured DNA visible as shoulders (Fig. 1b). The buoyant density of the hybrid was 1.736 g/cc, as expected. As in the previous case with *Acetobacter*, these experiments served a double purpose: hybridization showed that the DN^{15}-DNA was indeed derived from *X. pelargonii* P121 and not from a contaminant; the experiments also showed the optimal type of hybridization to be expected. The difference in buoyant density between DN^{15}-DNA and ordinary DNA was too small to allow a clean separation of the three peaks. A similar difficulty was previously encountered with *Acetobacter*.

Heating and annealing of DN^{15}-DNA from *X. pelargonii* together with ordinary DNA from *X. hederae* (Fig. 2), *X. juglandis*, and *X. carotae*

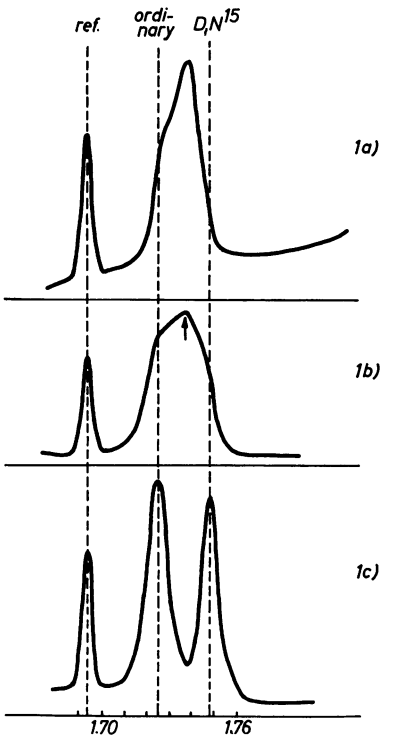

FIG. 1. *Hybrid formation between DN^{15}-DNA and ordinary DNA from Xanthomonas pelargonii P121; 1 μg of Cytophaga DNA was added as reference. (a) Both types of DNA were heated and annealed together, at 15* μg/ml each; not treated with phosphodiesterase; 5 *μg of DNA in the gradient. (b) Same but treated with phosphodiesterase; 12 μg of DNA in the gradient prior to enzyme hydrolysis. (c) Same as b but the two DNA preparations were heated and annealed separately.*

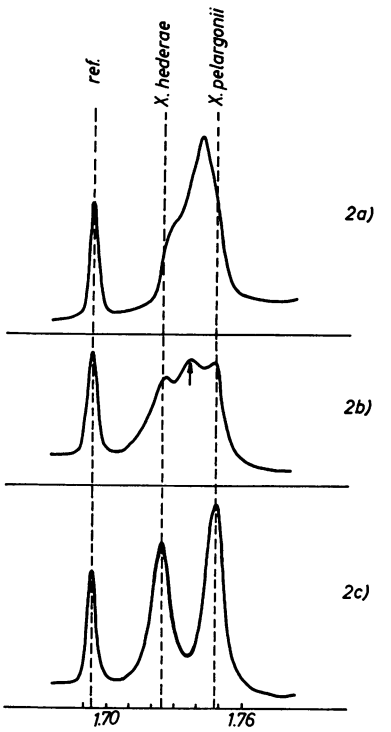

FIG. 2. *Hybrid formation between DN^{15}-DNA from Xanthomonas pelargonii P121 and ordinary DNA from X. hederae H1; 1 μg of Cytophaga DNA was added as reference. (a) Both types of DNA were heated and annealed together (15 μg/ml each); not treated with phosphodiesterase; 5 μg of DNA in the gradient. (b) Same but treated with phosphodiesterase; 12 μg of DNA in the gradient prior to enzyme hydrolysis. (c) Same as b but the DNA preparations were heated and annealed separately.*

(Fig. 3) showed quite distinct hybrid bands. These bands were less pronounced with *X. phaseoli* (Fig. 4b) and *X. begoniae* (Fig. 5d). No hybrid bands were formed with *X. tamarindi*, *X. campestris*, and *X. vesicatoria* (Fig. 5). It is possible, however, that a small amount of hybrid was formed but was not visible as a distinct band. Inspection of Fig. 5a or 4b and 4c shows that heating and annealing separately of ordinary and DN^{15}-DNA resulted in two distinct bands with a deep valley in between. Heating and annealing together of the same DNA types resulted in two confluent bands with little separation. For the last three strains, therefore, it seems likely that the valley between the two bands contains a small amount of hybrid.

The amount of hybrid formed in each case was roughly estimated by determining the areas, as

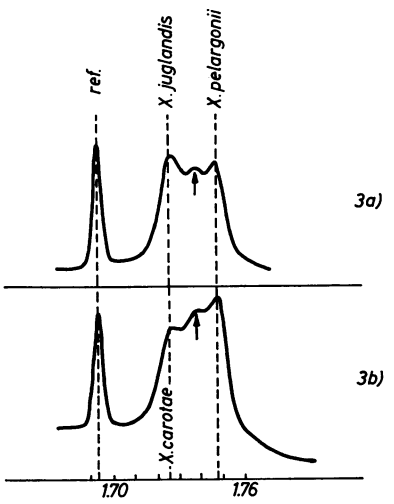

FIG. 3. *Hybrid formation between DN^{15}-DNA from Xanthomonas pelargonii P121 and ordinary DNA from either X. juglandis J107 (3a) or X. carotae C104 (3b) after treatment with phosphodiesterase. Methods as for Fig. 2b. The tracings without phosphodiesterase treatment or with the DNA preparations heated and annealed separately for each strain are similar to Fig. 2a and 2c, and are therefore not shown.*

described for *Acetobacter* (De Ley and Friedman, 1964).

DISCUSSION

It has been established that DNA from *X. pelargonii* P121 readily produces hybrids with that of other strains of the same genus, such as *X. hederae* H1, *X. juglandis* J107, *X. carotae* C104, and, less distinctly, with *X. phaseoli* P162 and *X. begoniae* B3. Although no separate hybrid peaks occurred with *X. tamarindi* T20, *X. campestris* C129, and *X. vesicatoria* V136, considerations of the depth of the valley between the renatured peaks and calculations of the percentage of hybridization favor the opinion that a small amount of hybrid might have been formed. This evidence, however, is only indirect and will need extension by a different experimental approach.

The compositional distribution curves of DNA from these strains (Fig. 6) provide one explanation for the quantitative aspects of hybridization. Strains H1, J107, and C104 have relatively broad DNA distributions which overlap considerably with strain P121. Extensive hybridization can be expected. These strains indeed formed visible hybrid bands, with the highest percentage of hybridization (43 to 52%). Strains P162, V136, and B3 have very narrow distributions of their DNA molecules. Not many hybrid molecules can be expected, therefore, in spite of the fact that their G + C content is close to that of strain P121. Finally, strains T20 and C129 have rather broad distributions, but there is little overlapping with DNA from strain P121 because their mean G + C content is slightly higher. The slight hybridization fulfilled expectations.

Taxonomic implications. As our group of strains was a random choice, it is likely that many more hybrids can be made between other "species" of *Xanthomonas*. The present results show that the structural similarity in DNA from several taxonomic species is considerable. All of the taxonomic implications which we set forth for *Acetobacter* (De Ley and Friedman, 1964) also hold here. The present results lend strength to the concept of a genetic species, rather than to the present division

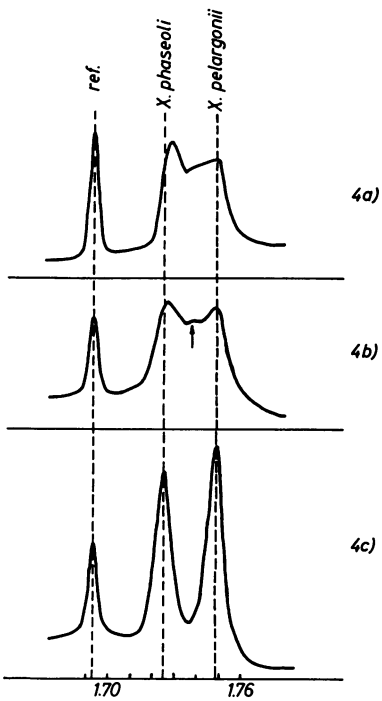

FIG. 4. *Weak hybrid formation between DN^{15}-DNA from Xanthomonas pelargonii P121 and ordinary DNA from X. phaseoli P162; 1 μg of Cytophaga DNA added as reference. (a) DNA preparations heated and annealed together (15 μg/ml each); not treated with phosphodiesterase; 3.8 μg of DNA in the gradient. (b) Same but treated with phosphodiesterase; 12 μg of DNA in the gradient prior to enzyme hydrolysis. (c) Same as b but the DNA preparations were heated and annealed separately.*

of the genus *Xanthomonas* into sharply delineated taxonomic species based largely on (possibly illusory) host specificity. These results established the need for a new approach to the taxonomy of *Xanthomonas*. The practical conclusion from our hybridization experiments seems to be that the great number of species names in *Xanthomonas* is perhaps no longer justified, and might be decreased to a few or perhaps even to only one.

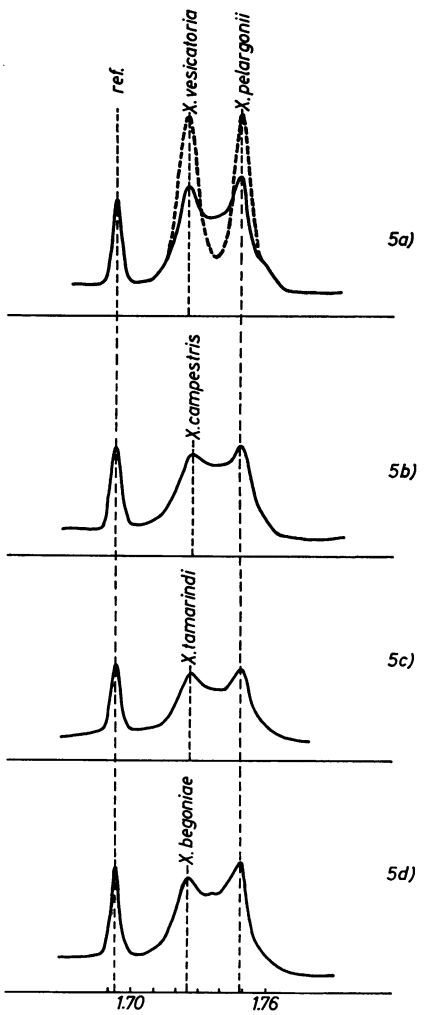

FIG. 5. *Attempted hybridization beteenw DN^{15}-DNA from Xanthomonas pelargonii P121 and ordinary DNA from several other Xanthomonas strains. Treated with phosphodiesterase. Methods as for Fig. 4. Since the controls (no phosphodiesterase treatment; each DNA heated and annealed separately) for each strain were similar to those in Fig. 4a and 4c, they are not included. For clarity, the dotted line in Fig. 5a represents each DNA after heating and annealing separately.*

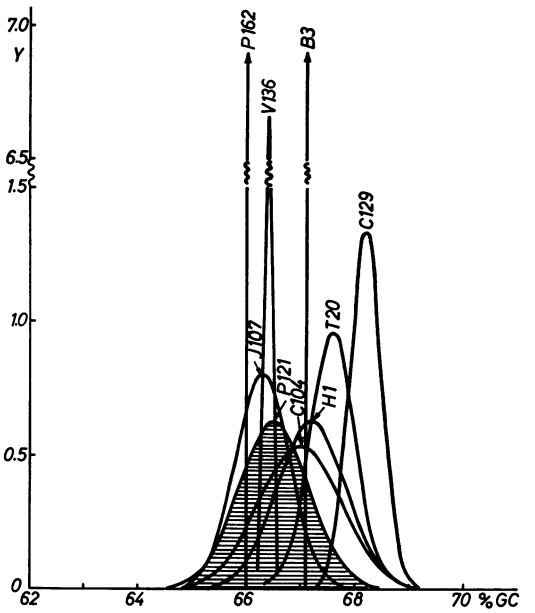

FIG. 6. *Approximation of the compositional distribution of the DNA molecules of the Xanthomonas strains used. The Gaussian distribution around the mean % (G + C) was calculated with the formula* $y = (1/\sigma\sqrt{2\pi}) \times e^{-x^2/2\sigma^2}$. *Values for* σ *were taken from Table 1. For x, values expressed as % (G + C) around the mean were used. The shaded area represents the compositional distribution of DN^{15}-DNA and ordinary DNA from Xanthomonas pelargonii P121.*

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