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Original article

Numerical evaluation of the species groupings among the classical propionibacteria

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Summary — Numerical analyses were used to evaluate the species groupings among the classical *Propionibacterium* species based on 147 strains from a dairy and an anaerobic environment. A data set of 74 phenotypic characters, determined using standardized API systems, were included. The Jaccard and Sokal and Michener coefficients as well as dendrogram distances were used to broaden the method of delineating the clusters. One-, two- and three-dimensional plots were created and each strain clustered in relation to its closest relatives. Numerical data at similarity levels of $S_J = 0.43$ and $S_{SM} = 0.71$, respectively, as well as PCR/RFLP data, revealed a separation of the 4 classical *Propionibacterium* species, confirming the major species as documented by Cummins and Johnson (1986). Each major grouping consisted of 2 or more clusters and many were linked at a lower level to loose lying strains. From the dendrogram groupings, it was also clear that even though these strains could be placed in 1 of the 4 classical species using the present identification key, the clustering positions were in many cases different. The strains with the lower similarities were identified to a specific species, but in many cases this did not agree with the clustering position. In the cases of the strains clustered away from the type strains, identification was confirmed using the PCR/RFLP data and this aided in confirming the identification status in terms of to which of the 4 classical species the strain was affiliated.

***Propionibacterium* / numerical analysis / species groupings / dairy / anaerobic environment**

Résumé — Évaluation numérique des espèces de bactéries propioniques classiques. *Des analyses numériques ont été utilisées pour évaluer les regroupements d'espèces parmi les bactéries propioniques classiques, à partir de 147 souches d'origine laitière ou provenant d'environnement anaérobie. Soixante-quatorze caractères phénotypiques, déterminés à l'aide de systèmes API standardisés, ont été inclus. On a utilisé les coefficients de Jaccard et de Sokal et Michener ainsi que les distances sur le dendogramme pour regrouper les souches en fonction de leur proximité. Les données numériques au niveau de similarité $S_J = 0,43$ et $S_{SM} = 0,71$ respectivement, ainsi que les données PCR/RFLP ont montré une séparation des 4 espèces classiques de* Propionibacterium *confirmant les espèces majeures décrites par Cummins et Johnson (1986). Chaque regroupement majeur consistait en 2 clusters ou plus et plusieurs d'entre eux étaient liés à un niveau plus bas à des souches dispersées. À partir des regroupements sur le dendrogramme, il était également clair que même si ces souches pouvaient être placées dans l'une des 4 espèces classiques à l'aide de la présente clé d'identification, les*

positions dans les clusters étaient dans plusieurs cas différentes. Les souches avec la plus faible similarité étaient identifiées à une espèce spécifique, mais, dans plusieurs cas, cela n'était pas en accord avec la position dans le cluster. Dans le cas des souches retirées des clusters des souches types, l'identification était confirmée à l'aide des données PCR/RFLP et cela permettait de confirmer l'identification en terme d'affiliation à l'une des 4 espèces classiques.

Propionibacterium / *analyse numérique / regroupement d'espèces / environnement laitier / environnement anaérobie*

INTRODUCTION

The propionibacteria are an important group not only in the dairy industry, where strains play a role in the ripening of Swiss-type cheeses, but also as members of the population of the human skin where their role as causes of disease is still a matter of interest.

As certain members of the genus may be pathogenic or may cause flavor and texture defects in a variety of foods, the correct identity of the strains is extremely important in improving the quality of dairy and other fermented products. At present, the separation of the different species is based on 5 phenotypic characteristics. However, in many cases it is difficult to assign strains to specific species (Cummins and Johnson, 1986) based purely on these characteristics. One of the major problems is that if one character differs, the strain will automatically be identified as a member of another species, even though the majority of the phenotypic characters determined differ from the description of the appropriate species (Riedel and Britz, 1993).

The aim of this study was to determine how serious this problem is among the classical propionibacteria by using numerical grouping based on phenotypic characteristics and then highlighting the problem clusters and strains. The phenotypic clusters could then be compared with data obtained from PCR/RFLP restriction profiles (Riedel *et al,* 1994).

MATERIALS AND METHODS

Bacterial strains and culture conditions

The 147 *Propionibacterium* used in this study comprised the 5 type and 22 reference strains from the ATCC, NCFB and NCIMB, as well as strains from the dairy industry, 60 from an anaerobic environment and 12 cutaneous strains. Details concerning the strains and their phenotypic identification is as previously given (Britz and Riedel, 1991, 1994; Riedel and Britz, 1992, 1993). All the classical strains were cultured anaerobically at 32°C for 4 d and the cutaneous strains at 37°C for 6 d in yeast extract lactate broth (YEL) prepared according to the method of Holdeman *et al* (1977). The YEL consisted of (g/l): yeast extract 5.0; sodium lactate (50% v/v) 20.0; peptone 2.0; KH_2PO_4 10.0; hemin 10.0 ml and Tween 80 1.0 ml.

Phenotypic characteristics

The 74 phenotypic characteristics were determined, as previously described (Britz and Riedel, 1994), in an anaerobic cabinet, using oxygen-free nitrogen (Holdeman *et al,* 1977) as gas phase. The API 50CH, 20E and 20A systems (API System SA, La Balme les Grottes, Montalieu Vercieu, France) were used to determine the substrate utilization and biochemical profiles. Carbohydrates that are important in the identification of propionibacteria were repeated using the conventional method described by Holdeman *et al* (1977). Each test was done in duplicate, and when different results were obtained, the test was repeated and the majority result was taken as being representative.

Computer analyses

Seventy-four characters were included in the data set and analyzed using the S_{SM} coefficient as well as the Jaccard (S_J) coefficient (Sokal and Michener, 1958). The unsorted similarity matrix was rearranged into groups by average linkage cluster analysis (Lockhart and Liston, 1970). Tests that gave uniform results for all the strains were excluded from the numerical analyses. Dendrogram distances (D_D) were also calculated for the 147 *Propionibacterium* strains using the Microlog Software System (Biolog, 1993) based on the phenotypic characteristics as calculation concept. One-, two- and three-dimensional data plots were created and each strain clustered in relation to its closest relatives. Ten strains, chosen randomly, were examined in duplicate to estimate the average probability of error *(P)* using the formula of Sneath and Johnson (1972).

Polymerase chain reaction

Genomic DNA was isolated as described by Riedel *et al* (1994). Strains were grown anaerobically at 32°C for 4 d in the medium of Charfreitag and Stackebrandt (1989) with 4% (v/v) mineral salts solution (Holdeman *et al*, 1977). The small subunit (16S rRNA) sequence data of specific *Propionibacterium* strains, as reported by Charfreitag and Stackebrandt (1989), were used as the basis of this study. The sequence data enabled the construction of PCR primers, as well as the identification of the correct amplified fragment. Four synthetic oligodeoxyribonucleotide primers: 16sP1 (5'-GGGTGACCGGCCACA-3'); 16sP3 (5'-AAGGTGGGGATGAGG-3'); and 16sP4 (5'-TCGGGTGTTACCGAC-3') were selected from the conserved regions of the 16S rRNA. The 16S rRNA genes from the genomic DNA were successfully amplified using the PCR process. Products of the predicted sizes, using primers 16sP1 and 16sP4 (approximately 1 100 bp) and 16sP3 and 16sP4 (approximately 251 bp) were obtained and subjected to restriction endonuclease digestion and optimized (Riedel *et al*, 1994). Visual differentiation between the various "classical" *Propionibacterium* species was possible after restriction endonuclease digestion of the PCR products, using *Hae*III and *Alu*I, as well as *Hpa*II.

RESULTS AND DISCUSSION

Clustering of strains

All the strains studied were gram-positive, nonmotile, nonsporing and produced propionic acid as major metabolite with lesser amounts of acetic acid and CO_2. No hydrogen was formed by any of the strains studied.

The outcome of any numerical groupings study will reflect the nature of both the statistical and phenotypic methods used. A legitimate criticism is the lack of standardization in methods, starting with the initial choice of operational taxonomical units (OTUs) and the numbers and types of characters to be used as well as the average probability of error. The probability of error found in this study, using the standardized API system, was found to be <3.0% and thus, the phenotypic character detection method would not present a serious distortion of the taxonomic structure. Furthermore, the similarity coefficient must be chosen to enable the delineation of clearly defined clusters of OTUs. On this basis, it was decided to include, as part of the OTUs, as many strains as possible from the dairy industry as well as type and reference strains. It soon became clear that the diversity of the dairy environment was rather limited (Britz and Riedel, 1994). Very few 'new isolates' are available to study and thus it was decided to include classical propionibacterial strains from a totally different anaerobic environmental niche (Riedel and Britz, 1993), giving a final number of 147 cultures.

The data set of 74 phenotypic characters, determined using the standardized API systems, was analyzed using both the S_J and the S_{SM} similarity coefficients as well as the D_D method so as to broaden the method of delineating the clusters. At similarity levels of 0.43 and 0.71, respectively,

phenotypic analyses based on the S_J and S_{SM} coefficients revealed a clear separation of the classical and the clinical *Propionibacterium* species, confirming the delineation of these 2 major groups as documented by Cummins and Johnson (1986). From the dendrogram groupings (table I), it was also clear that even though the classical strains could be placed in 1 of the 4 classical species as given by Cummins and Johnson (1986) using the present identification key, the clustering positions were in many cases different. This again illustrates the problem between the identification using the conventional system and the groupings obtained when using numerical analysis based on many characteristics.

When comparing the presence and structure of the classical clusters obtained using the S_J and S_{SM} similarity coefficients, it was found that the results were very similar with the major difference being the lower similarity when using the S_J, which was expected as a result of the exclusion of negative values between OTUs. The horizontal similarity between clusters within the major groups varied from 0.90–0.98 for the *P jensenii* and *P acidipropionici* groups to 0.89–0.82 for *P thoenii* and *P freudenreichii.* The vertical similarity was lower, ranging from 0.80–0.90, indicating a fairly wide inter- and intracharacter variation within the major groups. For both coefficients, it was found that each major group consisted of 2 or more clusters and each was linked at a lower level to loose lying strains. These strains, with the lower similarity, were identified, using the present identification key, to a specific species. In many cases, however, this identification did not agree with the general numerical clustering position. In the cases of the strains clustered away from the type strains, identification was confirmed using the PCR/RFLP data and this aided in confirming the identification status in terms of to which of the 4 classical species the strain was affiliated. Surprisingly, the isolates from the nondairy environment were found spread throughout the dendrogram, suggesting that the propionibacterial strains from other environments were closely related to the strains of dairy origin; thus, it might be of value to examine similar environments in order to widen the possible species diversity.

Major clusters

From the numerical data as well as from the PCR/RFLP profiles, it is clear that the 4 major classical species groupings can be delineated and that each major grouping consists of several subgroupings. With the

Table I. A list of the PCR profile and phenotypic groupings of the classical *Propionibacterium* strains. *Liste des regroupements par profil PCR et phénotypiques des souches de* Propionibacterium *classiques.*

Major groups	*Species*	*PCR-profiles**	*Pheno-clusters*	*Number of strains*
Acidipropionici	*P acidipropionici*	A	A1–A14	39
Jensenii	*P jensenii*	J	J1–J10	59
Thoenii	*P thoenii*	T	T1–T6	21
Freudenreichii	*P freudenreichii*	F	F1–F7	16

* Riedel *et al* (1994).

use of the two- and three-dimensional models based on the dendrogram distance method of calculation, the position of the major groups, with the internal clusters and loose strains, was clearer to view (fig 1).

From the data (table I), it was found that the 'Jensenii' major group contained at least 10 separate clusters of which 2 (J4 and J5) were made up of red-pigmented strains while the others were the typically white-pigmented strains. The grouping of the strains were confirmed by PCR/RFLP profiles as typical of the 'Jensenii' group. One red cluster was found to contain only isolates from the anaerobic environment and from Leerdammer cheese (cluster J4). No reference strains were closely related to this group. The type strain cluster (J2) was clustered separately from the other *P jensenii* clusters.

The 'Acidipropionici' major group was revealed as 14 somewhat closely related clusters with the type strain placed fairly centrally (cluster A4). However, the data showed several strains that clustered in the 'Acidipropionici' major group but were identified, using the standard key, as *P jensenii* strains. With the use of the PCR/RFLP profiles, these were correctly placed as part of the species *P acidipropionici.* Nitrate reduction was the varying 'key' character responsible for this confusion.

The red-brown pigmented 'Thoenii' major group, consisting of 2 large (T1 and T3) and 4 smaller clusters (T2, T4, T5 and T6), with the type strain centrally placed in cluster T1, were also well-defined clusters with the correct identification confirmed by the PCR/RFLP profiles.

The 'Freudenreichii' major group, with the appropriate type strain in cluster F1, was also found as 6 well-defined clusters (F1, F2, F3, F4, F5 and F6) and a single strain cluster (F7). The 3 subspecies, however, could not be positively separated. The PCR/RFLP profiles were similar for all the strains of this group.

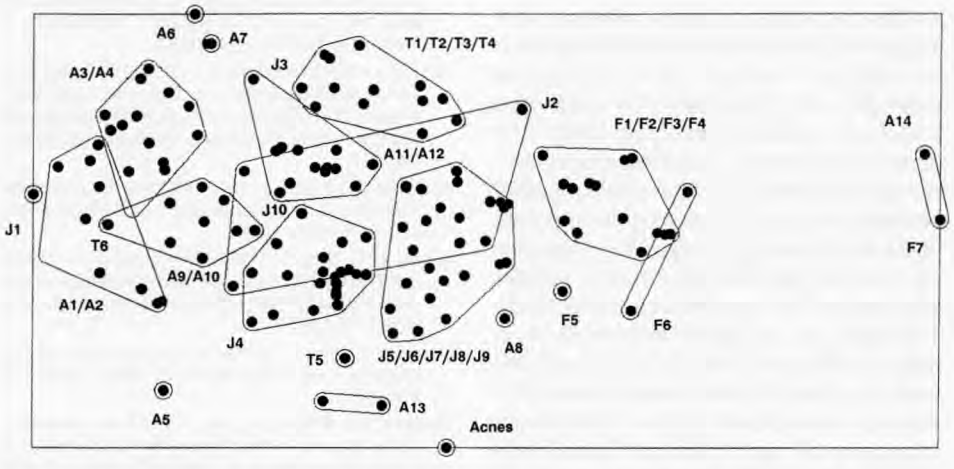

Fig 1. Two-dimensional plot, based on the dendrogram distances (D_D), showing the relationship between the *Propionibacterium* clusters. A = strains identified as *P acidipropionici;* T = *P thoenii;* J = *P jensenii* and F = *P freudenreichii.*
Tracé bidimensionnel basé sur les distances sur le dendrogramme (D_D), montrant la relation entre les clusters de Propionibacterium. *Les clusters dénommés A représentent les souches identifiées comme* P acidipropionici, *T,* P thoenii, *J,* P jensenii *et F,* P freudenreichii.

CONCLUSION

It was found that most of the strains from the dairy as well as from the anaerobic environment could be phenotypically assigned as members of the existing 4 species according to current taxonomy. From the cluster data, however, it is clear that even if strains are identified using the standard key, as a member of a specific species this was not always confirmed by the clustering position obtained when using numerical methods. In these cases, the species identification had to be confirmed by the use of PCR/RFLP profiles. Thus, it can also be concluded that the PCR/RFLP profiles are of great value in confirming the correct species identification of strains. Given the fact that the PCR/RFLP assays are both more sensitive and rapid compared to conventional culturing and biochemical characterization, these techniques can be applied successfully in the identification of members of the 4 major species of the genus *Propionibacterium*.

In this study, it was also found that within each major group 2 or more clusters, as well as single strain clusters, were present, suggesting that the spread of characters is so wide that the major groups might be extended and in the future be subdivided into more suitable taxonomic units. The dimensional models were essential in facilitating the positioning of the single member clusters which could possibly be the core positions for new groupings. This, of course, can only be confirmed when related isolates are obtained and included in a similar study. The basis for this statement can be illustrated by the positioning of the 60 isolates from the anaerobic environment among the dairy strains as part of this study where especially the type strains of *P acidipropionici* (cluster A4) and *P jensenii* (cluster J2) were strengthened into specific clusters. Similarly, new clusters (J3 and J4) consisting of only new cultures were also found.

One serious problem which still remains is the procurement of suitable distinguishing phenotypic characters for use at species-level separation; thus, additional work to facilitate identification at this level is essential.

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REFERENCES

Biolog Manual (1993) Biolog Inc, Hayward CA 94545, USA

Britz TJ, Riedel KHJ (1991) A numerical taxonomic study of *Propionibacterium* strains from dairy sources. *J Appl Bacteriol* 71, 407-416

Britz TJ, Riedel KHJ (1994) *Propionibacterium* species diversity in Leerdammer cheese. *Int J Food Microbiol* 22, 257-267

Charfreitag O, Stackebrandt E (1989) Inter- and intrageneric relationships of the genus *Propionibacterium* as determined by 16S rRNA sequences. *J Gen Microbiol* 135, 2065-2070

Cummins CS, Johnson JL (1986) Genus I. *Propionibacterium* Orla-Jensen 1909. *In: Bergey's Manual of Systematic Bacteriology, Vol. 2* (PHA Sneath, NS Mair, ME Sharpe, JG Holt, eds) The Williams & Wilkins Co, Baltimore, MD, USA

Holdeman JL, Cato EP, Moore WEC (1977) *Arachnia* and *Propionibacterium. In: Anaerobe Laboratory Manual, 2nd edn,* Virginia Polytechnic Institute and State University, Blacksburg, Southern Printing Co, VA, USA

Lockhart WR, Liston J (1970) *Methods for Numerical Taxonomy*. American Society for Microbiology, Washington DC, USA

Riedel K HJ, Britz TJ (1992) Differentiation of "classical" *Propionibacterium* species by numerical analysis of electrophoretic protein profiles. *Syst Appl Microbiol* 15, 567-572

Riedel K HJ, Britz TJ (1993) *Propionibacterium* species diversity in anaerobic digesters. *Biodiv Conserv* 2, 400-411

Riedel K HJ, Wingfield B, Britz TJ (1994) Justification of the "classical" *Propionibacterium* species concept by restriction analysis of the 16S ribosomal RNA genes. *Syst Appl Microbiol* 17, 536-542

Sneath PHA, Johnson R (1972) The influence on numerical taxonomic similarities of errors in microbiological tests. *J Gen Microbiol* 72, 377-392

Sokal RR, Michener CD (1958) A statistical method for evaluating systematic relationships. *Kansas Univ Sci Bull* 38, 1409-1438