Improved DNA-DNA Hybridization as a Taxonomic Tool for Phytopathogenic Bacteria*

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Phytopathogenic bacteria have often been characterized in terms of their specificity to host plants and named after their hosts. This has caused ambiguity and difficulty in clinical diagnosis as well as it has offered conveniences to a wide area of plant protection practices. It also has raised intense debate between different research groups on the validity of classification systems.

The seventh edition of Bergey's Manual (1957)[1], which generally conforms to the views of plant pathologists about such matters, listed about 60 nomenspecies in the genus *Xanthomonas* classified principally in terms of their specificity to host species. Numerical taxonomists were against such a system on their stand that organisms should be classified according to a number of characteristics weighted equally but not solely by phytopathogenicity.

The eighth edition of the same Manual (1974) lists only 5 species in *Xanthomonas*, comprising more than 100 species which have been recognized by plant pathologists in one species—*X. campestris*. The authors[3] claim that those nomenspecies included with *X. campestris* in the *X. campestris* group can be distinguished from *X. campestris* or from each other only by plant host reactions.

We have demonstrated that many *Xanthomonas* species in the seventh edition of Bergey's Manual (this classification is used in the present article for convenience) classified principally in terms of host specificity do show distinction from each other in their characteristic DNA segments differentiated with the host specificity and can be identified unequivocally by an improved DNA-DNA hybridization[4]. Further studies on *Erwinia* species and *Corynebacterium* species have confirmed that the phytopathogenicity closely interrelates with the differentiation or evolution of overall DNA base sequences[5),6)].

Thus, DNA hybridization, carefully calibrated for use under properly controlled experimental conditions, can be an additional and powerful tool for classifying and identifying phytopathogenic bacteria.

Methods of DNA-DNA hybridization in earlier reports

DNA-DNA hybridization was first applied to phytopathogenic bacteria by Friedman and DeLey[7] Their study was extended by DeLey and his co-workers[8),9),10)]. They reported high homology among many *Xanthomonas* species which they claimed to be synonymous and also very high homology between *Xanthomonas* and *Pseudomonas* species. Their studies on other genera also showed rather high homology among species in a genus. Their studies profoundly influenced the course of discussion on the taxonomy of phytopathogenic bacteria

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thereafter. Many bacteriologists were preoccupied by their view that classifying phytopathogenic bacteria on host specificity has little phylogenic ground and that a DNA homology test is of little use in classification.

Friedman and DeLey[7] used the method of Schildkraut et al.[11] in which reanealed segments of DNA formed between unlabelled DNA and DNA labelled with stable isotopes (^{15}N and deutrium) were resolved and quantitated by CsCl equilibrium density gradient centrifugation. DeLey and co-workers later adopted the agar method of McCarthy and Bolton[12] and nitrocellulose filter method of Denhardt[13] in which radioactive DNA is reanealed with DNA fixed on supporting materials.

These methods are valid in principle. However, microbiologists in earlier days were unaware of the importance of a proper setting of reanealing conditions for the resolution of homologous and non-homologous segments of DNA's. The conditions—temperatures as well as salt concentrations—that DeLey's group adopted in their earlier studies were apparently inadequate. Johnson and Ordal[14] examined the thermostability of duplexes formed on nitrocellulose filters at various temperatures. They determined the optimal condition for hybridization to be the incubation in 2×SSC (double the strength of SSC which comprises 0.15 M NaCl plus 0.015 M trisodium citrate) at 25°C below the melting temperature (Tm) measured in SSC. The duplex formed under the optimal condition is as thermostable as the native DNA, their Tm's being almost the same. By lowering the temperatures for reanealing, the duplexes formed contained an increasing quantity of non-specifically bound aggregates which is shown by the thermal transition curve with increasingly lower Tm than native DNA. They pointed out that the earlier results on phytopathogenic bacteria by DeLey's group were invalidated by the abundance of such non-specific binding.

Double-filter and recurrent hybridization method

We attempted to establish the method of DNA-DNA hybridization which would allow theoretically grounded evaluation of segmental homology of DNA (the proportion of homologous DNA segments in the entire length of reference DNA). We devised "double filter techniques" in which a pair of nitrocellulose filters, one loaded with reference DNA and the other with DNA to be tested, are together incubated with radioactive DNA in a vial[15),4)]. Homology is computed based on the ratio of reanealing on two filters by a formula, taking into account the change of efficiency of reanealing due to the incubation of two filters instead of one filter. In this method, we can check whether the experiment conducted under a given condition allows a valid interpretation of the segmental homology relationship. We do so by checking the coincidence of the effect of competitor DNA on the ratio of reanealing on two filters obtained experimentally and the effect of competitor calculated theoretically on the assumption that reassociation occurs on homologous segments but not on non-homologous segments. Experiments showed that the condition which is close to that of Johnson and Ordal gives clear resolution of homologous and non-homologous segments.

We further devised "recurrent hybridization" for identifying and assessing the segments of DNA shared by three or more organisms. We designate the three organisms as "primary reference," "secondary reference," and "tested organism." Radioactive DNA of primary reference organism is prepared.

In the first hybridization, the labelled primary reference DNA is reanealed with the secondary reference DNA fixed on nitrocellulose filter. The labelled DNA attached to the filter by forming duplexes with the secondary reference DNA (labelled) is then dissociated into buffer by heating and quenching. The segments of primary reference DNA (labelled)

selected for homology with the secondary reference DNA are thus prepared.

In the second hybridization, double filters, one loaded with the secondary reference DNA and the other with the third DNA of tested organism, are permitted to react with two batches of labelled primary reference DNA solution. One solution is enriched for the segments shared by primary and secondary references over the rest of the segments, and the other is with the original proportion of segments over the entire length.

By comparing the ratios of reassociation on the double filters in the two solutions, we can compute the relative size of the segments shared by three organisms, those with pairs of organisms, and the segments differentiated to each of the organisms. In computing, we take into consideration the possible contamination in the solution of the segments selected for the homology between the primary and secondary references by non-homologous segments.

With these two methods, we have clearly demonstrated that there is a large proportion of segments differentiated to species which have been classified principally in terms of host specificity in *Xanthomonas* and other genera. We also identified the segments of DNA characterizing genera or other genetic clusters.

Xanthomonas species

We examined 20 *Xanthomonas* species which are classified in *X. campestris* group in the eighth edition of Bergey's Manual, 4 species which the Manual distinguishes from *X. campestris* as true taxospecies—*X. fragariae, X. albilineans, X. axonopodis,* and *X. ampelina* —and 13 *Pseudomonas* species comprising saprophytes as well as phytopathogens for their genetic relatedness[4]. Species used as references were: *X. translucens, X. oryzae,* and *X. pruni* in the *X. campestris* group; *X. fragariae* in taxospecies outside the *X. campestris* group; and *Pseudomonas maltophilia.*

The experiment showed that all the *Xanthomonas* species possess DNA segments unique to the genus which reveal themselves in partial homology among *Xanthomonas* species. The existence of segments shared by three or more *Xanthomonas* species was confirmed by recurrent hybridization.

These segments were not found in *Pseudomonas* species in general. Only *Pseudomonas maltophilia* (and possibly *P. pseudoalcaligenes*) showed the partial homology with *Xanthomonas* species. The segments common to *Xanthomonas* do not exceed 50% of the entire genome, and each of the species possesses overwhelmingly abundant DNA segments which are differentiated according to species. The segments specific to nomenspecies are as large in those species classified principally in terms of host specificity and eventually grouped in *X. campestris* group as in *X. fragariae* and three other taxospecies which are accepted by the new Bergey's Manual (1974). Subspecies in *X. translucens* were not distinguished by the present method.

Erwinia species

Genus *Erwinia* belongs to Enterobacteriaceae, the family which includes enterobacteria parasitic animals. *Erwinia* species are often classified into three natural groups[16),2)]; soft rot group (or carotovora group), white non-pectolytic phytopathogen group (or amylovora group), and yellow saprophyte or phytopathogen group (or herbicola group). There are some reports on *Erwinia*-like organisms isolated from man and animals in connection with pathological conditions[17-20)]. They are generally named *Erwinia herbicola* (or *Enterobacter aggromerans* in Ewing and Fife's proposal)[21)].

Principal questions we sought to answer in DNA homology studies in *Erwinia* species[5)] were as follows: Is *Erwinia* genetically related with other enterobacterium genera? If so, do *Erwinia* phytopathogens form a genetic cluster which would justify the classification of the genus *Erwinia* within Enterobacteri-

aceae?; Do the three natural groups in *Erwinia* form respective genetic clusters identifiable in terms of higher partial homology?; Are nomenspecies distinguishable from each other in terms of DNA homology?

We designed the experiment paying particular attention to the clustering and dispersion of these organisms. *Erwinia* species in all natural groups were examined for their homology with one or more reference species in the same natural group. *Erwinia*-like organisms isolated from man were examined along with some yellow saprophyte or phytopathogen group organisms.

In all sets of experiments, some typical cultures of other natural groups and a few cultures of different genera in Enterobacteriaceae were included. We used as references the following species: *E. carotovora, E. aroideae,* and *E. cytolytica* in the soft rot group; *E. rubrifaciens, E. salicis,* and *E. amylovora* in the white non-pectolytic phytopathogen group; *E. herbicola, E. milletiae* in the yellow saprophyte or phytopathogen group; and three isolates from man and one plant epiphyte culture of *E. herbicola* for examining *Erwinia*-like organisms from man.

We obtained the following results:

1) *Erwinia* species share DNA segments —not exceeding 15 per cent of total genome— with members of other genera of Enterobacteriaceae, showing that *Erwinia* is truly enterobacterial.

2) There are no distinct DNA segments characterizing the entire genus *Erwinia* to distinguish the genus from other genera of Enterobacteriaceae. Rather, genetic clusters were found within all natural groups. These genetic clusters are, however, not consistent with any entire natural group.

3) In the white non-pectolytic phytopathogen group, where a host-parasite relationship is clear-cut, nomenspecies showed significant nomenspecies-specific DNA segments. Pathogenicity may be deeply involved in the evolution of DNA in this group as in xanthomonads. In the soft rot group and yellow saprophyte or phytopathogen group, high homology among some nomenspecies and a rather wide variation of DNA homology within a nomenspecies rendered the distinction of nomenspecies in terms of DNA homology ambiguous. Some nomenspecies, however, showed significant nomenspecies-specific DNA segments on the top of segments characterizing the genetic clusters.

4) *Erwinia*-like isolates from man share with the yellow saprophyte or phytopathogen group partial homology which is higher than the basic homology among enterobacteria. They also showed relatively wide variation in the rest of the DNA segments and in phenotypic characteristics.

5) It is, thus, possible by our methods of DNA homology testing, to identify an organism as being a member of an intrageneric cluster, and even as being a nomenspecies of *Erwinia* unless resolution of synonyms is required. It is not possible, however, to recognize an organism as being a member of *Erwinia* as distinct from other genera of Enterobacteriaceae by testing it against *Erwinia* species which do not happen to be cultures of the very nomenspecies or in the genetic cluster to which the specimen belongs.

Our results are essentially consistent with the studies by Brenner et al.[22),23)] and Gardner and Kado[24)] on soft rot and white non-pectolytic phytopathogen groups. Their methods, though different from ours, were carefully set by checking the thermostability of the heteroduplexes formed.

Corynebacterium species

The phytopathogenic coryneform bacteria, presently treated as species of the genus *Corynebacterium,* comprise a group which is physiologically, morphologically, and pathologically rather diverse. The propriety of classifying the phytopathogenic coryneforms in the genus *Corynebacterium* together with animal diphtheroids has been argued for and against. Proposals have been put forth to subdivide the coryneform bacteria into several genera, thereby excluding most plant patho-

gens from the genus *Corynebacterium*, and even to place the phytopathogenic *Corynebacterium* nomenspecies into more than one genus.

To throw some light on these conflicting situations, we examined 10 species of phytopathogenic corynebacteria (and a few organisms of other genera for comparison) for their genetic relatedness in terms of DNA homology. *C. poinsettiae*, *C. insidiosum*, and *C. fascians* were used as references.

Heterologous combinations between either of two references, *C. poinsettiae* and *C. insidiosum*, and any *Corynebacterium* species except for *C. fascians* revealed partial homology which is distinctly lower than perfect homology shown by homologous combinations but significantly higher than negligible homology of intergeneric combinations.

This shows that they form a genetic cluster which deserves the classification as a genus and that each nomenspecies, classified in terms of phytopathogenicity and other phenotypic characters, is differentiated from others in its major portion of DNA segments.

Debate has persisted on the classification of *C. fascians*, since this organism differs from other phytopathogenic coryneform bacteria in morphology and some other traits. Some have argued that this organism should be placed in the genus *Nocardia*. In our study, *C. fascians* did not show any homology with either *C. poinsettiae* or *C. insidiosum*, but rather showed low but significant homology with *Nocardia corallina*.

From the above results, we concluded that: 1) Phytopathogenic coryneform bacteria, except for *C. fascians*, can legitimately be classified in a genus. The present classification of nomenspecies reflects well the characteristic features of the entire DNA base sequence. (We did not study how these bacteria are related with animal diphtheroids, another major group in *Corynebacterium*.) 2) *C. fascians* should be reclassified in another genus with some *Nocardia* species. 3) Phytopathogenicity is deeply involved in the differentiation of major DNA segments as in xanthomonads and white non-pectolytic phytopathogens of *Erwinia*.

Conclusions

Classification of phytopathogenic bacteria has been the subject of intensive debate for years. Evaluation of phytopathogenicity or host-specificity as a major criterion of the classification is one of the main issues of the debate. Our studies showed that improved DNA-DNA hybridization studies could dissolve much of the ambiguity in both classification and identification of these organisms.

In classifying phytopathogenic organisms, DNA homology could be one of the most reliable measures of phylogenetic relatedness. Partial homology in a group of organisms could be the basis of genus or other taxonomic entities characterizing genetic clusters. If necessary, we can identify and assess the DNA segments common to three or more organisms by the recurrent hybridization technique. A larger quantity of differentiated DNA segments may be taken as the basis of taxonomic entity corresponding to the present nomenspecies. In practice, however, phenotypic characteristics, which provide us with useful information on the impact of the organisms on human life, may be relied on as has been done. However, each of the characteristics should be weighted according to how deeply it is involved in the differentiation of DNA. Our studies showed that phytopathogenicity is one of the most important characteristics.

DNA hybridization can be important in identifying unknown organisms according to nomenspecies, subgeneric clusters, or genera, if homology relationships among them have been explored. Most *Xanthomonas* species, for which no criterion but host-specificity has existed for identification, can now be identified in terms of DNA homology.

Studies on the interaction of pathogenicity and DNA differentiation may also lead us to discoveries regarding biochemical and physiological mechanisms of the host-pathogen relationship[25].

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