Thermal Stability of DNA: DNA Hybrids within the Genus *Agrobacterium*

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SUMMARY

Molecular hybrids were prepared between unlabelled DNA from representative strains of eleven genetic races of *Agrobacterium* and [¹⁴C]DNA from typical strains of each of the three main races. The thermal stability of each hybrid was determined. The nature of the hybrids formed varied with the incubation temperature and the kind of DNA used. Hybridization in 2 × SSC–30 % dimethylsulphoxide below 59 °C yielded two kinds of hybrids: a labile one of unknown nature, denaturing below 59 °C, and a more or less stable hybrid denaturing above that temperature. The latter was the only one formed in hybridizations at or above 59 °C. There were three kinds of stable hybrids. Within each of the main *Agrobacterium* races thermal stability of the molecular hybrid was about the same (within 2 °C) as for the homo-duplex. Between two races of 50 % DNA relatedness, the duplexes were about 6 °C less stable. Between races of 10 to 15 % DNA relatedness, the duplexes were weak, and the stability was at least 13 °C lower. The stability of the hybrids decreased concomitantly with the degree of DNA relatedness. The decreased hybrid denaturation curve is not due to AT-rich sequences. The less two races of *agrobacteria* appeared to be evolutionarily related, the more mutations occurred within the common part.

INTRODUCTION

From previous studies it appeared that the genus *Agrobacterium* is genetically very heterogeneous. DNA of cluster 1 (typical *Agrobacterium tumefaciens* and *A. radiobacter* strains), cluster 2 (*A. rhizogenes* and atypical *A. tumefaciens* strains), the ‘rubi’ group and two very small groups hybridize at about 10 to 15 %. Within cluster 1 the DNA of the seven groups hybridize at about 50 % and within each group at least 80 % (De Ley, 1972; Kersters, De Ley, Sneath & Sackin, 1973; J. De Ley, A. Reynaerts & H. Cattoir, unpublished).

Mutational events modified considerable parts of the genomes, preventing *in vitro* molecular hybridization. Considering the evolutionary history of a bacterial genus, one can wonder whether mutations also occurred within the common DNA parts. For an experimental answer to that question we prepared a number of DNA-hybrids between the genomes of different *Agrobacterium* groups and clusters, and determined their thermal stability which is interpreted as a measure of base-pairing imperfections, and mutational differences (Brenner & Cowie, 1967). We established that decreased hybrid denaturation curves were not due to preferential binding of heterologous AT-rich DNA sequences.

METHODS

Bacteria. The strains used are listed in Table 1; many of them are briefly described elsewhere (Kerstens *et al.* 1973). As controls we included *Salmonella typhimurium* 1 and *Acetobacter aceti* ch 31, which have a very low relationship with the agrobacteria (J. De Smedt & J. De Ley, unpublished).

The agrobacteria were grown on a medium of (g/l): yeast extract paste, 10; glucose, 10; $(\text{NH}_4)_2\text{SO}_4$, 1; and KH_2PO_4 , 0.25. *Salmonella* were grown on beef extract (Oxoid), 10; peptone, 10; and NaCl, 5. *Acetobacters* were grown on yeast extract paste, 10; glucose, 50; and CaCO_3 , 30. All media were made with 2.5 % agar in tap water. All bacteria were grown at 28 to 30 °C for 2 to 3 days, harvested and washed.

DNA preparation. DNA from all strains was extracted and purified as described by De Ley, Cattoir & Reynaerts (1970).

^{14}C -Labelled DNA from the reference strains *Agrobacterium tumefaciens* B6, TT111 and B2a, and *A. rhizogenes* TR7 were prepared as described previously (De Ley, Bernaerts, Rassel & Guilmet, 1966; Heberlein, De Ley & Tijtgat, 1967) with Marmur's (1961) method, supplemented by a pronase treatment of 40 µg/ml at 37 °C for about 15 h. The specific activity was 3700, 4000, 3600 and 5500 d.p.m./µg respectively.

Purified native DNA for fixation on a filter had a molecular weight of 5 to 10×10^6 . Labelled DNA for hybridization was sheared to a molecular weight of 4×10^5 by passage through a French pressure cell at 21 000 lb/in².

Fixation of denatured, high molecular weight DNA on membrane filters. This was carried out as described previously (De Ley & Tijtgat, 1970). PHWP Millipore filters were used, diam. 47 or 90 mm. Smaller filters of diam. 9 mm were punched out of them after fixation of the DNA, each containing usually 20 to 22 µg of DNA.

DNA: DNA hybridization. We used the method of Legault-Démare, Desseaux, Heyman, Séror & Ress (1967), in the conditions recommended by De Ley & Tijtgat (1970). The final volume was 0.8 ml $2 \times \text{SSC-DMSO}$, containing 3 µg of sheared, denatured, ^{14}C -labelled reference DNA and a 9 mm DNA-loaded filter. The optimal renaturation temperature of 59 °C was commonly used, although 42 °C, 62 °C and 72 °C were used occasionally. SSC buffer contained 0.15 M-NaCl in 0.015 M-sodium citrate, pH 7.0; $2 \times \text{SSC-DMSO}$ contains $2 \times \text{SSC}$ solution in a mixture of 70 % water and 30 % dimethylsulphoxide.

Determination of thermal stability of DNA: DNA hybrids. After hybridization the filters were removed and washed in $2 \times \text{SSC-DMSO}$. They were incubated for 15 min in each one of a series of small flasks, containing 2 ml of $2 \times \text{SSC-DMSO}$, in a series of water baths of increasing temperatures in 3 °C steps from the hybridization temperature up to 89 °C. No residual counts remained on the filters at 89 °C. The amount of labelled DNA removed was counted in a Tricarb 3310 Liquid Scintillation Spectrometer, by adding 0.5 ml portions of the washings to 19.5 ml of Bray (1960) solution, cooled to 2 °C for several hours and counted repeatedly for 20 to 50 min. $T_{m(50)}$ is the thermal elution midpoint, the temperature at which 50 % of the DNA bound (at the 59 °C hybridization temperature) is eluted.

RESULTS

Kinetics of DNA hybridization

In a previous paper (De Ley & Tijtgat, 1970) we established the optimal conditions for DNA hybridizations on membrane filters. We report here on the different types of *Agrobacterium* DNA hybrids which are formed as one varies the incubation time and temperature.

Fig. 1. Kinetics of homologous and heterologous *Agrobacterium* DNA hybridization. Thermal stability of the hybrids. Sheared, single-stranded [^{14}C]labelled DNA of *Agrobacterium tumefaciens* TTIII was incubated in $2\times\text{SSC}$ -DMSO with filter-fixed ordinary, high-molecular-weight, single-stranded DNA from either strain TTIII or *A. tumefaciens* B6 at either 42 °C, 62 °C or 72 °C for several lengths of time as shown on the graphs. The filter was removed and washed. The thermal stability was determined as described under Methods. The crosses (x) are the melting points of elution $T_{m(e)}$. Full lines are melting curves of the homoduplexes [^{14}C]TTIII:TTIII, broken lines of the heteroduplexes [^{14}C]TTIII:B6. The numbers next to the curves represent the time of hybridization in h. The incubation temperatures are shown in the upper left corners.

As a model system we prepared the homoduplex TTIII:[^{14}C]TTIII, and the heteroduplex B6:[^{14}C]TTIII in $2\times\text{SSC}$ -DMSO at 42 °C and 62 °C after 2, 4, 8 and 16 h of incubation, and at 72 °C after 16 h of incubation. The thermal stability of all these duplexes was determined. The results are shown in Fig. 1.

At 42 °C two types of duplexes are formed. The first is weakly bound and denatures below 59 °C. We shall call it here the 'labile duplex'. The second type displays a more stable denaturation profile. We shall call it here the 'stable duplex'. The labile duplex is formed much faster than the stable one. About one-half of the amount is formed after 2 h of incubation. There is more of the labile heteroduplex than of the labile homoduplex under identical conditions. The labile duplex is not formed when the hybridization is carried out at 62 °C, and *a fortiori* at 72 °C.

The formation of the labile duplex consumes a considerable amount of the available reference [^{14}C]DNA. This explains why the amount of stable duplex is about twice as much at 62 °C as it is at 42 °C. The midpoint of elution, $T_{m(e)}$, of the stable duplex is the same for each type of duplex at both working temperatures: 76 °C for the stable homoduplex TTIII:TTIII and 70 °C for the stable heteroduplex B6:TTIII. This difference of 6 °C between both types of duplexes was an important observation and led to all following experiments.

When hybridization is carried out at 72 °C, only a fraction of the stable duplex is formed: one-third of the homoduplex and only 11 % of the heteroduplex. The $T_{m(e)}$ of the homoduplex is now higher (77 °C) and that of the heteroduplex is 74 °C.

Table 1. $T_{m(e)}$ of the stable duplexes between DNA from a number of *Agrobacterium* strains with [^{14}C]DNA from the reference strains *A. tumefaciens* TT111 and B6 and from *A. rhizogenes* TR7

The results are expressed in °C. Abbreviations of strain numbers as in Kersters *et al.* (1973). 'Low' refers to a weakly stable molecular hybrid with $T_{m(e)} \leq 62$ °C. The division into clusters and groups was made according to De Ley (1972) and Kersters *et al.* (1973).

Bacteria (name as received)	DNA of strain on membrane filter	Reference [¹⁴ C]DNA		
		ICPB TT111	B6	ICPB TR7
	Cluster 1 TT111 group			
<i>A. tumefaciens</i>	ICPB TT111	75.4 ± 0.2	68.4	—
<i>A. radiobacter</i>	NCIB 8149	74.3	68.8	—
<i>A. tumefaciens</i>	NCPPB 1001	72.2	67.9	—
<i>A. tumefaciens</i>	A6	74.1	68.6	—
<i>A. tumefaciens</i>	II CHR	73.5	68.4	—
<i>A. tumefaciens</i>	ATB	74.0	68.7	—
<i>A. tumefaciens</i>	ATV	73.4	69.0	—
<i>A. tumefaciens</i>	Gembloux A	75.9	70.2	—
Presumed <i>Agrobacterium</i>	AB 1558	74.4	69.1	—
<i>A. tumefaciens</i>	NCPPB 4	75.5	—	—
<i>A. tumefaciens</i>	NCPPB 5	75.6	—	—
<i>A. tumefaciens</i>	ATCC 143	75.7	70.0	—
<i>A. tumefaciens</i>	ATCC 4720	75.6	—	—
<i>A. tumefaciens</i>	ICPB TT6	75.9	—	—
<i>A. tumefaciens</i>	NCPPB 398	74.6	69.4	—
<i>A. tumefaciens</i>	NCPPB 396	75	—	—
<i>A. tumefaciens</i>	ICPB TT5	72	69.4	—
<i>A. tumefaciens</i>	ICPB TT7	72	69.1	—
<i>A. tumefaciens</i>	ICPB TT8	71.9	69	—
<i>A. tumefaciens</i>	NCPPB 1674	74.9	69.4	—
<i>A. tumefaciens</i>	IIBV7	75.1	69.1	—
<i>A. tumefaciens</i>	Mushin 3	76	69.9	—
<i>A. tumefaciens</i>	Mushin 5	75.9	69.1	—
<i>A. tumefaciens</i>	181	—	—	low
<i>A. tumefaciens</i>	E. III 9.6.1	76	69.2	—
<i>A. tumefaciens</i>	IPO 417	75.2	68.7	—
<i>A. tumefaciens</i>	AT3	—	69.5	—
	B6 group			
<i>A. tumefaciens</i>	CIP B6	69.5	75.8	—
<i>A. tumefaciens</i>	B6-806	69	76	—
<i>A. tumefaciens</i>	B2a	69.3	75.6	—
<i>A. radiobacter</i>	ICPB TR6	68.6	—	—
<i>A. radiobacter</i>	S1005	69.1	75.5	—
<i>A. radiobacter</i>	ATCC 4718	69.4	—	—
<i>A. radiobacter</i>	ICPB TR1	69.6	—	—
<i>A. tumefaciens</i>	Delft F	69.9	75.1	—
<i>A. tumefaciens</i>	ATCC 4452	70.4	—	—
<i>Agrobacterium</i> sp.	Kerr 14	70.1	75.5	—
<i>A. tumefaciens</i>	ATCC 11156	69.9	—	—
<i>A. tumefaciens</i>	ATCC 11158	70.2	76	—
<i>A. tumefaciens</i>	ICPB TT2	68.8	75.5	—
<i>A. tumefaciens</i>	ICPB TT11	67.1	75.1	—
<i>A. radiobacter</i>	ATCC 13332	—	—	low
<i>Agrobacterium</i> sp.	O322	68.8	75.3	—
<i>A. tumefaciens</i>	NIAS I	—	76.3	—
<i>A. tumefaciens</i>	NIAS A-2-1-1	—	75.8	—
<i>A. tumefaciens</i>	H5	—	—	low
	RV3 group			
<i>A. tumefaciens</i>	CIP RV3	69.6	—	—
<i>A. tumefaciens</i>	Zutra 3/1	—	71.7	—
<i>A. tumefaciens</i>	NCPPB 1641	66.4	71.5	—

Table 1 (cont.)

Bacteria (name as received)	DNA of strain on membrane filter	Reference [¹⁴ C]DNA		
		ICPB TT111	B6	ICPB TR7
	0362 group			
<i>Agrobacterium</i> sp.	0362	67.5	70.9	low
<i>Agrobacterium</i> sp.	0363	65.7	70.5	low
	TT9 group			
<i>A. tumefaciens</i>	ICPB TT9	66.4	69.9	—
<i>A. tumefaciens</i>	T37	68.2	69.4	—
<i>A. tumefaciens</i>	Mushin 6	68.9	70.1	—
	F/1 group			
<i>A. tumefaciens</i>	Zutra F/1	—	70.1	—
<i>A. tumefaciens</i>	NCPBP 925	67.2	69.4	—
	M 2/1 group			
<i>A. radiobacter</i>	M 2/1	67.5	69.9	—
<i>A. radiobacter</i>	L 2/2/1	67.7	—	—
	Cluster 2			
<i>A. rhizogenes</i>	ICPB TR7	low	low	76.6
<i>A. rhizogenes</i>	ICPB TR101	low	low	75.9
<i>Agrobacterium</i> sp.	Kerr 38	low	low	75.9
<i>Agrobacterium</i> sp.	Kerr 39	low	low	76.5
<i>A. tumefaciens</i>	ICPB TT133	low	low	76.2
<i>A. tumefaciens</i>	NCPBP 1649	low	low	75.3
<i>A. tumefaciens</i>	NCPBP 1651	low	low	75.9
<i>A. rhizogenes</i>	ICPB TR107	—	—	76.6
<i>A. tumefaciens</i>	P2	—	—	76.5
<i>Agrobacterium</i> sp.	0321	—	—	76.1
<i>A. tumefaciens</i>	Apple 185	—	—	76.4
<i>A. rhizogenes</i>	NCIB 8196	—	—	75.4
<i>A. tumefaciens</i>	PPI 49b	—	—	76.2
<i>A. tumefaciens</i>	PPI 6	—	—	75.9
<i>A. tumefaciens</i>	PPI 1	—	—	76.1
<i>A. tumefaciens</i>	NIAS S1	—	—	75.8
<i>A. tumefaciens</i>	AG 6	—	—	76.1
<i>A. tumefaciens</i>	AG 1	—	—	75.6
<i>A. tumefaciens</i>	NCPBP 794	—	—	71.8
<i>A. tumefaciens</i>	NCPBP 223	—	—	75.7
<i>A. rhizogenes</i>	ATCC 11325	—	—	74.7
<i>A. rhizogenes</i>	ATCC 15834	—	—	74.2
	'rubi' group			
<i>A. rubi</i>	ICPB TR2	low	low	low
<i>A. rubi</i>	ATCC 13335	—	—	low
<i>A. tumefaciens</i>	EU 6	—	—	low
	Separate strains			
<i>A. tumefaciens</i>	NCPBP 1650	low	low	low
<i>A. tumefaciens</i>	NCPBP 1771	low	low	low
	Other organisms			
<i>Salmonella typhimurium</i>	1	low	—	—
<i>Acetobacter aceti</i>	ch31	low	—	—

Thermal stability of intra- and intergroup stable DNA: DNA hybrid

As reference strains for hybridization we selected a representative of each of the three major *Agrobacterium* groups: *Agrobacterium tumefaciens* B6 and TT111 from the groups of the same name in cluster 1 and *A. rhizogenes* TR7 from cluster 2 (De Ley, 1972; Kersters *et al.* 1973). In most cases only the stable duplex was allowed to form, by hybridization at 59 °C, followed by denaturing the hybrid. It is apparent (Table 1 and Fig. 2) that three kinds of stable hybrids are formed, within *Agrobacterium*. One type is usually almost

indistinguishable from the homologous duplexes with about 2000 c.p.m. of [^{14}C]DNA duplexed on the filter and a $T_{m(e)}$ of about 75 °C in $2 \times \text{SSC-DMSO}$. The second type hybridizes less, with about 1000 c.p.m. of duplexed labelled DNA, and has a $T_{m(e)}$ between 67 °C and 71 °C. The third type is formed in small amounts, about 200 c.p.m. being duplexed; it has no normal denaturation curve. A few examples are given in Fig. 2. Average values are calculated in Table 2.

The very small 'rubi' group and the individual strains NCPPB1650 and NCPPB1771 hybridize at about 10 % with clusters 1 and 2, and among themselves (De Ley, 1972; J. De Ley, A. Reynaerts and H. Cattoir, unpublished). The denaturation curve (Table 1) of the hybrids is very low.

The nature of the stable heteroduplex

There are two possible explanations for the low melting point of the heteroduplexes. One is that the molecular hybrid is formed by AT-rich sequences. Alternatively the presence of imperfectly matching base pairs may decrease the stability and $T_{m(e)}$ of heterologous duplexes. Experimental results favour the latter explanation. The hybrid fraction of [^{14}C]DNA from *Agrobacterium tumefaciens* B2a was prepared on a larger scale by hybridizing first in ten separate flasks, each containing 4 µg of labelled DNA, with 48 µg of *A. tumefaciens* A6 DNA on a membrane filter, for 16 h at 59 °C in $2 \times \text{SSC-DMSO}$. The hybrid fraction of [^{14}C]B2a DNA was then released by denaturing each filter separately for 15 min at 89 °C in the same 1.2 ml of $2 \times \text{SSC-DMSO}$. A fresh membrane filter with 46 µg ordinary denatured B2a-DNA was added to the latter solution and incubated for 16 h at 59 °C. Similar experiments were carried out with filter-fixed ordinary DNA from *Agrobacterium* sp. 0321. The results are shown in Fig. 3. In both cases the duplex of B2a: released [^{14}C]B2a had the same denaturation curve with exactly the same $T_{m(e)}$ as the original total homoduplex. This result excludes the possibility that the heteroduplexes are formed preferentially from AT-rich sequences.

Stability of molecular hybrids formed at 42 °C

In the cases of low hybridization at 59 °C or 62 °C we checked whether the shape of the stability curve would reveal the existence of another, weaker duplex, by hybridizing at a lower temperature. We determined the thermal stability of duplexes after hybridization at 42 °C between [^{14}C]DNA of *Agrobacterium tumefaciens* TT111 and filter-fixed DNA of several other strains from different groups (see Table 1). In each case there is a similar peak of the labile duplex below 55 °C. No new peaks appeared.

Percentage of DNA relatedness

Direct hybridization is not the best method for the determination of the percentage DNA relatedness (Hoyer, McCarthy & Bolton, 1964), and it was not our explicit purpose to measure it. The calculation of a high or moderate percentage DNA relatedness between individual strains relative to a reference strain is not meaningful in direct hybridization because of the high standard deviation of about 20 %. For strains with low relatedness (about 15 % or less) the absolute value of the error becomes negligible. Comparisons between group averages are statistically more meaningful. Table 3 shows that the DNA relatedness of the stable duplex between the groups TT111 and B6 is about 55 %. Between both groups on the one hand and either cluster 2, group TR2, strain 1650 or 1771 on the other hand, the percentage is about 12 %. This figure agrees excellently with results (J. De Ley *et al.* unpublished) using the initial renaturation rate method (De Ley, Cattoir & Reynaerts, 1970) where the data are respectively about 50 % and 10 to 15 %. With *Salmonella typhimurium*, *Aceto-*

Fig. 2. Typical differential denaturation curves of *Agrobacterium* DNA-hybrids. The three columns give examples of hybridizations with [^{14}C]DNA of *Agrobacterium tumefaciens* TT111, B6 and *A. rhizogenes* TR7, respectively. The top row represents homologous duplexes; the second row, duplexes with strains of the same group; the first and the second curves of the third row are of duplexes between the B6 and TT111 groups, all other cases represent different combinations between cluster 2 (TR7) and either the B6 or TT111 groups. Methods: see text.

Table 2. Average $T_{m(e)}$ in °C and standard deviation, of the intra- and intergroup stable heterologous DNA hybrids

Cluster or group	Reference [^{14}C]DNA		
	TT111	B6	TR7
TT111	74.6 ± 1.4	69.1 ± 0.6	low
B6	69.3 ± 0.9	75.6 ± 0.4	low
Cluster 2	low	low	75.7 ± 1
RV3	68.0 ± 1.6	71.6 ± 0.1	—
O362	66.6 ± 0.9	70.7 ± 0.2	low
TT9	67.8 ± 1.3	69.8 ± 0.4	—
F/1	67.2	69.8 ± 0.4	—
M2/1	67.6 ± 0.8	69.9	—

Fig. 3. Denaturation curves of the hybrid fraction after heteroduplexing. Curve 2: the heteroduplex of *Agrobacterium tumefaciens* [^{14}C]DNA of B2a with $\Lambda 6$ DNA. After denaturing at 89 °C the labelled B2a fraction from this hybrid was collected and hybridized with another filter of native B2a DNA. The latter duplex denatures as shown in curve 3. Curve 4: the heteroduplex of *A. tumefaciens* B2a [^{14}C]DNA with *Agrobacterium* sp. 0321. Curve 5: after treatment as above, the duplex of the released [^{14}C]B2a fraction: native B2a. Curve 1: the denaturation curve of the homoduplex [^{14}C]B2a: native B2a as a control. The crosses (x) are the melting points of elution, $T_{m(e)}$.

Table 3. *A semi-quantitative estimate of the degree of DNA relatedness among agrobacteria and two control bacteria, from direct molecular hybridization on membrane filters*

The results are expressed in percentages of the average relatedness within each of the three main homologous groups taken as 100 ($\pm$ standard deviation). The standard deviation includes both experimental error and genetic heterogeneity within the group.

Bacteria	Reference [^{14}C]DNA		
	TT111	B6	TR7
Agrobacterium TT111 group	100 $\pm$ 27	58 $\pm$ 17	13
Agrobacterium B6 group	51 $\pm$ 12	100 $\pm$ 20	12 $\pm$ 1
Agrobacterium cluster 2	7	11	100 $\pm$ 21
<i>A. tumefaciens</i> NCPPB 1650	—	—	15 $\pm$ 3
<i>A. tumefaciens</i> NCPPB 1771	—	—	11
<i>Salmonella typhimurium</i> 1	3	—	13
<i>Acetobacter aceti</i> ch31	3	—	—

bacter aceti (Table 3), and many other organisms (J. De Smedt and J. De Ley, unpublished), the relatedness is less than 5 %. Whether this indicates some remote phylogenetic ties, or the zero level of the scale, cannot be decided.

DISCUSSION

The most striking conclusion is the excellent agreement between the present data and the division of the agrobacteria into clusters and groups, on the basis of phenotypic and genotypic criteria (De Ley, 1972; Kersters *et al.* 1973). The strains with a hybrid- $T_{m(e)}$ around 75 °C belong in the same group or cluster as the reference strain. Within the groups of cluster 1 the standard deviation of $T_{m(e)}$ is about 1 °C. Between the groups B6 and TT111 $\Delta T_{m(e)}$ is about 6 °C. Between the small groups RV3, O362, TT9, F/1, M2/1, and both large groups, $\Delta T_{m(e)}$ is 4 °C to 8 °C.

Each of the small groups RV3, etc., with $T_{m(e)}$ between 67 °C and 71 °C (Tables 1 and 2) are genotypically closer to the B6 and TT111 groups than to cluster 2 (with TR7). It is very interesting to note, however, that their $T_{m(e)}$ values are always 2 °C to 4 °C higher with [^{14}C]B6 DNA than with [^{14}C]TT111 DNA. DNA hybridization studies (De Ley, 1972; J. De Ley, A. Reynaerts and H. Cattoir, unpublished) showed that each of these five small groups has about half of its chromosomes in common with the others and with the B6 and TT111 groups. The present paper provides some additional information (Tables 1 and 2); for every strain the stability of the hybrid with B6 is higher than that with TT111. This difference indicates that those five small groups still have a slightly greater relationship with the B6 group. There is another point of agreement; the RV3 group is phenotypically indistinguishable from the B6 group (Kersters *et al.* 1973), and its average $T_{m(e)}$ value of 71.6 ± 0.1 °C is also the highest of those of the five small groups.

There are several possible explanations for the decreased melting point of heteroduplexes. In our case we have shown experimentally that the decrease is not due to duplexes of AT-rich sequences. This possibility is also unlikely on purely theoretical grounds. The average $\Delta T_{m(e)}$ for both types of heteroduplex is 6 °C and > 13 °C. Assuming that the decrease concerns AT-rich hybrids, their average % GC would be 44 and 26, respectively, since the mean % GC of agrobacteria is about 59. From the melting curve of native *Agrobacterium* DNA we know that the chromosome contains no or not enough fragments with 26 % GC, and not enough with 44 % GC to explain the hybridization pattern. Brenner & Cowie

Fig. 4. Relation between the decrease in melting point $\Delta T_{m(e)}$ of heterologous hybrids and the % DNA relatedness, determined at about $T_m - 25^\circ\text{C}$. Most values are group averages. A, *Agrobacterium* (this paper); V, *Vibrio* (Anderson & Ordal, 1972); s, *E. coli* K12 with other coli strains, *Alkalescens-Dispar*, *Shigella*, and presumed *Citrobacter* and *Enterobacter* (Brenner *et al.* 1972); L, *Leptospira* (Haapala *et al.* 1969); N, *Neisseria* (Kingsbury *et al.* 1969); S, *E. coli*:*S. typhimurium* (Brenner & Cowie, 1968).

(1968), in an experiment similar to ours, attempted to explain the low melting point of the DNA hybrid *Escherichia coli*:*Salmonella typhimurium*. The $T_{m(e)}$ of the reisolated *E. coli*:*E. coli* duplex was far below that of the homologous native duplex; therefore the participation of AT-rich sequences was not convincingly ruled out. In *Agrobacterium* the situation is quite clear-cut. It is thus most likely that low denaturation curves for the present heterologous hybrids are caused by imperfect base matchings, which results from evolutionary divergence of the clusters and groups of strains.

In the evolution of the agrobacteria, mutational changes also happened in the common parts, frequently to a considerable extent. The less two DNAs hybridize, the less stable is their common part. According to McCarthy & Farquhar (1972) a change in 1 % of the base pairs decreases $T_{m(e)}$ by about 1.6°C . The total molecular weight of the *Agrobacterium* genome DNA is 3.7×10^8 . DNA relatedness between the B6, TT111 and some small groups is about 50 %. Single-stranded stretches totalling about 2.8×10^6 bases are still able to hybridize. About 3.75 % or 10^5 of these bases would be mismatched. The duplexing stretches between cluster 1 and 2 total about 7×10^5 bases. Because the denaturation curve is flat, $T_{m(e)}$ is difficult to estimate. It is 62°C or less. The mis-matching in the common part would be over 8 % or 50000 bases.

The phenomenon of decreased molecular hybrid stability of less related organisms seems to be widespread in bacterial evolution (Fig. 4). It occurs in *Neisseria* (Kingsbury, Fanning, Johnson & Brenner, 1969), *Leptospira* (Haapala, Rogul, Evans & Alexander, 1969), the Enterobacteriaceae (Brenner & Cowie, 1968; Brenner, Fanning, Skerman & Falkow, 1972; Brenner, Fanning & Steigerwalt, 1972), and in *Vibrio* (Anderson & Ordal, 1972).

Two more conclusions can be drawn from our data. (i) Since $T_{m(e)}$ of the released hybrid fraction is always the same (Fig. 3), no high or low % GC fractions remained untouched after evolutionary mutation, and mutations happened all over the genome, even in the common part which may be as small as 15 %. (ii) During hybridization the $T_{m(e)}$ of the

stable homo- or heteroduplex remains unchanged, within experimental error (Fig. 1). Thus no high or low % GC fractions react preferentially.

The nature of the labile duplex below T_{or} (temperature of optimal renaturation, De Ley *et al.* 1970) is not known. It is commonly dismissed (e.g. Johnson & Ordal, 1968) as due to unimportant unspecific bonds, but this is very likely only partially true. In many organisms homologous cistrons differ in over 20 % of their base pairs outside the active centre still produce homologous enzymes. However, these cistrons will not hybridize at T_{or} , and they would be dismissed in the unimportant fraction. Undoubtedly quite a number of such cases exist in evolutionarily distant organisms. Our results, as well as a critical examination of published data on the effect of temperature on the hybridization and the stability of hybrids, show that it is frequently unrealistic to hybridize at temperatures of $T_m - 25^\circ\text{C}$ or higher, as advocated by Johnson & Ordal (1968). De Ley & Tijtgaat (1970) have warned against this procedure because of the uncontrollable release of unlabelled DNA from the filters. In addition, one artificially selects for a stable fraction of the hybrid which may be unrepresentative of the total possible stable duplex, but the less stable fractions, which may be much more important phenotypically, are not detected. The resulting homology has a limited value, particularly for organisms with a homology of less than 70 % at $T_m - 25^\circ\text{C}$. For example, how can one decide without bias what temperature to use for homology measurements between cluster 2 and the B6 or T111 group? The results will vary with the temperature and the salt concentration. It seems almost certain that in many cases homology determined at temperatures below $T_m - 25^\circ\text{C}$ is taxonomically more meaningful.

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