NN8201 595 C

Genetic, morphological and physiological relationships among coryneform bacteria

BIBLIOTHEEK DER LANDBOUWHOGESCHOOL WAGENINGEN

NN08201,595

W. H. J. Crombach

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Genetic, morphological and physiological relationships among coryneform bacteria

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus, prof. dr. ir. H. A. Leniger,
hoogleraar in de technologie,
in het openbaar te verdedigen
op woensdag 9 oktober 1974 des namiddags te vier uur
in de aula van de Landbouwhogeschool te Wageningen

Centre for Agricultural Publishing and Documentation

Wageningen – 1974

Stellingen

1

Tussen grond-arthrobacters en corynebacteriën van kaas ('cheese coryneforms') bestaat slechts een geringe verwantschap.

Dit proefschrift.

2

Gezuiverd DNA, ingevroren bij −70°C en bewaard bij −21°C, blijft gedurende tenminste een half jaar geschikt voor thermische denaturatie en hybridisatie-experimenten.

Dit proefschrift.

3

De grootte van het bacterie-genoom moet worden betrokken bij de interpretatie van DNA-DNA hybridisatie-experimenten.

Dit proefschrift.

4

De opvatting van Platt, dat somatische mutaties waarschijnlijk geen rol van betekenis spelen bij het verouderingsproces, is voor kritiek vatbaar.

D. Platt, 1973. Münch. med. Wschr. 115:1881–1884.

5

Bij de interpretatie van het thermische elutie-profiel van DNA, geadsorbeerd aan hydroxy-apatiet, dient rekening te worden gehouden met de mogelijkheid dat bij relatief hoge elutie-temperaturen ongedenatureerd DNA elueert.

H. G. Martinson, 1973. Biochemistry 12:139–145, Biochemistry 12:145–150.

6

Een juiste waardering van de geschiktheid van verschillende conserveringsmethoden voor zuursels vereist naast een onderzoek naar de wijziging in de bacteriologische samenstelling een kwantitatieve controle op de zuringsactiviteit. Tevens dient daarbij een eventuele verandering in de gevoeligheid voor fagen te worden betrokken.

M. S. Reddy, E. R. Vedamuthu, C. J. Washam & G. W. Reinbold, 1974. J. Dairy Sci. 57:124–127.

7

In de gebruikelijke reclame-campagnes, waarin sterk het accent wordt gelegd op bier als genotmiddel, wordt aan de gunstige werking van bier op fysiologische processen te weinig aandacht geschonken.

8
De bij vele duivenliefhebbers levende opvatting dat een goede rui van een duif de waarborg is voor een goede gezondheid, is slechts in omgekeerde zin juist.

9
Een daadwerkelijke herindustrialisatie op zeer korte termijn van de Mijnstreek is dringend gewenst om de ex-mijnwerkers elk argument te ontnemen zich als een vergeten groep te beschouwen nu voor hen de uitdrukking 'het zwarte goud' alleen nog maar historische waarde heeft.

10
De bekwame bedrijfsdirecteur leidt zijn stafleden zodanig op dat hij voor het bedrijf in wezen overbodig wordt.

Proefschrift van W. H. J. Crombach
Wageningen, 9 oktober 1974

Abstract

Crombach, W. H. J. (1974) Genetic, morphological and physiological relationships among coryneform bacteria. Doctoral thesis, Wageningen, (x) + 27 p., 110 refs., 1 fig., + offprints Antonie van Leeuwenhoek 38 (1972) 105-120, 39 (1973) 249-255, 40 (1974) 133-144, 40 (1974) 347-359, 40 (1974) 361-376, Dutch summary.
An abridged version is also published as: Agric. Res. Rep. (Versl. landbouwk. Onderz.) 824.

The DNA base composition of the soil arthrobacters tested (65.3 – 67.0% GC) suggests that this group is genetically homogeneous. Hybridization experiments, however, revealed clear differences between the *Arthrobacter simplex* and the *Arthrobacter globiformis* strains. The orange cheese coryneforms were fairly homologous, which was shown by the narrow % GC range (63.2 – 63.8, except one strain), the significant hybridization with the type species *Brevibacterium linens,* and the mutual morphological and physiological resemblance. The majority of the orange sea-fish coryneforms resembled both morphologically and in their GC contents the *Brevibacterium linens* group; the results of hybridization experiments and physiological studies indicated, however, that only a minority of the sea-fish strains are closely related to the orange cheese coryneforms. With the exception of four strains tested, the majority of the non-orange cheese strains were closely related as concluded from their GC contents (65.5 – 66.9%) and their high degree of hybridization. The soil arthrobacters, the orange cheese and sea-fish coryneforms, and the non-orange cheese coryneforms were found to be only remotely related because of the poor hybridization between the DNAs of the respective reference strains in addition to differences in GC contents or morphological and physiological characters, or both. In general, the results obtained from DNA analysis and DNA-DNA hybridization experiments and those from morphological and physiological studies were found to be fairly well correlated. A comparative study with native and deep-frozen DNA revealed that freezing at $-70°C$ and subsequent storing at $-21°C$ for at least half a year had no significant effect on thermal denaturation and hybridization. In the initial renaturation phase, mismatching in hybrids of closely related DNAs was restricted to a few %, and in those of moderately related DNAs to approximately 10%.

Voorwoord

Bij de presentatie van dit proefschrift zou ik gaarne allen willen bedanken die het hunne hebben bijgedragen aan de totstandkoming hiervan.

Mijn vrouw Marlies ben ik veel dank verschuldigd voor het getoonde begrip voor het feit dat zij tijdens de voorbereiding van dit proefschrift de kinderen vaak alleen moest opvangen.

Mijn promotor Professor Dr. Ir. E. G. Mulder ben ik dankbaar voor de vrijheid die hij mij heeft geboden om in het Laboratorium voor Microbiologie van de Landbouwhogeschool te Wageningen dit onderzoek te verrichten. Zijn zeer kritische instelling en ervaring hebben bij de beoordeling en correctie van de manuscripten er mede toe bijgedragen dat deze als onderdelen van dit proefschrift gepubliceerd konden worden.

Tevens gaat mijn dank uit naar Professor Dr. J. De Ley en zijn medewerkers voor de gastvrijheid en prettige samenwerking tijdens mijn verblijf in het Laboratorium voor Microbiologie en Microbiële Genetica, Gent, België. Door hun bereidwilligheid en waardevolle suggesties ben ik de aanloopperiode van dit onderzoek versneld doorgekomen.

De redaktie van het tijdschrift Antonie van Leeuwenhoek ben ik erkentelijk voor het snelle publiceren van mijn laatste twee artikelen. Met name wil ik mijn dank betuigen aan Dr. A. L. Houwink voor zijn opbouwende kritiek en geboden hulp bij de uiteindelijke vormgeving van de manuscripten.

Een woord van dank ben ik zeker verschuldigd aan de heer J. Antheunisse, van wiens rijke ervaring ik heb mogen profiteren.

Dr. Maria Deinema, Dr. A. D. Adamse, Dr. C. J. E. A. Bulder, Dr. J. L. M. Huntjens, Dr. T. A. Lie en Dr. L. P. T. M. Zevenhuizen dank ik voor het kritisch doorlezen van een of meerdere manuscripten.

Verder dank ik alle dames van de administratie voor de geboden hulp; met name ben ik Mevr. C. Möller-Mol en Mevr. J. Philippi-Haagen zeer erkentelijk voor de correcte en snelle wijze waarop zij een aantal manuscripten getypt hebben.

Mevr. M. Kokke-Hopstaken en Mej. A. Scholten zeg ik dank voor hun medewerking bij een aantal experimenten.

De heren M. van de Kolk, C. F. Schouten, J. C. van Velzen en A. Wessels ben ik zeer erkentelijk voor het verlenen van technische assistentie en het geven van praktische adviezen tijdens dit onderzoek.

De medewerkers van Pudoc ben ik dank verschuldigd voor hun raadgevingen bij de samenstelling van dit boekje.

Curriculum vitae

In 1961 behaalde de auteur, geboren op 4 mei 1943 te Gulpen, het HBS-B diploma aan het St. Bernardinus College te Heerlen. Daarna heeft hij aan de Landbouwhogeschool te Wageningen Zuivelbereiding en Melkkunde gestudeerd. Het ingenieursdiploma werd behaald in januari 1968, waarna de schrijver als wetenschappelijk assistent met een promotie-onderzoek is begonnen in het Laboratorium voor Microbiologie van de Landbouwhogeschool. Hij is sedert januari 1970 aan dit laboratorium verbonden als wetenschappelijk medewerker.

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1 Introduction

Coryneform bacteria, showing a pleomorphic morphology, constitute an important proportion of the microflora present in a variety of habitats.

A comparative study of the morphology and physiology of coryneforms isolated from soil, the surface of cheeses and the skin of sea fish (Mulder & Antheunisse, 1963; Mulder et al., 1966) revealed some distinct physiological differences among these bacteria, in spite of their morphological resemblance.

In comparative studies on morphology and physiology only a small part of the total bacterial chromosome is involved. DNA base composition, hower, is related to the total genome and is a stable characteristic of an organism. Therefore, in any classification of micro-organisms the GC contents should be implicated.

In this study the DNA base composition of a number of soil, cheese and sea-fish coryneforms was determined according to the thermal denaturation method (De Ley & Van Muylem, 1963).

In addition to the GC content the chromosomal DNA is further characterized by its nucleotide sequence and its genome size. DNAs of evolutionary closely related micro-organisms are almost identical with respect to these three parameters. The similarity of the nucleotide sequence among the coryneforms and their genome sizes were determined by the renaturation-rate method (De Ley et al., 1970; Gillis et al., 1970).

A reliable evaluation of the results of hybridization experiments requires the determination of the thermal stability of the formed hybrids, which declines by mismatching (pairing of DNA fragments of non-complementary nucleotide sequences) and unpairing. The extent of mismatching in homologous DNA duplexes (formed from fragments of one type of DNA) was calculated from the decrease of the melting point (thermal stability) of renatured duplexes compared with that of native DNA. The mismatching in heterologous DNA duplexes (formed from fragments of the two component DNAs) was estimated by comparing the melting point of the mixture of heterologous and homologous duplexes with the average value of the melting points of the component DNA duplexes.

The keeping qualities of deep-frozen bacterial DNA were studied by comparing the results of thermal denaturation and hybridization experiments carried out with both native and deep-frozen DNA.

The aim of this investigation was to determine the genetic as well as the morphological and physiological relationships among the coryneform bacteria from different habitats, and further, to study the correlation between the results obtained from investigations on morphology and physiology and from those carried out at the DNA level.

2 Review of literature on coryneform bacteria

2.1 Introduction

Coryneform bacteria are mainly characterized by their pleomorphic morphology which is more or less evident in the different genera. When the coccoid or short rod-shaped cells of coryneform bacteria are transferred to an appropriate fresh medium, they swell and give rise to rods which on ageing change more or less completely into cocci. This vague criterion implies that Gram-positive micro-organisms of the genera *Corynebacterium, Listeria, Erysipelothrix, Microbacterium, Cellulomonas, Arthrobacter, Bevibacterium,* and presumably several species of the genera *Mycobacterium* and *Nocardia* may be designated as coryneform bacteria.

In the present review, however, attention will only be paid to the genera *Corynebacterium, Arthrobacter, Brevibacterium, Mycobacterium* and *Nocardia.*

2.2 Ecology

Representatives of several genera of the coryneform bacteria are the cause of serious infections (Bergey's Manual, 1957; Tomlinson, 1966; Smith, 1966; Smith et al., 1973). In addition to the pathogenic types, saprophytic coryneform bacteria are widely distributed in soil (Winslow et al., 1920; Conn & Dimmick, 1947; Mulder & Antheunisse, 1963; Keddie et al., 1966; Skyring & Quadling, 1970; Bowie et al., 1972; Bousfield, 1972), in dairy waste activated sludge (Mulder & Antheunisse, 1963; Adamse, 1966, 1970) and in pond water (Vanderzant et al., 1972). They are further found on fresh herbage and leaves of Gramineae (Keddie et al., 1966; Bessems, 1973), in milk (Abd-El-Malek & Gibson, 1952), on the surface of hard and soft cheeses (Mulder & Antheunisse, 1963; El-Erian, 1969), on the skin of sea fish (Shewan et al., 1960; Mulder et al., 1966; Shewan, 1971), in poultry deep litter (Schefferle, 1966), in poultry, eggs and meat (Kraft et al., 1966).

In these environments coryneform bacteria may represent a large part of the bacterial flora; e.g. 10^3 to 10^6 arthrobacters per gram of soil have been found, accounting for up to 50% of the soil isolates (Mulder & Antheunisse, 1963; Somare & Blondeau, 1972). Coryneform bacteria constitute similar percentages of the flora found in dairy waste activated sludge, in poultry deep litter, on sea fish and on wooden fish boxes (Spencer, 1959).

Many species of coryneforms are able to decompose a variety of materials, e.g. aliphatic hydrocarbons (Foster, 1962; Jurtshuk & Cardini, 1972), aromatic hydrocarbons (Stevenson, 1967) including herbicides (Jensen, 1966), pyridine derivatives (Ensign & Rittenberg, 1965), DNA and some DNA derivatives (Davis & Newton, 1969; Antheunisse, 1972). In addition, the strong proteolytic activity of soil arthrobacters and coryneforms derived from activated sludge (Mulder et al., 1966; Adamse, 1970; Crombach, 1974c)

may also indicate their important role in decomposition processes in soil and waste water. This is promoted further by the pronounced ability of arthrobacters to withstand drying in soil for more than 10 months without a considerable drop in viability (Mulder & Antheunisse, 1963). Coryneform bacteria presumably also play a role in the ripening of soft cheeses as they predominate in the microflora of cheese surfaces. Part of these bacteria is proteolytic (Mulder et al., 1966; El-Erian, 1969).

2.3 Taxonomy

2.3.1 Morphology

2.3.1.1 Corynebacterium

The straight or slightly curved rods of the *Corynebacterium* species show a tendency to form club and pointed forms. Snapping division may occur, which results in angular (V formation) and sometimes palisade arrangement of the cells (Bergey's Manual, 1957). Bowie et al. (1972) described corynebacteria as being Gram-positive micro-organisms with a tendency to pleomorphy but without showing a mycelium or branching. Bowie et al. obviously limited the term 'branching' to true or secondary branching, for Winslow et al. (1920) and Kuhn & Starr (1960) had already reported primary branching in plant pathogenic *Corynebacterium species.*

2.3.1.2 Arthrobacter

This genus comprises species showing the most pronounced growth cycle. When cocci of these species are transferred to a fresh, appropriate medium, one, two or three germination tubes per cell arise, which develop into rods. These rods are frequently irregular and may show primary branching; snapping division regularly occurs (Mulder et al., 1966; Crombach, 1974c) True or secondary branching has never been observed in *Arthrobacter* (Stevenson, 1968). On ageing, the rods of typical *Arthrobacter* species change nearly completely into cocci. *Arthrobacter* species take a central position between the less pleomorphic *Corynebacterium* species and the mycelial *Nocardia* species. In Bergey's Manual (1957) the *Arthrobacter* species are described to be in general non-motile. Occasional motility has been observed in *A.atrocyaneus* (Clark & Carr, 1951) and in *A.simplex* and *A.citreus* cultures (Kuhn & Starr, 1960). Recently, J. Antheunisse (pers. commun.) found the majority of the *A.simplex* strains, in contrast to the *A.globiformis* strains, to be motile.

2.3.1.3 Brevibacterium

The slender, mostly regular, Gram-positive rods of some *Brevibacterium* species change upon ageing to a certain extent into cocci. In contrast to the description in Bergey's Manual (1957), branching may occasionally occur in some *Brevibacterium* cultures. The growth cycle of the type culture *B.linens* resembles that of soil arthrobacters (Mulder et al., 1966; Crombach, 1974c). However, the complete cycle of development takes more time and the rods which show frequently V formation are more slender than those of the

Arthrobacter species. No clear germination tubes become visible when the cocci are transferred to fresh media. This phenomenon is probably due to the small size of the cocci, rendering the germination tubes less conspicuous.

2.3.1.4 *Mycobacterium*

Mycobacterium species are characterized as acid-fast, straight or slightly curved rods which upon ageing may shorten to cocci; occasionally, slender filaments may be observed. *Mycobacterium* species may show a true branching mycelium at some growth stages (Bowie et al., 1972). *Mycobacterium* and *Corynebacterium* species resemble each other morphologically except for the lesser tendency of the former to form swollen cells. V formation has been observed by J. F. van Delden (Veldkamp, 1970) in *M. rhodochrous* cultures.

2.3.1.5 *Nocardia*

This genus is very ill-defined as can be concluded from the long introduction in Bergey's Manual (1957). *Nocardia* species exhibit a growth cycle reminiscent of that occurring in the preceding genera. In the early growth stage the nocardiae form mycelia of abundantly branched hyphae, the branching being true. On ageing the mucelia break up into short cylindrical cells which subsequently change into cocci. The latter germinate into mycelia after transfer to a fresh medium. V formation has been observed in two *Nocardia* species by J. F. van Delden (Veldkamp, 1970). Recently, Kwapinski & Horsman (1973) categorized the *Nocardia* species in six cultural groups, viz. (1) organisms forming a hard pellicle with an aerial mycelium in Kwapinski's *Nocardia* medium (KNM), but not in Kwapinski's *Actinomyces* medium (KAM); (2) strains producing a hard pellicle in KAM; (3) strains producing a soft *Mycobacterium*-like pellicle; (4) strains with a similar gross appearance to those of group (3) but able to grow in KAM; (5) strains producing a flaky white or gray deposit; (6) strains forming a dispersed growth.

2.3.1.6 *General conclusions on the morphology of coryneform bacteria*

The coryneform genera are morphologically not sharply delineated. Many bacteria become pleomorphic under conditions of unbalanced growth (Müller, 1957; Stevenson, 1961; Chan, 1964); moreover the extent of conversion from rods into cocci is extremely dependent on cultural conditions and may even vary widely within a genus (Skyring & Quadling, 1970; Crombach, 1974c). As morphology is often used as sole criterion for classification, it is not surprising that the coryneform genera are heterogeneous as to non-morphological characteristics (Bowie et al., 1972; Bousfield, 1972; Crombach, 1972). Species of *Corynebacterium, Arthrobacter, Mycobacterium* and *Nocardia* are morphologically akin (Mulder et al., 1966; Gordon, 1966), and simply for that reason it is illogical that the former two and the latter two genera belong to two different orders. Thus, the classification of *Arthrobacter* and *Corynebacterium* in the family Mycobacteriaceae, order Actinomycetales, as given by Conn & Dimmick (1947) was not so objectionable.

Gordon (1966) suggested that the genus *Nocardia*, at present a 'catch-all', should be limited to strains which form filamentous colonies and macroscopically visible hyphae.

Kurup & Schmitt (1973) attributed the confused classification of this genus partly to the disagreement of the results of tests carried out in different laboratories.

2.3.2 V formation, transformation from rods into cocci, and difference in cell-wall composition of cells in either the rod or the coccoid shape

V formation can arise from germination of adjacent cocci, from subpolar germination of rods, or from post-fission movement. The latter mechanism was studied by Krulwich & Pate (1971) who found the cell walls of *Arthrobacter crystallopoietes* to consist of two layers. The inner one invaginated during cell division, whereas the outer layer did not. During further growth the outer layer ruptured at one point and the daughter cells moved within 15 sec or shorter like a closing pocket-knife, which resulted in V formation (snapping division).

It has been reported that rods revert to cocci in two different ways. On ageing, the rods change into cocci either by a gradual reduction in rod length as they divide, or by a simultaneous deposition of cross septa along the rods, which upon rupture gives rise to small oval cells. These cells change into spheres. The latter mechanism was observed in cultures of *A. pascens* containing long rods that had arisen from the germination of cystites (Chaplin, 1957).

Clark (1972) mentioned structural differences in the cell walls of spheres and rods of *A.crystallopoietes.* In addition, the specific activities of several enzymes were found to change during the growth cycle. It was suggested that, at fragmentation, the cells revert to an endogenous metabolism and that the permeability of the membrane is reduced. DNA-RNA hybridization experiments indicated a significant change in the portion of the genome that is read during the various stages of the growth cycle.

It should be stressed, however, that no qualitative differences between spheres and rods have been found in the pattern of major cell-wall compounds (Gillespie, 1963a, b; Keddie et al., 1966) and fatty acid components (Bowie et al., 1972) of several *Arthrobacter* species; Gillespie reported only some quantitative differences in polysaccharides and amino acids.

2.3.3 Various taxonomical tests

As the various genera of coryneform bacteria could not be clearly differentiated by morphology alone, different tests and experiments have been used to elucidate the taxonomy of named coryneform bacteria, viz. cell-wall analysis (Cummins, 1962; Cummins & Harris, 1956, 1958, 1959; Keddie et al., 1966; Yamada & Komagata, 1970a), determination of fatty acid compositions (Bowie et al., 1972; Lechevalier et al., 1973; Minnikin et al., 1974), physiological studies (Mulder & Antheunisse, 1963; Mulder et al., 1966; Gordon, 1966; Crombach, 1974c), serological studies (Cummins, 1962; Lazar, 1968; Kwapinski et al., 1973), and numerical taxonomy (Da Silva & Holt, 1965; Harrington, 1966; Goodfellow, 1967; Davis & Newton, 1969; Masuo & Nakagawa, 1969a; Tsukamura, 1969; Skyring & Quadling, 1970; Yamada & Komagata, 1970a; Bousfield, 1972).

2.3.3.1 Corynebacterium

As the pattern of major cell-wall components of *Corynebacterium* species of animal origin, viz. DL-diaminopimelic acid, glumatic acid and alanine in association with some sugars, arabinose and galactose, was also found to be present in rapidly growing *Mycobacterium* and in *Nocardia* species, Cummins and Harris (1956, 1958) concluded that these three genera should be gathered into the family Mycobacteriaceae. In addition, Cummins (1962) pointed at some serological relationship among these genera. Harrington (1966) even proposed to gather the mentioned genera into one genus. However, this was in deisagreement with the results of numerical taxonomic studies (Davis & Newton, 1969; Bousfield, 1972) showing a clear distinction between *Corynebacterium* and *Mycobacterium-Nocardia* species.

In addition to having similar mureins (peptidoglycans, muropeptides, Barksdale, 1970), species of *Corynebacterium, Mycobacterium* and *Nocardia* also incorporate mycolic acids in their cell walls. These acids are α-branched, β-hydroxy fatty acids. The corynomycolic acids associated with *Corynebacterium* have carbon skeletons of about 30 atoms; those fatty acids associated with *Mycobacterium*, the mycolic acids sensu stricto, contain skeletons of 80 carbon atoms and those associated with *Nocardia*, nocardomycolic acids, have skeletons of about 50 carbon atoms (Lechevalier et al., 1973)

The plant pathogenic corynebacteria were found to be different from the animal and human *Corynebacterium* species with respect to cell-wall composition; the former group of strains contained neither arabinose nor galactose (Cummins, 1962; Cummins & Harris, 1956, 1958) and its majority possessed either LL-diaminopimelic acid or lysine in their cell walls, whereas the majority of the latter group contained DL-diaminopimelic acid (Yamada & Komagata, 1970a). It should be stressed that re-examination of the cell-wall components of several plant pathogenic *Corynebacterium* species revealed that *C.tritici, C.betae, C.flaccumfaciens* and *C. poinsettiae* did not contain lysine as was generally accepted, but ornithine (Perkins & Cummins, 1964).

Serological studies of Cummins (1962) and Lazar (1968) indicated differences between the plant and the animal *Corynebacterium* species; Lazar found that the plant pathogenic *Corynebacterium* species could be divided into five subgroups, while the animal *Corynebacterium* species belonged to at least two serological subgroups. *C.fasciens* appeared to occupy serologically a central position between the plant pathogenic *Corynebacterium* species and the animal *Corynebacterium* ones. All these studies indicated that the former species should be excluded from the genus *Corynebacterium*, but they did not provide clear data for a satisfactory alternative classification; more detailed taxonomic information is needed.

2.3.3.2 Arthrobacter

The genus *Arthrobacter* also appeared to be heterogeneous as to cell-wall composition (Keddie et al., 1966; Yamada & Komagata, 1970a). *A.globiformis, A.pascens, A.aurescens* and *A.ureafaciens* contained alanine, glutamic acid, lysine and galactose as major cell-wall components, whereas *A.simplex* and *A.tumescens* contained alanine, glutamic acid, glycine, LL-diaminopimelic acid, galactose and rhamnose. *A.flavescens* and *A.terregens* had a cell-wall component pattern similar to that of the herbage strains studied by Keddie

et al. (1966) and contained among others an unidentified ninhydrin-positive compound.

The eccentric position of *A.simplex* and *A.tumescens* within their genus was accentuated by their aberrant fatty acid composition (Bowie et al., 1972) and by data of numerical taxonomic studies (Da Silva & Holt, 1965; Goodfellow, 1967; Davis & Newton, 1969; Skyring & Quadling, 1970a; Bousfield, 1972). These species (including *A.marinus*, Oliver & Colwell, 1973) were found to contain different types of C 16 and C 18 fatty acids as major fatty acids, whereas all the other *Arthrobacter* species tested possessed major fatty acids of the C 15:0 anteiso-branched type. The absence of a serological relationship between *A.globiformis* and *A.simplex* was in agreement with the mentioned results, but a certain serological relatedness between the former species and *A.tumescens* was somewhat unexpected (Katznelson & Mason, 1962).

Arthrobacter species can be classified into two groups with respect to their nutritional requirements. First, the species which are able to utilize inorganic nitrogen and citrate as sole N and C sources, respectively, and secondly, the nutritionally more exacting species (Bergey's Manual, 1957). The heterogeneity of the genus is also reflected in carbohydrate metabolism. One group of *Arthrobacter* species (*A.globiformis, A.ureafaciens*) studied by Zagallo & Wang (1962) catabolized glucose mainly by the Embden-Meyerhof pathway, whereas the other group (*A.simplex, A.pascens* and *A.atrocyaneus*) metabolized this carbohydrate primarily by the Entner-Doudoroff and pentose phosphate-pathway.

2.3.3.3 Brevibacterium

The variation in cell-wall components found in some *Brevibacterium* species matches that occurring in *Corynebacterium* and *Arthrobacter* species. Yamada & Komagata (1970a) found that 45 *Brevibacterium* strains could be divided into two main groups: 30 strains showing the presence of lysine and 12 strains containing DL-diaminopimelic acid in their cell walls. The three remaining strains possessed LL-diaminopimelic acid. Even within the species *B.linens* there were representatives which contained either lysine or DL-diaminopimelic acid. Further, a numerical taxonomic study showed the heterogeneity of *Brevibacterium* (Chatelain & Second, 1966).

A comparison of the pattern of major fatty acids (Bousfield, 1972) revealed that *B.linens* and *B.sulphureum* possessed C 15:0 anteiso-branched acids, as did the majority of the *Arthrobacter* strains. These results and those of a numerical analysis (Skyring & Quadling, 1970) support the opinion of Da Silva & Holt (1965) and Davis & Newton (1969) that at least some species of *Brevibacterium* belong to *Arthrobacter;* Da Silva & Holt proposed *B.linens* be renamed *Arthrobacter linens.* This would imply that *Brevibacterium* becomes illegitimate because it then lacks its type species. This reallocation, however, was opposed by Mulder et al. (1966) mainly on a physiological basis and by Crombach (1972, 1974b) primarily because of % GC values and hybridization data. In spite of the morphological resemblance, *B.linens* differs clearly from *Arthrobacter* species in being nutritionally more exacting, less proteolytic and showing a much greater salt tolerance and a much less pronounced tendency to synthesize polysaccharides (Mulder et al., 1966).

Even though some species of the ill-defined genus *Brevibacterium* might be classified into related coryneform genera, the species *B.linens* should remain in its genus.

2.3.3.4 *Mycobacterium*

The *Mycobacterium* species can be divided into rapidly and slowly growing ones. The latter have rarely been involved in taxonomic studies as the generally used tests provide little differentiation among slowly growing *Mycobacterium* species (Wayne, 1967). A numerical taxonomic study (Tsukamura, 1966) clearly showed two distinct clusters within the slowly growing *Mycobacterium* species and five recognizable groups within the rapidly growing ones. The slow-growers in contrast to the rapid-growers were not resistant to 0.1% picric acid and were unable to utilize fumarate, malate and succinate as sole carbon source, and L-glutamate as sole nitrogen as well as sole carbon source.

Mycobacterium phlei was found to contain palmitic acid (C16:0) and oleic acid (C18:1) as major fatty acids (Lennarz et al., 1962) which were also found to be present in the *Norcardia* strains tested by Bowie et al. (1972). However, the difference between the genera *Mycobacterium* and *Nocardia* was shown by the results of a numerical taxonomic study (Goodfellow et al., 1972) and by the absence of the lipid LCN-A (unidentified Lipid Characteristic of *Nocardia asteroides*) in *Mycobacterium* in contrast to the presence of this compound in *Nocardia* species (Mordarska, et al., 1972).

A very unclear position among the *Mycobacterium* strains is occupied by a group of strains tentatively designated by Gordon & Mihm (1959) as *Mycobacterium rhodochrous*. Because of their cell-wall composition, these bacteria might be included in the genera *Corynebacterium, Mycobacterium* or *Nocardia* (Cummins, 1962). Though *M.rhodochrous* and *Corynebacterium* strains share many features, Gordon (1966) decided not to place the former group of strains in the genus *Corynebacterium* because of the supposed difference in glucose metabolism between the type species *C.diphtheriae* and *M.rhodochrous* strains. Veldkamp (1970) commented, however, that both *C.diphtheriae* and *M.rhodochrous* strains are able to oxidize glucose.

The presence of nocardomycolic acids (Lechevalier et al., 1971) and the occurrence of the lipid LCN-A (Mordarska et al., 1972) in *M.rhodochrous* strains indicated a possible relation of these strains with *Nocardia* species. However, in certain cases the characteristic LCN-A spot had a somewhat lower Rf value than that of the reference LCN-A, and it is therefore questionable whether these LCN-A lipids, slightly differing in Rf values, provided reliable evidence for a relationship. Moreover, the data of numerical taxonomy revealed that *M.rhodochrous* strains formed a cluster quite distinct from those clusters accommodating *Nocardia* strains (Goodfellow, 1971; Goodfellow et al., 1972). These authors also pointed at the absence of clustering of strains of the '*rhodochrous* complex' with rapidly growing *Mycobacterium* species. The detection of aberrant mycolic acids in a representative of the mentioned complex (Minnikin et al., 1974) in addition to the variation in Rf values, accentuates the heterogeneity of the complex. More characteristics should be available before the '*rhodochrous*' strains can be assigned to a suitable genus.

2.3.3.5 *Nocardia*

The genus *Nocardia* includes all types of bacteria which exhibit fragmenting mycelia. But fragmentation is not always clear and it is often dependent on the strain under study. The border-line between *Nocardia* and *Mycobacterium* is sometimes difficult to establish (Bradley, 1972); cell-wall composition does not differentiate *Nocardia* species from

rapidly growing *Mycobacterium* species. However, lipid characterization (e.g. mycolic acids and the lipid LCN-A) may be useful for differentiating both genera (Lechevalier & Lechevalier, 1970; Mordarska et al., 1972). From a comparative study on lipids Pommier & Michel (1973) concluded that true *Nocardia* species (*N.asteroides, N.caviae, N.erythropolis* and *N.farcinica*) were very homogeneous in spite of the difference in mycolic acid composition between one *N.farcinica* strain and four other *Nocardia* strains. Lechevalier et al. (1973) found all the *N.farcinica* strains tested to contain mycolic acids sensu stricto. These data in addition to the absence of the lipid LCN-A in *N.farcinica* (Mordarska et al., 1972) provide evidence that this species belongs to *Mycobacterium* and support the suggestion of Lechevalier et al. (1971) that *N.farcinica* should be considered a nomen dubium. Then it would pave the way for considering *N.asteroides* as the type species as was proposed by Gordon & Mihm (1962).

The difference between *Nocardia* and *Mycobacterium*, and the heterogeneity within the former genus as described in Bergey's Manual (1957), were also demonstrated by a study on phage sensitivity (Prauser & Falta, 1968); the genus *Nocardia* should be divided into at least four subgenera.

Immunologically the *Nocardia* strains appeared to be heterogeneous; however, on the whole, these bacteria, in contrast to the findings of Prauser & Falta, were found to be related to fastly growing *Mycobacterium* strains (Kwapinski et al., 1973).

Extensive numerical taxonomic studies with 'nocardioforms' (bacteria which produce a mycelium that fragments into rods and cocci) revealed seven major clusters (Tsukamura, 1969; Goodfellow, 1971; Kurup & Schmitt, 1973). As the first and the last mentioned study revealed that *N.asteroides* is highly heterogeneous, more data are needed before a sharp delineation of this species can be made which is essential for a type species.

In conclusion: the present taxonomy of nocardiae is confused and the genus *Nocardia* should be revised.

2.3.3.6 Coryneform isolates.

The coryneform isolates derived from different soils, dairy waste activated sludge, the surface of cheeses and the skin of sea fish were found to be morphologically related and to resemble closely the *Mycobacterium* and *Brevibacterium* species (Mulder & Antheunisse, 1963). Most of the activated sludge coryneforms should be classed in the genus *Arthrobacter* even though they differ in a few respects from the soil arthrobacters (a somewhat higher salt tolerance and a ready utilization of lactate (Adamse, 1970). The soil and activated sludge coryneforms are physiologically distinguished from the cheese and orange sea-fish strains by their nutritionally non-exacting requirements, their low salt tolerance and their ability to produce large amounts of polysaccharides, (Mulder et al., 1966). This distinction was emphasized by the ability of the soil and activated sludge isolates, in contrast to the cheese and sea-fish ones, to utilize choline as sole carbon and nitrogen source (Kortstee, 1970) and to decompose both uric acid and urea (Antheunisse, 1972). Consequently, the cheese and sea-fish coryneforms should not be placed together with the soil isolates in the genus *Arthrobacter*. This was corroborated by %GC values, data obtained from hybridization experiments and morphological and physiological investigations (Crombach, 1972, 1974b, 1974c). The orange sea-fish coryneforms differed physiologically somewhat from the cheese ones. The latter could be divided into two

groups: viz. the orange strains of the *Brevibacterium linens* type and the non-orange strains (Mulder et al., 1966; Crombach, 1974c). Though both types of strains resembled each other in some respects, the non-orange strains showed a more pronounced tendency to form cocci on ageing and had a somewhat lower salt tolerance, while their nitrogen requirement was more or less intermediate between that of the soil arthrobacters and that of the exacting orange cheese strains.

Mulder et al. (1966) stated that the orange coryneforms from poultry deep litter (see also Schefferle, 1966) showing the *Arthrobacter* morphology were probably closely related to the cheese coryneforms as most of the former strains also required organic nitrogen.

Keddie et al. (1966) and Owens & Keddie (1969) found a notable proportion of the coryneform isolates from soil and herbage displaying the characteristic *Arthrobacter* morphology, especially the nutritionally non-exacting or only biotin-requiring soil coryneforms. Ninety percent of the soil coryneforms were able to utilize inorganic nitrogen when provided with the necessary vitamins, whereas only 30% of the herbage coryneform isolates showed this capacity (Owens & Keddie, 1969). The non-exacting or only biotin-requiring soil coryneforms had the same combination of major cell-wall components as present in *A.globiformis,* whereas none of the herbage coryneforms had a cell-wall component pattern characteristic of these soil isolates.

2.3.4 DNA base composition

An extensive review of DNA base compositions has been published by Hill (1966), but only a few GC contents of coryneform bacteria were compiled. Recently, several articles dealing with DNA base compositions of various coryneform bacteria have been published.

Some discrepancies in % GC values of certain bacteria do occur in the literature (De Ley, 1970; Bowie et al., 1972). These are partly caused by the variety of formulae used for conversion of melting points or buoyant densities to % GC. Therefore, some caution is needed when interpreting DNA base compositions determined according to different methods.

2.3.4.1 Corynebacterium

Marmur et al. (1963) and Bouisset et al. (1963) found DNAs of both plant pathogenic and animal *Corynebacterium* species to contain 52 to 60% GC. Schuster et al. (1968), Yamada & Komagata (1970b), Bowie et al. (1972), Bousfield (1972) and Crombach (1972), however, found significantly higher GC contents in plant *Corynebacterium* species (68 – 76%) than in animal *Corynebacterium* species; GC contents in the latter were close to 60 rather than to 70%. Masuo & Nakagawa (1969b) found all of the animal *Corynebacterium* species tested to range from 48 to 58% GC. Especially the marked difference in % GC between the type species *C.diphtheriae* and plant pathogenic *Corynebacterium* species corroborates the suggestion, based on cell-wall analysis and numerical taxonomy, that the latter group should be excluded from the genus *Corynebacterium.*

2.3.4.2 Arthrobacter

Until recently, the DNA base composition of *Arthrobacter* species has been determined for only one species, *A.globiformis.* It was found to range from 62 to 64% GC (Marmur et al., 1963). Lately, Skyring & Quadling (1970) and Yamada & Komagata (1970b) found GC contents of *Arthrobacter* species ranging from 59 to 74%. This wide range shows that several species, e.g. *A.tumescens* and *A.simplex* (72 – 74% GC) stand out from the genus as they are quite dissimilar from the type species *A.globiformis.* A similar difference of 6 to 8% GC between the former two and the latter species was also published by Bowie et al. (1972). These DNA data correspond with the differences between the species in cell-wall and fatty-acid compositions.

The arthrobacter-coryneform soil isolates studied by Skyring & Quadling (1970) ranged from 40 to 74% in GC content, in contrast to those studied by Crombach (1972) which covered a range from 65.3 to 67.0% GC. This difference in range is partly because the former isolates were selected merely on the possession of the pleomorphic 'arthrobacter-coryneform' morphology, whereas the latter group contained only those strains identified as *A.globiformis* and *A.simplex* (Crombach, 1972, 1974b); the latter author found no significant difference in % GC between both species.

2.3.4.3 Brevibacterium and coryneform isolates from cheese and sea fish

The wide range, 46 to 73% GC, found in *Brevibacterium* species (Yamada & Komagata, 1970b; Bousfield, 1972) indicates that several species are evolutionarily far removed from each other because DNAs differing 10 to 20% in GC content can only share a limited number of complementary nucleotide sequences (De Ley, 1969). The GC content of *B.linens* was found to range from 61 to 64% (Skyring & Quadling, 1970; Bousfield, 1972; Crombach, 1972). Yamada & Komagata (1970b), however, reported a remarkably wider range, viz. 60 to 68% GC, within the species *B.linens.*

The DNA base composition of six out of seven orange cheese coryneforms of the *B.linens* type and that of the majority of the orange sea-fish coryneforms covered the same range being 63 to 64% GC (Crombach, 1972, 1974b). This similarity, in addition to the morphological and physiological resemblance, suggested a genetic relationship.

The non-orange cheese coryneform isolates (Crombach, 1972) were heterogeneous as their DNA base compositions ranged from 56 to 67% GC; the majority of the strains, however, showed GC contents of around 66%.

2.3.4.4 Mycobacterium

The % GC values of 30 *Mycobacterium* strains, ranging from 64 to 70, were reported by Wayne & Gross (1968) to be bimodally distributed: a cluster exhibiting GC values within the range 64.0 to 66.4%, and a cluster containing GC values in excess of 66.5%. This bimodality which seems to be somewhat exaggerated, did not correspond with the division into slow and fast growers. Šlosárek (1970) found a nearly equal % GC range among 20 fast growing *Mycobacterium* strains, viz. 63.3 to 67.9. The % GC values of three *M.smegmatis* strains were found to be close to those reported by Crombach (1972), viz. 68. The GC contents of the *M.phlei* species covered the whole range, in contrast to

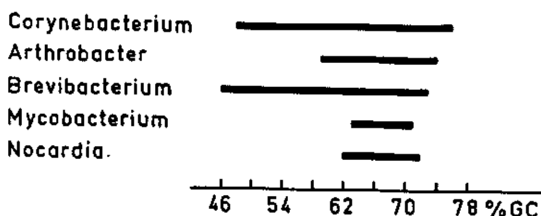

Fig. 1. Range of DNA base composition of several coryneform genera.

those given by Tewfik & Bradley (1967), Masuo & Nakagawa (1969b), Crombach (1972) and Bradley et al. (1973) which were in the vicinity of 69%.

The GC contents in the *M.rhodochrous* complex were found to be in the range of 66 to 71% (Wayne & Gross, 1968; Bousfield, 1972; Crombach, 1972).

2.3.4.5 Nocardia

The GC contents found so far in this genus were between 62 and 72% (Tewfik & Bradley, 1967; Wayne & Gross, 1968). Three *N.asteroides* strains exhibited a GC content between 64 and 69%, and *N.brasiliensis* had a GC value of 65% (Hill, 1966). Eleven *Nocardia* strains studied by Tewfik & Bradley (1967) and Bousfield (1972) fell apart into two groups due to their DNA base composition: first, strains with a GC content of 62 to 64% and secondly, strains exhibiting 68 to 69% GC. The DNA base composition of *Nocardia* strains studied by Bowie et al. (1972) and Crombach (1972) and being 66.8 – 71.8% GC, partly overlapped those of the latter group. The majority of the *Nocardia* species tested exhibited GC contents close to 70 rather than to 60%.

It can be concluded that the five genera considered in this review can not be differentiated by their DNA base composition as the GC contents overlap each other more or less (Fig. 1.). However, a coryneform bacterium with a % GC value less than 60 probably belongs neither to the mycobacteria and nocardiae, nor to the phytopathogenic corynebacteria.

So far, DNA base compositions of only a limited number of coryneforms have been determined, probably because of the difficulties encountered when lysing the cells of many coryneform bacteria.

2.3.5 DNA–DNA hybridization and genome size

Although DNA base composition is a very useful tool in classification, it can only provide evidence of a dissimilarity between bacteria differing sufficiently in % GC. With DNA-DNA hybridization experiments, however, the similarity in the nucleotide sequence among organisms can be estimated, which gives an impression of the evolutionary relationship; heterologous DNAs are only capable of reassociating if they share sufficient complementary base sequences. Homologous bacterial chromosomes have similar genome sizes.

Several hybridization techniques have been employed (De Ley et al., 1970; Crombach, 1974a). Recently, the membrane-filter method and the hydroxyapatite method have been

used most frequently (Brenner et al., 1972). A third one, the renaturation-rate method, has the advantage of using unlabelled DNA, but the disadvantage of requiring relatively large amounts of reference DNA.

So far, only a few articles dealing with result of hybridization experiments with coryneform bacteria have been published. Hybridization experiments with DNA of coryneforms derived from soil, cheese and sea fish, including some named coryneform cultures, have been carried out by Crombach (1974b). The results exhibited a low degree of binding, D, (below 30%) between *A.globiformis* on the one hand and a plant-pathogenic *Corynebacterium* and *B.linens* on the other. This supported the objection of Mulder et al. (1966) to the suggestion of Da Silva & Holt (1965) to reallocate *B.linens* in the genus *Arthrobacter.* In spite of a fair hybridization between *A.globiformis* and both a *Mycobacterium* and a *Nocardia* strain (D was 44 and 52%, respectively), a close evolutionary relationship between these organisms is unlikely because of their marked differences in genome sizes. The low degree of binding between *B.linens* and *C.bovis*, in addition to their different genome sizes, indicates only a remote relationship.

The hybridization data obtained from soil coryneforms corresponded with the division of these strains into an *A.simplex* and an *A.globiformis* group; the strains of the latter group appeared to be either moderately or closely related. The strains of the simplex group hybridized only to a low degree with the reference strain of the globiformis group (Crombach, 1974b).

The orange cheese coryneforms, in contrast to the orange sea-fish coryneforms, appeared to be homogeneous as concluded from their high degree of binding with *B.linens*. Only a minority of the orange sea-fish coryneform bacteria were found to be closely related with the orange cheese strains.

Most of the non-orange cheese coryneforms were mutually closely related, in spite of their different origin.

The hybridization data supported the opinion of Mulder et al. (1966) and Crombach (1974c) that the cheese and orange sea-fish coryneforms should remain separate from the soil arthrobacters. Further study is required in search of an appropriate genus for the majority of the orange sea-fish coryneforms.

Hybridization experiments revealed little homology between the fast growing *Mycobacterium* species (*M.phlei* and *M.smegmatis*) and the slow growers (*M.bovis* and *M.tuberculosis*) (Gross & Wayne, 1970; Bradley, 1972, 1973); however, the latter two species exhibited an extensive homology (D was 91%). The heterogeneity among the *Mycobacterium* species was also demonstrated by the wide range of their genome sizes, being from 2.5 to 4.5 x 10^9 dalton (Bradley, 1972).

There are many difficulties when the usual diagnostic tests are used to differentiate between the genera *Mycobacterium* and *Nocardia*. These are due to the defective delineation of both genera. Reassociation studies demonstrated that mycobacterial reference DNA bound only poorly with that of various *Nocardia* species, which held also for nocardial reference DNA and various *Mycobacterium* species (Bradley, 1973; Bradley et al., 1973). This indicates the lack of a close genetic relationship between both genera.

The latter authors found two groups of *Nocardia* species on the basis of their relatedness with *N.erythropolis,* which corresponded closely with the division into strains with high and low GC contents. Even within the species *N.asteroides* at least two subspecies were present. Further evidence of heterogeneity in the genus *Nocardia* was

provided by reassociation studies (Clark & Brownell, 1972), which demonstrated little relatedness between two *Nocardia* species in spite of their sensitivity to the same phage.

More detailed studies especially at the DNA level have to be carried out for elucidating the confused taxonomy of coryneform bacteria.

Antonie van Leeuwenhoek **38** (1972) 105–120

DNA base composition of soil arthrobacters and other coryneforms from cheese and sea fish

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CROMBACH, W. H. J. 1972. DNA base composition of soil arthrobacters and other coryneforms from cheese and sea fish. Antonie van Leeuwenhoek **38:** 105–120.

The DNA base composition of 34 coryneforms isolated from different sources, and those of 20 named cultures of the genera *Arthrobacter*, *Brevibacterium*, *Mycobacterium*, *Corynebacterium* and *Nocardia* has been determined.

A preliminary study of the morphological and physiological characteristics of the new isolates, and some named cultures, led to a division into three groups:
1) Soil coryneforms identified as arthrobacters, completed with the *Arthrobacter globiformis* strains ATCC 8602 and 8010.
2) Orange coryneforms and one white isolate from cheese, and orange coryneforms including one yellow isolate from sea fish, completed with two *Brevibacterium linens* strains ATCC 9174 and 9175.
3) Non-orange cheese coryneforms.

DNA base composition of group (1) ranges from 65.3 to 67.0 molar % GC, suggesting that this group is genetically homogeneous. % GC values of group (2) range from 62.6 to 64.0 except for one isolate (65.6), suggesting that this group is also homogeneous. DNA base composition of group (3) ranges from 65.5 to 66.9 % GC, except for three isolates (56.5, 60.1, 60.6). It is concluded that as far as their % GC is concerned, the strains of group (3), except the threem entioned ones, may be closely related to the arthrobacters of group (1). The strains of group (2) are probably less closely related to those of the groups (1) and (3).

INTRODUCTION

The coryneform bacteria are an important group widely distributed in nature, *viz.* in soil, on plants, in waste water, in activated sludge and in poultry deep litter, on cheese and on sea fish. They may degrade a variety of materials including aromatic compounds occurring in several herbicides (Mulder and

Antheunisse, 1963) or play a role in the ripening of soft cheeses (Mulder et al., 1966; El Erian, 1969).

For the classification of coryneform bacteria several taxonomic methods have been used. Assuming that the pattern of principal amino acids in the cell wall of gram-positive bacteria is characteristic of groups approximately at the level of the genus (Cummins and Harris, 1956), the taxonomic position of coryneforms together with arthrobacters was investigated (Cummins and Harris, 1958, 1959). In these studies an investigation about the composition of cell-wall sugar compounds was involved. It was concluded to reallocate the genera *Mycobacterium*, *Corynebacterium* and *Nocardia* into one family: Mycobacteriaceae (see also Harrington, 1966). The genus *Arthrobacter* was found to be heterogeneous (Cummins and Harris, 1959). This was confirmed by Keddie, Leask and Grainger (1966) using cell-wall composition as a taxonomic tool, by Goodfellow (1967) and Davis and Newton (1969) using numerical taxonomy and by Yamada and Komagata (1970*a*) on the basis of the pattern of principal amino acids in the cell wall.

However, the data from cell-wall composition should not be used too directly for classification of microorganisms (Cummins, 1962) unless they are reinforced by other tests.

Studies of the morphological and physiological characters of coryneform bacteria including the arthrobacters make the latter appear to be related to the mycobacteria, corynebacteria and nocardiae (Mulder and Antheunisse, 1963; Mulder et al., 1966; Gordon, 1966). This conclusion is partly supported by Masuo and Nakagawa (1969*b*) who claimed that many cultures of the corynebacteria are akin to certain mycobacteria. Davis and Newton (1969) made an attempt to gain a deeper insight into the relationship between several groups of coryneforms with the aid of numerical taxonomy. According to their findings the arthrobacters, together with the genera *Brevibacterium* and *Cellulomonas* should be placed into a newly created family Arthrobacteriaceae, belonging to the order Eubacteriales. The fast growing species of the genus *Mycobacterium* and the species of the genus *Nocardia* should be placed into the family Mycobacteriaceae, and the corynebacteria, listeriae and kurthiae into the family Corynebacteriaceae, both families to be inserted into the order Eubacteriales. More numerical taxonomic studies about coryneforms have been reported by Skyring and Quadling (1969) and Masuo and Nakagawa (1969*a*).

Morphological and physiological studies (Mulder and Antheunisse, 1963; Mulder et al., 1966) showed that the orange coryneform bacteria from sea fish resemble *Brevibacterium linens* in the nitrogen and vitamin requirements, and that the orange cheese coryneforms isolated from the surface of both hard and soft cheese are identical with *Brevibacterium linens*, which resembles the

arthrobacters, having more exacting nutritional demands. Abe, Takayama and Kinoshita (1967) are of the opinion that the difference between the genus *Brevibacterium* and related genera including *Corynebacterium* is not clear. Hence, the strains classified into the genus *Brevibacterium* may possibly be transferred into a related genus. Davis and Newton (1969) reported that most of the strains of the genus *Brevibacterium* are probably closely related with the arthrobacters.

For obtaining an objective and better justified taxonomical classification of the coryneforms, it seemed advisable to carry out DNA base composition determinations of different strains. When this study was in progress, a number of articles dealing with DNA base composition and taxonomy of coryneforms appeared (Skyring and Quadling, 1969; Masuo and Nakagawa, 1969*b*; Yamada and Komagata, 1970*b*). One of the striking results of these studies is the heterogeneity of the genera *Arthrobacter*, *Brevibacterium* and *Corynebacterium* as they are classified in Bergey's Manual (Breed, Murray and Smith, 1957). Further, Skyring and Quadling (1969) and Masuo and Nakagawa (1969*b*) found that the clusters, obtained with numerical taxonomy, contained some strains which, according to their DNA base composition, were not related to the other strains in the same cluster. The latter results would mean that numerical taxonomy should not be overestimated in bacterial classification.

MATERIALS AND METHODS

Microorganisms. The newly isolated strains and the named cultures of the genera *Arthrobacter*, *Brevibacterium*, *Mycobacterium*, *Corynebacterium* and *Nocardia*, together with colour and source, are listed in Table 2. For checking the procedure employed, the strains *Escherichia coli* B and *Agrobacterium tumefaciens* B 6, with known DNA base composition, have been included.

Cultivation of organisms. All the strains under investigation, except those belonging to the genera *Mycobacterium*, *Corynebacterium* and *Nocardia*, were cultivated on a solid medium containing yeast extract, 0.7% (Oxoid) and glucose, 1.0%. The bacteria of the genera *Mycobacterium*, *Corynebacterium* and *Nocardia* were grown on a solid medium containing nutrient broth, 1.3% (Oxoid) and glycerol, 7%. Strains C 28 and C 29 were grown on a medium composed of soja tryptone, 3% (Oxoid), Tween 80, 1% and agar 1.2%. All the strains were cultivated at 25 or 30 C. They were harvested during the logarithmic phase and washed twice with 0.01 M phosphate buffer adjusted to pH 7.0. The cell mass was stored at –30 C.

Isolation of DNA. DNA was isolated and purified according to the method

of Marmur (1961) slightly modified by the present author. For lysing the cells, they were suspended in Tris-EDTA (0.033 M Tris(hydroxymethyl)-aminomethane and 0.001 M ethylenediaminetetraacetate, pH 8.0) to which lysozyme (5mg/g wet packed cells) was added. This mixture was incubated for 30 min at 37 C. After lysis, 0.1 volume of sodium lauryl sulfate, 25 %, was added and the mixture was heated for 10 min at 60 C. The dissociation of the protein from thy DNA was achieved by adding $NaClO_4$ to a final concentration of 1M. Be shaking the hot mixture to foam, the recovery of DNA was increased (Prof. Dr. J. De Ley, personal communication). For deproteinizing, the mixture was gently shaken with an equal volume of chloroform–isoamyl alcohol (24:1, v/v) for 30 min. The phases were separated by centrifugation for 10 min at 10,000 $\times g$ and the upper phase was pipetted off. The deproteinization was repeated twice. The nucleic acids were precipitated by 2 volumes of ethyl alcohol (96 %), spooled on a rod and sterilized by exposure to ethyl alcohol, 70 %, for several hours. For a further elimination of protein, a step with pronase (Calbiochem, B grade), final concentration 50 μg/ml, for 16 hr at 37 C was included after the treatment with ribonuclease (Calbiochem, A grade). These two enzyme reactions were carried out in 1 × SSC (0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0) sterilized by Chamberland candle filtration, in which the precipitated nucleic acids were redissolved. In this incubation mixture no bacterial growth was noticed. After the step with pronase the mixture was deproteinized thrice in the manner already described. The nucleic acid was precipitated with ethanol (96 %) and redissolved in 9 ml of 0.1 × SSC. Then 1.0 ml acetate-EDTA (3.0 M sodium acetate and 0.001 M EDTA) was added and the nucleic acid precipitated by 0.54 volume of isopropyl alcohol. After redissolving in 0.1 × SSC this step was repeated. Subsequently the DNA was washed in four solutions of ethyl alcohol of increasing concentration (70, 80, 90 and 96 %). The purified DNA was dissolved to a high concentration in 1 × SSC in the presence of a few drops of chloroform and stored at 4 C.

Molecular weight of the DNA fragments. The molecular weight (M.W.) of the isolated DNA fragments can be estimated with the relationship

$$S_{20,w} = 0.063\ M_w^{0.37} \text{ (Doty, McGill and Rice, 1958)}$$

$S_{20,w}$ stands for the sedimentation coefficient, expressed in Svedberg units, at 20 C in water. M_w represents the M.W. of the double-stranded DNA fragments. This sedimentation coefficient was determined in the Spinco Analytical Ultracentrifuge Model E. The rotor cell was filled with DNA dissolved in 1 × SSC to a concentration of 50 μg/ml. After equilibrium, several pictures were taken every 4 minutes using ultraviolet optics. The sedimentation coefficient was calculated from

$$S_{20,w} = \frac{r_2 - r_1}{\frac{r_1 + r_2}{2} \cdot (t_2 - t_1) \cdot w^2 \cdot 60}$$

in which r represents the distance from the boundary between the DNA solution and the DNA-free solvent, to the centre of the rotor at time t and w stands for the rotation velocity. To facilitate the determination of these distances, the pictures were screened with the Photovolt Microdensitometer Model 520 A.

DNA concentration. The concentration of the DNA samples for analytical use was determined according to Burton (1956). Deoxyribose standard solutions of five different concentrations were included in every experiment. The DNA content of the samples, expressed as mg/ml can be calculated with the aid of the factor 4.9, converting the amount of apparent deoxyribose to DNA.

Protein concentration. The ratios of the absorption of a DNA solution at 280/260 nm and at 230/260 nm give a rough indication on the protein contamination of the DNA. For a more exact determination of the protein content of the DNA samples, the method of Ramachandran and Fraenkel-Conrat (1958) was used. The standard curve was made with crystalline bovine albumine. The protein concentration was determined in 1 ml DNA sample containing 700 to 1000 μg DNA. Three standard samples were included in every experiment. The reaction and colour development were performed at 37 C. The colour intensity was read at 700 nm.

RNA concentration. The determination of the RNA concentration of the DNA samples was performed with the orcinol reagent according to the method of Ceriotti (1955).

Because the DNA itself offers some contribution to the colour, it is necessary to correct for it. A correction curve was made for five different concentrations of pure DNA (BDH, Thymus Gland). Their concentrations had to be in the same range as those of the samples of which the RNA concentrations were measured.

Thermal denaturation curve. The thermal denaturation curve was determined according to the method of De Ley and Van Muylem (1963) with an adapted Gilford spectrophotometer Model 2400, provided with three sets of thermospacers. The DNA was redissolved in 1 × SSC to a concentration of about 50 μg/ml. The DNA solution in the quartz cuvettes (1 cm pathway and closed with teflon stoppers) was heated by a hot mixture of glycerol–water flowing through a double set of thermospacers. The temperature could be programmed at any desired value by a Neslab temperature-programmer. The temperature of the cuvette house was determined by a calibrated platina resistance and recorded through the Gilford Thermosensor, which was calibrated against a very accurate

thermometer, placed in a cuvette with 1 × SSC solution. By this calibration the temperature of the DNA solutions in the cuvettes during the thermal denaturation could be measured. After equilibrium, the temperature of the DNA solution was increased from 80 to 100 C with a velocity of 0.1 C/min. During the heating, the optical density (O.D.) of the DNA solution was recorded against a blank, consisting of guanine dissolved in 1 × SSC, O.D. of about 1. The blank was compensated for drift in the apparatus and thermal expansion of the solution by the Gilford Reference Compensator. The photomultiplier and the split were cooled with water of 25 C instead of tap water to prevent formation of condensate on the photomultiplier (Prof. Dr. J. De Ley, personal communication). The rubber pipes and the cuvette house were carefully isolated. The thermosensor was calibrated every fortnight. Some DNA samples with known thermal denaturation curve were regularly included as a control.

Mean guanine plus cytosine content. The mean guanine plus cytosine (GC) content of DNA is expressed as molar %:

$$\frac{\text{guanine} + \text{cytosine}}{\text{guanine} + \text{cytosine} + \text{adenine} + \text{thymine}} \times 100.$$

The % GC can be estimated with the formula of Marmur and Doty (1962) recalculated by De Ley (1970):

$$\%\,\text{GC} = (\text{T}_\text{m} - 69.4) \times 2.44$$

T_m, the melting point of DNA, equals the temperature at which the relative absorbance rise has reached its half value of total increase during thermal denaturation.

Compositional distribution. The standard deviation σ, expressed as % GC, of the compositional % GC distribution around the mean, was calculated according to the modified formula of De Ley (1969):

$$\sigma = (\Delta\text{T} - 0.6) \times 1.25$$

ΔT is the temperature interval between 15.9 and 84.1 % of the total relative increase of the O.D.

The whole experimental procedure was checked by comparing the % GC of a distinct purified DNA sample and that of two named cultures (*Escherichia coli* B and *Agrobacterium tumefaciens* B 6) as found in the present study with that obtained in different laboratories (Table 1). It is clear that the results are in good agreement with those of the other investigators.

Table 1. Mean DNA base composition of a certain DNA sample and two named cultures, determined by different investigators

	% GC		
	DNA[1])	*Escherichia coli* B	*Agrobacterium tumefaciens* B 6
Heberlein et al. (1969)	–	52.5	61.4
v.d. Plaat (1969)	–	52.0	61.3
De Ley (pers. comm.)	60.2	–	–
The author	60.4	52.1	61.5

[1]) DNA sample, kindly supplied by Dr. J. De Ley, Gent, Belgium.

RESULTS AND DISCUSSION

A preliminary study has shown that according to morphological and physiological characters the strains under investigation may be divided into three groups. The morphological characters studied include the shape of the cells and the velocity of transformation from rod into coccus at different temperatures and at different nutritional and culture conditions (solid or liquid media); the physiological characters include colour production, salt-tolerance, hydrolysis of casein, gelatin, and starch, and utilization of a number of carbon compounds such as glucose, lactose, saccharose, lactate, acetate, citrate, and glycerol.

Group one contains the strains isolated from soil, together with *Arthrobacter globiformis* ATCC 8602 and 8010. They are grey-white, and salt-intolerant. On ageing they form cocci from rods fairly quickly. Mulder et al. (1966) showed that the soil arthrobacters have a pronounced tendency to accumulate glycogen as reserve material and can grow on ammonium salts as the sole nitrogen source.

Group two comprises orange coryneforms and one white isolate from cheese, and orange coryneforms including one yellow isolate from sea fish, completed with two *Brevibacterium linens* strains ATCC 9174 and 9175. They are salt-tolerant; the rods tend less to transform into cocci and are more regular and thinner than those of the strains of group one. The strains of group two do not accumulate glycogen as reserve material, and require amino acids as the nitrogen source (Mulder et al., 1966).

Group three comprises the non-orange cheese coryneforms, which in their main physiological characters resemble the strains of group two, but morphologically there are differences, e.g., at 15 C the tendency of the strains of group three to form cocci is more pronounced than that of the strains of group two, but less than that of the soil arthrobacters. However, at 25 C, the non-orange cheese strains turn into cocci even more readily than do the soil arthrobacters.

This agrees with the results of Mulder et al. (1966). Also the shape of the cells is more like that of the soil arthrobacters than like that of the strains of group two.

For optimal recovery of DNA the cells must be lysed completely. Because most of the arthrobacters and other coryneforms have stronger cell walls in the coccoid than in the rod stage, only cells in the latter stage have been used. Lyophilizing the deep-frozen cells of the strains tested did not promote lysis, but resuspending them in Tris-EDTA, pH 8.0, did. The use of Tris-EDTA was a marked improvement as compared to saline-EDTA (Marmur, 1961). The favourable effect of Tris on solubilization of cell walls is in accordance with the results of Cox and Eagon (1968) who reported a promoting effect of Tris on solubilization of cell walls of *Pseudomonas aeruginosa*, suspended in a mixture of EDTA-lysozyme.

The width of the thermal denaturation curve is known to be nearly independent of the size of the DNA fragments, if the M.W. of the latter is within the range 1 to 25 $\times 10^6$ dalton (De Ley, 1969). Hence the M.W. of fourteen representative DNA samples was determined and found to be within the range 6 to 13 $\times 10^6$ dalton, which agrees with reports of Marmur (1961), Heberlein, De Ley and Tytgat (1967) and van der Plaat (1969).

Another important parameter of the physical condition of isolated DNA is the value of the hyperchromic effect which equals the relative increase of absorbance due to melting of DNA, expressed as a percentage of the $O.D._{260\,nm}$ of DNA at 25 C. During denaturation of DNA the O.D. increases due to the transition of the double helix into the random coil. Hydrogen bridges between regularly placed bases in the double strands break and the strong interaction among the π-electrons of the bases disappears. The increase of the absorbance depends rather on the physical condition of DNA than on the DNA base composition. It is clear that hyperchromicity will decrease if many hydrogen bridges have already got broken before denaturation occurs, e.g. by rough isolation and purification of DNA.

It is seen from Table 2, that the hyperchromic effects of all DNA isolates cover a range from 30 to 37%. This is in accordance with the values reported in literature, which are generally in the vicinity of 35%.

To obtain an impression of the purity of the DNA isolates, their protein and RNA content have been determined. The average protein content of ten randomly selected DNA samples was found to be 1.8% estimated with the method of Ramachandran and Fraenkel-Conrat (1958). The protein content of twelve other DNA samples has been determined spectrophotometrically, assuming that 46 µg DNA/ml gives an $O.D._{260\,nm} = 1$. With this less accurate method, the average protein content of the twelve samples was found to be 1.5%. These values agree with those of van der Plaat (1969) and Loeb and Chauveau

(1969) (less than 2%). The low protein contamination of the DNA isolates is also shown by the ratios of O.D. at 280 and 260 nm and those at 230 and 260 nm. These ratios cover the range 0.50 to 0.52 and 0.45 to 0.47 respectively, and agree with the results of Marmur (1961) and those of van der Plaat (1969).

To demonstrate the effect of protein contamination on thermal denaturation, protein was added to a number of DNA samples to a final concentration of 2 and 5%, respectively. This did not result in any significant shift in the thermal denaturation curve. Although the protein added was not identical to that present in DNA isolates, it may be concluded that the low protein content of the DNA isolates has not interfered with the thermal denaturation.

Residual RNA content in the DNA samples has been determined with the method of Ceriotti (1955). The mean RNA concentration of ten DNA samples was 1.5%. However, this method is not very reliable as high DNA concentrations interfere with the colour formation.

Another test for demonstrating the presence of RNA in DNA preparations is based on the fact that the absorbance of a DNA solution which is not free from RNA increases distinctly when the temperature rises from 25 to 80 C. This is mainly due to the melting of the RNA inter- and intrastrand hydrogen bridges between 25 and 60 C (Hastings and Kirby, 1966). In the present investigation this phenomenon was never observed. These results and those of the chemical analysis support the conclusion that the DNA samples were free from interfering amounts of RNA.

The DNA base composition of the strains under investigation are recorded in Table 2 and the distribution is shown in Fig. 1. The melting point of each sample of DNA was ascertained fivefold. The standard deviation of all the melting point determinations was 0.15 C. The DNA of twelve strains was isolated twice and the mean deviation of DNA base composition between different isolates of the same strain was 0.4% GC.

As will be seen from Fig. 1 and Table 2, there is a clear difference between the % GC of the strains of groups one and three on the one hand and those of group two on the other. The % GC of the strains of groups one and three, except strains EC 14, EC 20 and AC 253, are in the vicinity of 66, while the orange strains of group two, including strains B 4 and AC 481, have a mean of 63.7% GC.

The strains of group one (soil coryneforms, identified as arthrobacters) are probably genetically closely related as the % GC is within the range of 65.3 to 67.0. This may be inherent to the isolation method, albeit the strains have been isolated from different soils. This homogeneity of the soil arthrobacters contrasts with the results of Skyring and Quadling (1969) who found a heterogeneity in the coryneform soil isolates in respect of the % GC, ranging mainly from 60 to

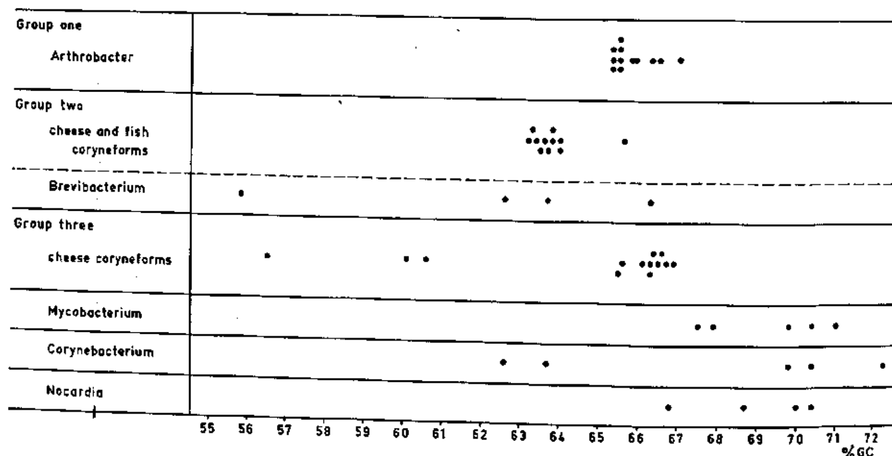

Fig. 1. Survey of the mean DNA base composition of strains tested, belonging to different groups.

74. However, in their investigation the most important criterion for identifying the strains as arthrobacter-coryneform soil isolates was the pleomorphic "arthrobacter-coryneform" morphology. This may account for the large variation in % GC found by these authors as contrasted with the narrow range found in the present study.

Considering the % GC of the strains of group two, it will be clear that this group, except isolate B 3, may be genetically homogeneous which would mean a high degree of relationship between the orange cheese coryneforms including the white isolate B 4, and the orange coryneforms from sea fish including the yellow isolate AC 481.

During the above-mentioned morphological and physiological investigation of the various coryneform organisms, three strains of group three were found to deviate from the majority. The strains EC 20 and AC 253 are distinguished by a much more pronounced tendency to transform into cocci on ageing; they are strongly proteolytic, decompose starch and can utilize saccharose. The deviation of these two strains of group three is confirmed by their DNA base composition, *viz.* 60.6 and 60.1% GC, respectively. Strain EC 14, a grey-white cheese coryneform, stands out from the other strains of group three by the ability of using ammonium salts in the presence of added vitamins (El Erian, 1969)

Table 2. List of microorganisms, together with their mean DNA base composition, standard deviation σ of the compositional % GC distribution in the bacterial chromosome, and the hyperchromic effect of the DNA samples.

Microorganism	Strain number	Colour	Origin	Mean %GC	σ, as %GC	Hyp. effect
Group one						
Arthrobacter spec.	AC 1	grey-white	acid soil	65.5	3.2	32%
Arthrobacter spec.	AC 4	grey-white	acid soil	65.5	3.2	34%
Arthrobacter spec.	AC 8	grey-white	alkaline soil	65.3	4.6	34%
Arthrobacter spec.	AC 11	grey-white	alkaline soil	65.3	4.1	34%
Arthrobacter spec.	AC 16	grey-white	alkaline soil	67.0	3.9	34%
Arthrobacter spec.	AC 29	grey-white	neutral soil	65.3	3.5	34%
Arthrobacter spec.	AC 157	grey-white	alkaline soil	66.3	3.9	33%
Arthrobacter spec.	AC 158	grey-white	alkaline soil	65.8	4.0	34%
Arthrobacter spec.	AC 166	grey-white	acid soil	65.5	4.2	33%
Arthrobacter spec.	AC 206	grey-white	dried alkaline soil	65.9	3.7	31%
Arthrobacter globiformis	AC 403	grey-white	ATCC[1]) 8602	66.5	4.1	34%
Arthrobacter globiformis	AC 405	grey-white	ATCC 8010	65.5	4.5	35%
Group two						
cheese coryneform	B 3	orange	Limburger cheese	65.6	3.4	34%
cheese coryneform	B 4	white[2])	Limburger cheese	63.6	3.5	35%
cheese coryneform	AC 251	orange	Meshanger cheese	63.8	3.1	34%
cheese coryneform	AC 252	orange	Meshanger cheese	63.8	3.2	34%
cheese coryneform	AC 275	orange	Meshanger cheese	63.2	3.1	35%
cheese coryneform	AC 423	orange	Gouda cheese	63.7	3.2	35%
cheese coryneform	AC 448	orange	Edam cheese	63.4	3.2	34%
fish coryneform	AC 470	orange	sea fish	64.0	3.1	34%
fish coryneform	AC 471	orange	sea fish	64.0	3.7	35%
fish coryneform	AC 480	orange	sea fish	63.5	3.6	35%
fish coryneform	AC 481	yellow	sea fish	63.3	4.6	34%
Brevibacterium linens	B 41	orange	ATCC 9174	62.6	3.2	35%
Brevibacterium linens	B 42	orange	ATCC 9175	63.7	3.5	34%
Group three						
cheese coryneform	EC 7	light-red	Limburger cheese	65.6	3.5	34%
cheese coryneform	EC 9	light-red	Limburger cheese	66.3	3.4	34%
cheese coryneform	EC 10	light-red	Limburger cheese	66.3	3.5	34%
cheese coryneform	EC 14	grey-white	Limburger cheese	56.5	4.5	36%
cheese coryneform	EC 15	grey-white	Limburger cheese	66.7	3.6	33%
cheese coryneform	EC 16	grey-white	Limburger cheese	66.5	3.2	33%
cheese coryneform	EC 20	grey-white	Limburger cheese	60.1	4.7	36%
cheese coryneform	AC 253	grey-white	Meshanger cheese	60.6	5.0	37%
cheese coryneform	AC 256	grey-white	Meshanger cheese	66.1	4.0	35%
cheese coryneform	AC 261	grey-white	Meshanger cheese	66.9	3.0	33%
cheese coryneform	AC 262	grey-white	Meshanger cheese	65.5	4.0	35%
cheese coryneform	AC 263	grey-white	Meshanger cheese	66.4	3.4	35%
cheese coryneform	AC 278	light-red	Meshanger cheese	66.6	4.1	34%

[1]) American type culture collection, Rockville, Maryland, U.S.A.
[2]) orange in yeast extract glucose medium plus 4% of added NaCl.

(Table 2, continued)

Microorganism	Strain number	Colour	Origin	Mean %GC	σ, as %CG	Hyp. effect
Named cultures						
Brevibacterium						
B. ammoniagenes	B 47	white	NCIB[3]) 8143	55.8	4.2	37%
B. vitarumen	B 48	yellow-grey	NCIB 9291	66.3	5.6	36%
Mycobacterium						
M. phlei	M 9	orange	Lab. coll.[4])	69.8	4.1	30%
M. phlei	M 10	orange	ATCC 354	70.4	3.1	33%
M. rhodochrous	M 14	orange	Lehman and Neumann. R. E. Gordon, Inst. of Microbiology, Univ. of New Jersey, New Brunswick.	71.0	3.5	31%
M. smegmatis	M 25	light-pink	ATCC; E.O. Jordon.	67.5	3.6	33%
M. smegmatis	M 29	grey-yellow	ATCC; Lehmann and Neumann	67.9	3.1	33%
Corynebacterium						
C. bovis	C 28	brown-red	ATCC 7715	62.6	2.9	33%
C. bovis	C 29	brown-red	NCTC[5]) 3224	63.7	2.7	33%
C. flaccumfaciens	C 33	brown-yellow	v.d. Want, Lab. of Virology, Wageningen	69.8	3.4	30%
C. michiganense	C 34	yellow	la Rivière, Lab. of Microbiol. Delft	72.3	5.0	36%
C. tritici	C 37	yellow	NCPPB[6]) 471	70.4	4.6	33%
Nocardia						
N. convoluta	N 2	light-pink	ATCC 4275	66.8	3.2	32%
N. spec.	AC 86	red	peaty soil, Lab coll.	68.7	2.6	32%
N. spec.	AC 555	yellow-green	clay soil	70.0	3.1	31%
N. spec.	AC 556	yellow-green	clay soil	70.4	3.4	32%
Escherichia coli B			Dr. J. De Ley[7])	reference strain		
Agrobacterium tumefaciens B6			Dr. J. De Ley	reference strain		

[3]) National collection of industrial bacteria, Torry Research Station, Aberdeen, Scotland
[4]) Culture collection of the Laboratory of Microbiology, Agricultural University, Wageningen, The Netherlands.
[5]) National collection of type cultures, London, England.
[6]) National collection of plant pathogenic bacteria, Harpenden, Hertfordshire, England.
[7]) Laboratory of Microbiology, Gent, Belgium.

and by a slightly less retarded transformation from rods into cocci. These differences are attended by a marked deviation of % GC of this strain from the mean % GC of group three.

The molar % GC values of the strains of group three, except the strains EC 14, EC 20 and AC 253, cover a narrow range from 65.5 to 66.9. This means that the strains of this group may be genetically closely related. This is also true of the strains of group one on the one hand and of the majority of strains of group

three on the other. Consequently, in regard of the % GC, the non-orange cheese coryneforms of group three may be related to the soil arthrobacters, corroborating the expectation based on morphological characters, but the agreement of % GC between the two groups fails to reflect the differences in physiological characters of the strains of the two groups. On the basis of differences in mainly physiological characters Mulder et al. (1966) concluded that the cheese coryneforms should not be placed into the genus *Arthrobacter*. However, it should be stressed that these characters are the expression of only a small part of the genome.

To gain more information concerning the relationship between soil arthrobacters (group one) and cheese and fish coryneforms (groups two and three) on the one hand and representatives of the genera *Brevibacterium*, *Mycobacterium*, *Corynebacterium* and *Nocardia* on the other, the DNA base composition of a number of named cultures of these genera have been determined and listed in Table 2 and a survey of % GC is given in Fig. 1. From the DNA base compositions it can be concluded that the possibility of a close genetic relationship between the strains of group two, except isolate B 3, and the named cultures, except the two *Corynebacterium bovis* strains (C 28 and C 29), is not very likely. However, the soil arthrobacters and the non-orange cheese strains, except EC 14, EC 20 and AC 253, may be genetically moderately related with the mycobacteria, corynebacteria and nocardiae.

The % GC values of the four named cultures of the genus *Brevibacterium* — covering a range from 55.8 to 66.3 — show that this genus is not very homogeneous. The heterogeneity of the genus has already been signalized by several investigators (Hester and Weeks, 1969; Komagata, Yamada and Ogawa, 1969), and was corroborated by the findings of Yamada and Komagata (1970*b*) who reported GC values of some named cultures of the genus *Brevibacterium* covering a range of 25%.

It is remarkable that the hyperchromic effects of the DNA samples isolated by Yamada and Komagata cover a very wide range, *viz.* from 20 to 38%. Further they reported a % GC of *Arthrobacter globiformis* ATCC 8010 and *Brevibacterium linens* ATCC 9175 differing about 3.5% from the values published by Skyring and Quadling (1969) and from those of this study. So, it is difficult to compare the absolute values of the DNA base composition of their strains with those of the present work.

Although the GC values of only five *Corynebacterium* strains were investigated, it is interesting to stress the difference in DNA composition between the two corynebacteria from animal origin, *Corynebacterium bovis* C 28 and C 29, (62.6 and 63.7% GC) and the plant pathogenic corynebacteria, *Corynebacterium flaccumfaciens* C 33, *Corynebacterium michiganense* C 34 and *Corynebacterium*

tritici C 37 (69.8, 72.3 and 70.4% GC). This demonstrates the slight relationship between both types of corynebacteria.

The DNA base composition of a microorganism is a very useful tool in taxonomy and can be used for preliminary screening a number of microorganisms the genetic relationship of which is being studied. For establishing the degree of genetic relatedness among a number of microorganisms however, also the base sequences in the bacterial chromosome must be compared by carrying out hybridization experiments. Results of such experiments will be published in a subsequent paper.

The author is grateful to Professor E. G. Mulder for stimulating discussions and critical corrections of the manuscript and to the Instituut voor Plantenziektenkundig Onderzoek, Wageningen, for the use of the Analytical Ultracentrifuge and to Mr. D. Maat for his help in operating this apparatus. The author is also much indebted to Professor J. De Ley, Gent, for the hospitality met with during his stay at the Laboratory for Microbiology, Gent, Belgium, for helpful discussions and kind supply of a reference DNA sample and some reference strains. The present investigation was in part supported by a grant from the Landbouwhogeschoolfonds.

Received 11 *June* 1971

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Deep-freezing of bacterial DNA for thermal denaturation and hybridization experiments

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What effect freezing purified DNA at –70 C and then keeping it at –21 C for several months might have on the thermal denaturation and hybridization was investigated. One part of each DNA sample was stored at 4 C, and the other frozen at –70 C and stored at –21 C, and the results of thermal denaturation and hybridization experiments were compared. They show that freezing at –70 C and then storing at –21 C for half a year or probably even one year do not significantly affect thermal denaturation and hybridization.

INTRODUCTION

Purified bacterial deoxyribonucleic acid (DNA) intended for use in thermal denaturation and hybridization experiments is generally stored as a concentrated salt solution in the presence of a few drops of chloroform. The keeping qualities mainly depend on the kind of DNA and on its concentration. DNA solutions can be stored for several months if the absorbance of the solution at 260 nm is at least 20 (De Ley, Cattoir and Reynaerts, 1970). It would be more convenient if the DNA samples could be stored for a longer time. Van der Plaat (1969) and Citarella and Colwell (1970) stored purified DNA at –20 C. However, little is known about the effect of storage at –20 C on the physical state of purified DNA. Possible changes might have consequences for the determination of DNA base composition according to the thermal denaturation method, and for hybridization.

The aim of this study is to investigate the effects of freezing purified DNA at –70 C and storing it at –21 C for several months, as compared to the effects of storing it at 4 C for some time, on thermal denaturation and hybridization. Furthermore, the molecular weight (M.W.) of purified DNA fragments of

both types of samples was estimated as this is an important parameter of the physical condition of purified DNA.

MATERIALS AND METHODS

Microorganisms. DNA of the following microorganisms was investigated: *Arthrobacter* spec.: AC 1, AC 166; *Arthrobacter globiformis*: AC 405 (ATCC 8010): cheese coryneforms: EC 14, EC 15, EC 20, AC 253, AC 256, AC 263, AC 275, AC 448, B 3, B 4; fish coryneform: AC 471; *Brevibacterium linens*: B 42 (ATCC 9175); *Agrobacterium tumefaciens*: B6; *Nocardia* spec.: AC 555; *Escherichia coli*: B, K12 (Crombach, 1972).

Purification of DNA. DNA intended for thermal denaturation and estimation of M.W. was purified as described by Crombach (1972). It was redissolved in 1 × SSC (0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0) to an absorbance of about 40 at 260 nm. A few drops of chloroform were added for conservation. The purified DNA solution was stored at 4 C, but within about 6 weeks a part was frozen at –70 C (mixture of acetone and dry ice). These samples were stored at –21 C.

The purification of DNA intended for hybridization experiments was carried out according to a combination of the methods of De Ley et al. (1970) and Crombach (1972). The latter method was followed up to and including three washings after the pronase treatment. Then, according to De Ley et al., NaCl was added to the DNA solution to a final concentration of 3%. The purified DNA was redissolved in 0.1 × SSC and treated further as described above for DNA for thermal denaturation. However, the DNA samples of the strains AC 253 and AC 263 were kept as a concentrated solution at 4 C for six months before division into two parts.

Determination of mean DNA base composition. The determination of the mean DNA base composition (%GC), the standard deviation σ_1 of the compositional %GC distribution around the mean, and the hyperchromic effect of the DNA samples was carried out according to the thermal denaturation method (Crombach, 1972). The thermal denaturation of the DNA samples stored at –21 C was performed after storage for several months and that of samples stored at 4 C within about one month after purification, except the samples of strains AC 448, B 3 and AC 555, which were stored at 4 C for about 2 months.

Molecular weight of DNA fragments. Molecular weight of DNA fragments of various DNA samples was calculated from the sedimentation coefficient determined with the Analytical Ultracentrifuge (Crombach, 1972).

Hybridization. Hybridization experiments were carried out according to the renaturation method of De Ley et al. (1970).

RESULTS AND DISCUSSION

Thermal denaturation. The %GC values, the standard deviation σ_1 and the hyperchromic effect of DNA stored at 4 C and at –21 C are recorded in Table 1.

No significant differences in %GC value can be seen between DNA stored at 4 C and that stored at –21 C. The average difference between both types of samples is 0.4%, which is close to the standard deviation of 0.35% found in a previous investigation (Crombach, 1972).

Neither the standard deviation σ_1 of the compositional %GC distribution around the mean, nor the hyperchromic effect is affected by the method of storage (Table 1).

Molecular weight. The estimated M.W. of the DNA fragments of both groups of samples are recorded in Table 2. The greatest difference in M.W. between the DNA fragments stored in different ways, is 3×10^6 daltons which is most probably due to the experimental error. Consequently, freezing at –70 C

Table 1. Influence of method of storage of purified DNA on its mean DNA base composition, %GC, standard deviation σ_1 of the compositional %GC distribution around the mean, and hyperchromic effect

Strain number	Storage of DNA						
	at 4 C			frozen to —70 C, stored at —21 C			
	Mean %GC	σ_1 as %GC	Hyp. effect (%)	Time of storage (months)	Mean %GC	σ_1 as %GC	Hyp. effect (%)
EC 15	66.7	3.6	33	12	66.1	4.0	35
A. tumefaciens B6	61.6	3.6	33	12	61.6	4.2	36
AC 555	70.0	3.1	31	11	70.6	3.6	35
AC 405	65.5	4.7	37	6	66.3	4.2	36
AC 256	66.1	4.0	35	6	66.2	3.9	34
AC 448	63.4	3.3	35	6	63.6	3.4	37
B 3	65.8	3.5	34	6	65.4	3.6	37
E. coli B	52.1	5.7	40	6	52.5	6.0	40
AC 166	65.5	4.7	35	2.5	64.7	4.7	35
EC 20	60.4	5.2	37	2	59.7	4.1	37
AC 275	63.4	3.8	36	2	62.8	3.4	36
AC 471	63.8	4.0	36	2	63.6	4.0	36

Table 2. Molecular weight of DNA fragments stored as a concentrated solution at 4 C, and in the frozen state at −21 C, respectively. In parentheses, months of storage

Strain number	Molecular weight (dalton)	
	4 C	−21 C
EC 14	12×10^6 (4)	12×10^6 (12)
E. coli K12	12×10^6 (5)	9×10^6 (11)
AC 405	n.d.[1]	10×10^6 (6)
AC 166	9×10^6 (3)	7×10^6 (3)
EC 20	10×10^6 (3)	10×10^6 (2)
B 4	8×10^6 (3)	7×10^6 (2)
AC 275	9×10^6 (3)	9×10^6 (2)

[1] not determined

and keeping at −21 C of purified DNA has no significant influence on the size of the DNA fragments. In this connection it can be stated that a variation of the M.W. of the DNA fragments within the range 1 to 25×10^6 daltons has no effect on thermal denaturation of DNA (De Ley, 1969).

Hybridization. The effect of freezing purified DNA at −70 C and keeping it at −21 C for several months on its suitability for hybridization experiments was studied by comparing the degree of duplexing (D_1) between DNA samples stored at 4 C with that (D_2) between corresponding DNA samples stored at −21 C. The degree of duplexing (D_3) between DNA samples of which one had been stored at 4 C and the other at −21 C was also determined (Table 3).

Considering the reliable D values (degrees of duplexing below some 20% have probably only a semi-quantitative value, De Ley et al., 1970), it appears that no great difference exists between the corresponding degrees of duplexing, except between D_1 and D_3 of strains 263 and 256. The absolute differences are within the range of 6%, slightly exceeding the experimental error of 4.7% (De Ley et al., 1970). The above mentioned exception may be due to an altered physical condition of the purified DNA of strain AC 263, resulting either from the 6-months storage at 4 C before freezing, or from the effects of freezing on such an old DNA sample. Therefore, it is recommendable to freeze purified DNA immediately after its redissolution.

Apparent reaction rate constant. The degree of duplexing was calculated from the renaturation velocity of denatured DNA (De Ley et al., 1970). If freezing purified DNA at −70 C and keeping it at −21 C affected renaturation, this would find expression in the apparent reaction rate constant k′ of the individual DNA samples (Table 4). ($k' = v'/c'^2$; v′ stands for the renaturation velocity expressed as decrease in absorbance at 260 nm per minute, and c′ represents the concentration of DNA expressed as mmolar nucleotide pairs).

Table 3. Degree of duplexing of DNA stored in two different ways. In parentheses, months of storage of the respective DNA samples.

Strain numbers	Degree of duplexing D (in % of the total haploid genome)					
	No. of determinations	D_1	No. of determinations	D_2	No. of determinations	D_3
B 42 × AC 448	3	82 ± 2 (2; 1)	4	78 ± 4 (12;5)		
B 42 × AC 275	4	86 ± 3 (2; 2)	4	80 ± 2 (12;5)		
AC 275 × AC 448	2	91 ± 1 (2;1.5)	4	87 ± 2 (5;5)		
AC 263 × AC 256	4	88 ± 5 (5; 5)			4	103 ± 4 (6; 2)
AC 253 × AC 256	3	13 ± 2 (5; 5)			3	21 ± 5 (6; 3)
AC 1 × AC 1	0	100[1]			2	98 ± 1 (1.5; 3)
AC 1 × B 42	4	11 ± 2 (3;1.5)			1	13 (1.5;1.5)
B 42 × AC 253	2	43 ± 3 (1.5;1.5)			1	38 (1;1.5)

D_1: Both DNA samples stored at 4 C; D_2: Both DNA samples stored at –21C; D_3: First-mentioned DNA sample stored at –21 C, the other at 4 C.

[1] Theoretical D value.

Table 4. Apparent reaction rate constant k′ and its standard deviation σ_2 of the renaturation of DNA stored at 4 C and at −21 C, respectively. In parentheses, number of determinations.

Strain number	DNA stored at 4 C			DNA stored at −21 C		
	Mean $k' \times 10^2$	$\sigma_2 \times 10^2$	Average time of storage (months)	Mean $k' \times 10^2$	$\sigma_2 \times 10^2$	Time of storage (months)
B 42	16.5 (61)	2.5[1]	2.5	14.6 (8)	0.5[2]	12
AC 253	11.5 (6)	1.0	3.5	11.3 (4)	0.8	6
AC 263	19.3 (4)	2.1	5	12.1 (4)	0.4	6
AC 448	15.9 (6)	0.7	1.5	15.6 (8)	1.1	5
AC 275	16.9 (6)	1.5	2	15.9 (11)	1.2	5
AC 1	11.7 (10)	1.1	2.5	12.1 (3)	0.4	1

[1] σ_2 of the k′ value derived from several batches of DNA of strain B 42.
[2] σ_2 of the k′ value derived from one batch of DNA of strain B 42.

From the data in Table 4 no significant differences can be seen between the k′ values calculated from the same denatured DNA stored in two different ways, except for strain AC 263. Concerning this exception the same explanation holds as described for the difference between the degrees of duplexing D_1 and D_3, of the DNA samples from the strains AC 256 and AC 263.

The comparatively high standard deviation σ_2 of the k′ values (Table 4) are mainly due to the experimental error connected with the determination of the DNA concentration, in spite of its eight-fold determination (Gillis, De Ley and De Cleene, 1970). Therefore, for calculating the D values from renaturation experiments, it is advisable to use only DNAs of which the concentration has been determined in the same batch.

From the results of this study, in which frozen DNA of 19 strains was involved, it may be concluded that freezing purified DNA at −70 C and keeping it at −21 C for half a year, or probably even one year, have no significant effect on thermal denaturation and on hybridization according to the renaturation method.

The author is greatly indebted to Professor E. G. Mulder for helpful criticism and advice during the preparation of the manuscript. He is also grateful to Dr. J. L. M. Huntjens for reading the manuscript and to the Instituut voor Plantenziektenkundig Onderzoek, Wageningen, for the use of the Analytical Ultracentrifuge and to Mr. D. Maat for his help in operating this apparatus.

Received 18 *October* 1972

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Thermal stability of homologous and heterologous bacterial DNA duplexes

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Thermal stability of homologous and heterologous DNA duplexes renatured according to the renaturation-rate method of De Ley et al. (1970) for 35 min or 17 hr, was estimated from the melting profiles of the duplexes. Comparison of the melting points of native and renatured DNA revealed that in the first 35 min of renaturation highly stable homologous duplexes were mainly formed, whereas up to 7% mismatching occurred in duplexes renatured for 17 hr. Up to 8% more mismatching was found in heterologous DNA duplexes of moderately related coryneform bacteria than in homologous ones after 35 min renaturation. It can be concluded that mismatching in heterologous hybrids of closely related DNAs had been restricted to a few % and of moderately related DNAs to approximately 10% in the initial renaturation phase.

INTRODUCTION

DNA-DNA hybridization methods are widely used to determine the genetic relatedness of microorganisms. The agar method of Bolton and McCarthy (1962) and more recently the membrane-filter method of Gillespie and Spiegelman (1965) are mostly employed.

De Ley, Cattoir and Reynaerts (1970) introduced a method based on the renaturation rate of denatured DNA fragments. The degree of reassociation between two DNAs depends largely on the stringency of the incubation conditions. The most stringent conditions are at high temperatures and low concentrations of salt. This results in significantly less binding between polynucleotide sequences of both moderately and remotely related microorganisms (Brenner, Fanning and Steigerwalt, 1972*a*).

For a reliable evaluation of the degree of binding, various workers have determined the thermal binding index TBI. This index is the ratio of the degree of binding at the optimum reassociation temperature to that at a higher, more

restrictive, temperature, at which instable duplexes are incapable of surviving (Citarella and Colwell, 1970; Brenner et al., 1972*a*; Palleroni et al., 1972). The TBIs follow the degrees of binding; the TBIs of duplexes with a degree of binding higher than 70% range from 0.85 to 1.0, those of duplexes with a degree lower than 60% ranging from 0.4 downwards (Citarella and Colwell, 1970; Brenner et al., 1972*b*).

An other parameter of the stability can be obtained from the thermal elution profiles of reassociated duplexes (Brenner and Cowie, 1968; Citarella and Colwell, 1970; Okanishi and Gregory, 1970; Anderson and Ordal, 1972; Brenner et al., 1972*a*, 1972*b*). Reassociated DNA fragments are dissociated by increasing the temperature in steps, followed by eluting and assaying each fraction. The differences between the Tm_e – the temperature at which 50% of the duplexes have been dissociated – of a heterologous duplex and that of the homologous reference indicates the thermal stability of the heterologous duplex. ΔTm_e ranges from 0 to about 18 C with thermal stability closely following the reassociation data obtained at the optimum renaturation temperature (Anderson and Ordal, 1972; Brenner et al., 1972*b*). At a more stringent incubation temperature, thermal stability of duplexes may increase, resulting in a significant drop in the degree of duplexing (Brenner et al., 1972*a*). However, it should be emphasized that at stringent temperatures a considerable amount of DNA will leach from the membrane filters, viz. 40 to 80% at 70 to 80 C (Ballard et al., 1970; De Ley and Tijtgat, 1970), which invalidates the interpretation of the degree of duplexing.

Studies with synthetic polynucleotides revealed that the thermal stability of reassociated duplexes is extremely sensitive to small amounts of mismatching and unpairing of bases (Bautz and Bautz, 1964; Kotaka and Baldwin, 1964). Laird, McConaughy and McCarthy (1969) and Anderson and Ordal (1972) estimated 0.7 and 1.6°C ΔTm_e, respectively, per 1% mispairing. These values were obtained from studies on chemically altered, – e.g. deaminated – base pairs, which are not the same as the mismatched base pairs in reassociated duplexes of unaltered DNA. For that reason these studies can only provide an approximation of the relationship between ΔTm_e and per cent mismatching. At present, 1°C ΔTm_e per 1% mismatching is generally accepted. It should be emphasized that a mismatched base pair at the end of a stable duplex – stable minimum length is about 20 nucleotide pairs – does not affect the thermal stability (Walker, 1969).

The aim of this study was to investigate the thermal stability of duplexes from closely as well as from moderately and remotely related DNAs. The renaturation was performed during 35 min and 17 hr. All of the D values were calculated from the former renaturation time.

MATERIALS AND METHODS

Microorganisms. DNAs of 23 coryneform bacteria from our collection and of *Pseudomonas putida*, CCEB 520, strain No. 18 were used in this study (CCEB = Culture Collection of Entomogenous Bacteria, Prague, Czechoslovakia).

Cultivation of microorganisms. Strains 1 to 9, inclusive, 19, 20 and 22, were cultivated in a slightly modified medium of Yamada and Komagata (1970), consisting of casitone (Difco), 5 g; yeast extract, 5 g; Bacto peptone (Difco) 10 g; malt extract, 100 ml; Tween 80, 0.05 g; glycine, 5 g; distilled water, 900 ml. The pH was adjusted to 7.4 before sterilization.

The remaining strains were grown in a medium containing glucose, 10 g and yeast extract, 7 g per 1000 ml of distilled water. All the cultures were strongly aerated.

Isolation of DNA. DNA was isolated and purified according to a combination of the methods of De Ley et al. (1970) and Crombach (1972), as described previously (Crombach, 1973).

DNA concentration. The concentration of the DNA samples was assayed according to the chemical method of Burton (1956). In all the experiments five different standard solutions of 2-deoxy-D-ribose were included.

Shearing of DNA. DNA dissolved in 0.1 × SSC (0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0) to a concentration suitable for hybridization experiments, was sheared by passing through the Aminco French pressure cell at 20 000 psi in the liquid.

Molecular weight and hyperchromic effect of DNA fragments. The average molecular weight (M.W.) and hyperchromic effect of sheared DNA fragments were estimated as described previously (Crombach, 1972).

Thermal denaturation. The melting point of sheared native DNA and that of DNA duplexes renatured for 17 hr, both dissolved in 2 × SSC to a concentration of about 80 μg/ml, were determined with the adapted Gilford spectrophotometer (Crombach, 1972). The melting profile was performed in the temperature span of $T_{or}+5$ to 100 C. For the determination of the melting curves of duplexes renatured for 35 min, the temperature of the cuvette house was immediately increased to $T_{or}+5$ C, except for the first two experiments recorded in Table 3. In these experiments the temperature was increased gradually – 0.1 °C/min – starting from the renaturation temperature to 100 C

Compositional distribution. The left, low-temperature side standard deviation σl of the compositional %GC distribution was calculated from the formula of De Ley (1969).

Optimum renaturation temperature. The optimum renaturation temperature

T_{or} was calculated from the equation of Gillis, De Ley and De Cleene (1970):

$$T_{or} = 0.51 \times \%GC + 47.0$$

Apparent reaction rate constant k'. The k' values were calculated from the equation:

$$k' = v'/c'^2$$

v' stands for the renaturation velocity expressed as decrease in absorbance per min, and c' represents the DNA concentration expressed as mmolar nucleotide pairs (Gillis et al., 1970).

Hybridization. Hybridization experiments were carried out according to the renaturation-rate method of De Ley et al. (1970). Those DNA samples of which melting curves were determined before renaturation, were further denatured in the cuvettes at 105 C for 15 min. Subsequently, the denatured samples were briefly chilled in a waterbath at 82 C and renatured at T_{or}.

RESULTS AND DISCUSSION

The addition of glycine to the culture medium can improve the sensitivity of bacterial cells to lysozyme (Yamada and Komagata, 1970). This was confirmed by our preliminary experiments: 0.5% glycine markedly increased the lysozyme sensitivity of the cells, with maintenance of an acceptable cell yield.

For satisfactory reproducibility of renaturation rates, the double-stranded DNA fragments have to be sheared to a M.W. of about 400000 dalton (Gillis et al., 1970). The M.W. of DNA fragments of three sheared samples was found to be in the range of 377 to 480 × 10^3 dalton.

Before interpreting the results of hybridization experiments, it is advisable to measure the thermal stability of the formed duplexes. Under certain conditions considerable mismatching or unpairing may occur, resulting in unreliable degrees of binding, which can lead to erroneous conclusions.

Thermal stability of homologous DNA duplexes

In the present study the thermal stability of renatured homologous DNA duplexes was investigated by comparing the melting point Tm and the standard deviation σl of sheared native and renatured DNA (Table 1).

Mismatching in duplexes results in a decrease of their melting points and is also evidenced by a broader melting profile resulting particularly in greater σl values.

In all cases the data of each strain, except strain number 11 (Table 1), were obtained from a single experiment to eliminate the possibility of errors arising from adjusting the DNA-salt solution to 2 × SSC. The salt concentration affects the thermal denaturation. (The same does hold for the data of the

Table 1. Melting point Tm, and standard deviation σl of the compositional %GC distribution of native and renatured DNA

Strain number	Native DNA		DNA renatured for				ΔTm_{nr}[3]	$\Delta\sigma l_{nr}$[4]
			35 min		17 hr			
	Tm_n[1] (°C)	σl_n[2] (%GC)	Tm_r[1] (°C)	σl_r[2] (%GC)	Tm_r (°C)	σl_r (%GC)		
4	98.8	5.2	98.0	5.7			0.8	−0.5
5	98.3	5.2	97.9	6.0			0.4	−0.8
6	98.4	5.0	98.1	6.4			0.3	−1.4
6	99.1	5.2	98.4	5.7			0.7	−0.5
11	97.7[5]	5.6	96.9[6]	5.5			0.8	+0.1
16	98.5	4.5	97.5	6.2			1.0	−1.7
17	97.3	5.2	96.4	5.7			0.9	−0.5
20	98.1	5.2	97.4	5.7			0.7	−0.5
21	97.2	4.7	96.9	5.0			0.3	−0.3
22	96.6	6.0	96.1	6.5			0.5	−0.5
23	97.0	4.5	96.7	4.7			0.3	−0.2
9	98.4	5.2			96.9	10.5	1.5	−5.3
12	97.8	6.0			95.2	13.2	2.6	−7.2
15	98.1	5.7			95.9	9.0	2.2	−3.3
17	97.2	5.0			95.7	10.2	1.5	−5.2
19	97.0	6.7			93.7	13.5	3.3	−6.8
21	97.7	5.8			95.8	8.4	1.9	−2.6

[1] Tm_n and Tm_r represent the melting points of native and renatured DNA, respectively.
[2] σl_n and σl_r stand for the left, low-temperature side, standard deviation of %GC distribution of native and renatured DNA, respectively.
[3] ΔTm_{nr} represents Tm_n minus Tm_r.
[4] $\Delta\sigma l_{nr}$ represents σl_n minus σl_r.
[5] These data were determined two-fold; average deviation of Tm_n 0.1°C.
[6] These data were determined four-fold; average deviation of Tm_r 0.3°C.

hybridization experiments recorded in the Tables 2 and 3).

The ΔTm_{nr} and $\Delta\sigma l_{nr}$ values of Table 1 reveal little mismatching in homologous duplexes renatured for 35 min, whereas there was a distinct increase of mismatching in duplexes formed during a renaturation period of 17 hr.

The decreased thermal stability of duplexes formed by the renaturation-rate method, is mainly due to mismatching. This is concluded from the fact that the hyperchromic effect of duplexes formed during 17 hr renaturation was about equal to that of sheared native DNA – about 30% – which implies that there are not many more free ends and open loops in renatured homologous DNA than in sheared native DNA. Assuming that at most half of the instability of duplexes obtained from membrane-filter methods (Laird et al., 1969; Anderson and Ordal, 1972), is due to open loops and free ends, 1°C decrease of thermal

stability of duplexes formed according to De Ley's method during 17 hr, might correspond with at most 2% mismatching. This implies a maximum of about 7% mismatching in homologous duplexes formed during 17 hr renaturation. As it seems illogical to suppose that free ends and open loops occur much more frequently in the duplexes formed during 35 min than in those formed during 17 hr, mismatching was assumed to be restricted to at most 2% in the former. This means that hybrids of high stability had particularly been formed in the initial phase of renaturation. This is in agreement with the findings of Krueger and McCarthy (1970) obtained from DNA-RNA hybridization on membrane filters.

Thermal stability of heterologous duplexes formed during 17 hr of renaturation

To study whether perfect or imperfect matching had occurred during reassociation of polynucleotide sequences of two different DNAs, a comparison was usually made between the thermal stability of the heterologous duplexes and the homogolous ones of the reference DNA (e.g. Anderson and Ordal, 1972; Brenner et al., 1972*b*). In this study the thermal stability of the duplexes in the mixture was compared with that of the component DNAs.

The $\triangle Tm_r$ and $\triangle \sigma l_r$ values in Table 2 reveal that the thermal stability of the renatured duplexes in the mixture of very closely related DNAs – D higher than 90% – and that of the renatured component DNAs did not differ markedly. However, this does not simply imply that in the duplexes of the mixture no mismatching had occurred at all, as is always tacitly assumed (e.g. Anderson and Ordal, 1972; Brenner et al., 1972*a*). For, some mismatching will have occurred in the renatured component DNAs.

There was somewhat more mismatching in the duplexes of the mixture of moderately related DNAs – D between 75 and 30% – than in the renatured component DNAs; the $\triangle Tm_r$ and $\triangle T\sigma l_r$ values being limited to 0.4°C and —1.8%, respectively (Table 2).

It must be stressed that there are relatively few heterologous hybrids in the renatured mixture; viz. 35 and 20% at D values of 70 and 40%, respectively. The effect of a possible thermal instability of the heterologous hybrids on Tm_r will be diminished by the large proportion of the more stable homologous duplexes. Therefore, the mismatching in heterologous hybrids cannot be exactly quantified from $\triangle Tm_r$ values. However, the thermal instability of the heterologous hybrids will raise the σl_r value of the mixture's melting profile. For example, when about one third of the hybrids are heterologous, which occurs in a mixture at D values between 60 and 70%, this results in a $\triangle \sigma l_r$ value of -1.0 to -1.8%. These values correspond with about equal mismatching – 2% – which

Table 2. Degree of duplexing D, melting point Tm_r, and standard deviation σl_r of the compositional %GC distribution of DNA duplexes renatured at T_{or} C during 17 hr

Strain number	D[1] (%)	Tm_r (°C)	σl_r (%GC)	ΔTm_r[2]	$\Delta \sigma l_r$[3]
8		96.6	11.0		
8 × 9	100	96.8	10.7	0.0	0.0
9		97.0	10.5		
8		97.4	10.0		
8 × 7	94	97.1	10.7	0.2	−0.2
7		97.1	10.5		
17		95.5	8.1		
17 × 21	85	95.4	8.9	0.2	−0.7
21		95.8	8.4		
17		95.7	10.2		
17 × 12	73	95.2	13.5	0.2	−1.8
12		95.2	13.2		
17		95.5	10.5		
17 × 19	69	94.2	13.0	0.4	−1.0
19		93.7	13.5		
17		96.6	8.7		
17 × 14	64	96.0	11.0	0.2	−1.6
14		95.8	10.2		
17		96.4	9.5		
17 × 13	23	95.3	14.2	0.8	−4.5
13		95.8	10.0		
17		96.4	9.0		
17 × 10	19	96.0	11.2	0.4	−1.5
10		96.4	10.5		
17		95.4	9.0		
17 × 15	17	95.3	11.7	0.3	−2.7
15		95.9	9.0		

[1] D expressed as % of total haploid genome.
[2] Difference between the average Tm_r of renatured component DNAs and the Tm_r of the duplexes formed in the mixture.
[3] Difference between the average σl_r of renatured component DNAs and the σl_r of the duplexes formed in the mixture.

occurs in homologous hybrids formed during 35 min renaturation (Table 1). Thus, in the case of renaturation during 17 hr, it can be estimated that mismatching in heterologous hybrids of moderately related DNAs exceeded that in homologous duplexes by about 6% ($^{100}/_{33}$ × 2%, calculated for 100% heterologous hybrids).

The argument that the effect of the instability of heterologous hybrids of moderately related DNAs is not expressed in σl_r values because the major part of these hybrids would melt outside the range of 15.9 to 84.1% of the absorbance increase during melting, is disposed of by the unimodal shapes of the melting curves obtained from all renatured duplexes (Figs. 1 and 2).

In the case of distantly related DNAs – D lower than 30% – the mismatching in heterologous duplexes cannot be estimated from the melting profiles, as the effect of a possible instability of the heterologous hybrids may be nullified by the relatively many homologous duplexes. However, the $\Delta\sigma l_r$ values of Table 2 clearly show that mismatching in heterologous duplexes of remotely related DNAs is greater than in heterologous hybrids of closely and moderately related DNAs.

Thermal stability of heterologous duplexes formed during 35 min of renaturation

When determining the melting profiles of the renaturation products of the first two experiments of Table 3 the temperature was gradually increased by 0.1°C/min, starting from the renaturation temperature. Consequently, it took a long time before the duplexes started to melt (Fig. 1). During that time some renaturation continued, as is shown by a slight decrease of the absorbance at 260 nm. Ultimately, it resulted in a slightly less perfect matching occurring in the heterologous duplexes of the mixture as compared with the renatured component DNAs; the corresponding ΔTm_r and $\Delta\sigma l_r$ values were not more than 0.3°C and −0.8%. Considering the other experiments of Table 3, the temperature of the cuvette house was immediately increased to T_{or} +5 C after renatura-

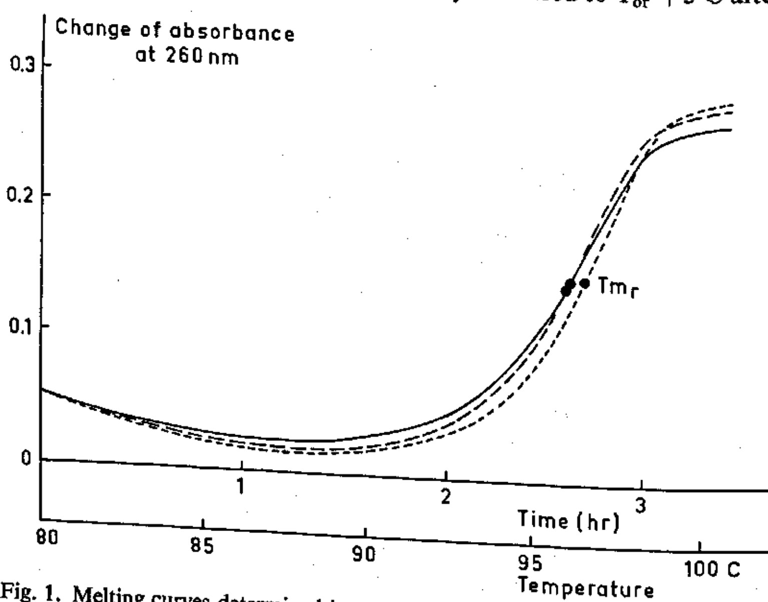

Fig. 1. Melting curves determined immediately after renaturation.
——— melting curve of the duplexes in the mixture,
– – – – melting curve of the renatured component DNA of strain 17,
.... melting curve of the renatured component DNA of strain 11.

Table 3. Degree of binding D, melting point Tm_r and standard deviation σl_r of the compositional %GC distribution of DNA duplexes renatured during 35 min at different temperatures.

Strain number	D[1] (%)	Tm_r (°C)	σl_r (%)	ΔTm_r[2]	$\Delta \sigma l_r$[3]	Renaturation temp. (°C)
11		96.5	5.2			
11 × 17	83	96.1	6.0	0.3	−0.8	78
17		96.3	5.3			
11		97.2	6.5			
11 × 17	78	97.0	7.0	0.2	−0.7	69
17		97.3	6.2			
6		98.1	6.0			
6 × 5	97	98.0	6.0	0.0	0.0	80
5		97.9	6.0			
21		96.9	5.0			
21 × 23	77	96.5	5.1	0.3	−0.3	77
23		96.7	4.7			
21		96.8	4.7			
21 × 23	81	96.2	4.9	0.3	−0.3	68
23		96.2	4.5			
19		97.2	5.1			
19 × 20	70	96.6	7.2	0.7	−1.8	80
20		97.4	5.7			
22		96.1	6.5			
22 × 23	53	96.2	7.7	0.2	−1.9	76
23		96.7	5.2			
21		98.4	4.5			
21 × 22	49	97.8	5.9	0.2	−0.9	77
22		97.7	5.6			
18		96.9	6.2			
18 × 11	27	97.1	6.0	−0.1	−0.3	78
11		97.1	5.2			
18		96.3	6.2			
18 × 11	19	96.5	6.0	0.0	−0.3	69
11		96.7	5.2			
18		96.2	6.0			
18 × 17	25	96.3	5.9	0.0	−0.2	78
17		96.4	5.4			
18		96.0	6.2			
18 × 17	27	96.1	5.7	0.0	0.0	69
17		96.2	5.2			
6		96.8	5.7			
6 × 3	23	96.4	6.2	0.1	−0.5	80
3		96.2	5.7			
6		98.4	5.7			
6 × 4	23	98.1	5.7	0.1	0.0	80
4		98.0	5.7			

[1] D expressed as % of total haploid genome.
[2] ΔTm_r equals the average Tm_r of renatured component DNAs minus Tm_r of the duplexes in the mixture.
[3] $\Delta \sigma l_r$ equals the average σl_r of renatured component DNAs minus σl_r of the duplexes formed in the mixture.

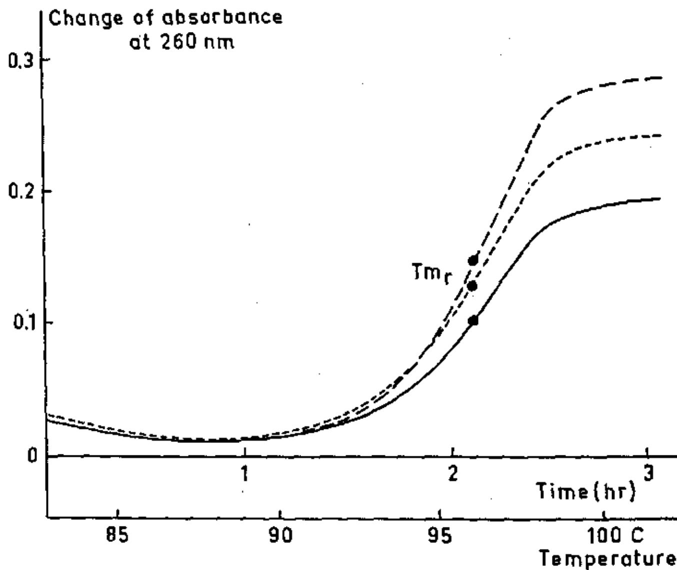

Fig. 2. Melting curves determined immediately after renaturation.
——— melting curve of the duplexes in the mixture,
- - - - melting curve of the renatured component DNA of strain 17,
.... melting curve of the renatured component DNA of strain 18.

tion. A significant additional duplexing did not occur in the short period of time between the end of renaturation and the beginning of the melting (Fig. 2). Consequently, almost all of the duplexes involved in the thermal stability measurements were formed during the initial renaturation period of 35 min. Considering the low $\triangle Tm_r$ and $\triangle \sigma l_r$ values in the case of closely related DNAs – D greater than 75% – (Table 3), it can be concluded that the mismatching in the heterologous duplexes exceeded that in the homologous hybrids by only a few per cent. It should be emphasized that at D values between 75 and 100% the mixture contains about 37 to 50% of heterologous duplexes, respectively.

The mismatching in the heterologous duplexes in the mixture of moderately related DNAs of Table 3 exceeded that in the homologous hybrids by about the same extent as was the case during renaturation for 17 hr (Table 2); the $\triangle Tm_r$ and $\triangle \sigma l_r$ values being limited to 0.7°C and −1.9, respectively.

Taking into account the mismatching in renatured hybrids, the total mismatching in the heterologous duplexes was not more than about 10%.

From the $\triangle Tm_r$ and $\triangle \sigma l_r$ values of Tables 2 and 3 it can be concluded that the mismatching in heterologous hybrids of distantly related DNAs formed during 35 min of renaturation is very probably less than in those formed during 17 hr of renaturation.

Effect of renaturation temperature on D *and* k′ *values*

The data of Table 3 confirm the results of De Ley et al. (1970), that the D value is nearly independent of the renaturation temperature within a range of 15°C, below T_{or}. However, the apparent reaction-rate constant k′ was affected by the temperature, as the k′ values determined at T_{or} minus 10°C were 13 to 32% lower than the corresponding ones at T_{or} (Table 4).

The decrease in k′ values confirms that the flat temperature optimum of reaction rate extends only over approximately 5 to 10°C (Gillis et al., 1970).

Table 4. Apparent reaction-rate constant k′ of the renaturation of DNA at different temperatures

Strain number	Renaturation at 68–70 C		Renaturation at 79–80 C	
	number of determinations	Mean $k' \times 10^2$	Number of determinations	Mean $k' \times 10^2$
1	2	7.2 ± 0.8	3	10.4 ± 1.0
2	2	10.8 ± 0.0	2	14.0 ± 0.4
6	4	9.0 ± 1.3	5	13.2 ± 1.0
11	4	14.5 ± 0.7	4	16.6 ± 1.1
17	4	14.9 ± 1.2	4	17.3 ± 0.8
18	4	11.9 ± 0.4	4	13.6 ± 0.6

It should be emphasized that the D values of the present study are not corrected for the unequal genome sizes of the two strains involved in the hybridization experiments. Unequal k′ values indicate unequal genome sizes. However, the fact that the D values are not corrected for unequal genome sizes does not affect the thermal stability data.

The results of this investigation show that the mismatching in heterologous DNA duplexes of closely and moderately related coryneform bacteria, when renatured during the initial renaturation phase, is at most 6% higher than in homologous ones. Strong evidence was provided that equal melting points of duplexes in the mixture of two different DNAs and their renatured component DNAs do not imply the absence of mismatching in the heterologous renaturation products. Mismatching also occurs in homologous hybrids, although to a low extent in the initial renaturation phase.

The author wishes to thank Professor E. G. Mulder for valuable criticism and advice during the preparation of the manuscript. He is also greatly indebted to Professor J. De Ley, Gent, for his interest and very helpful discussions, and to Dr. T. A. Lie and Dr. L. P. T. M. Zevenhuizen for critically reading the manuscript.

Received 14 *May* 1973

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Relationships among coryneform bacteria from soil, cheese and sea fish

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Crombach, W. H. J. 1974. Relationships among coryneform bacteria from soil, cheese and sea fish. Antonie van Leeuwenhoek **40**: 347–359.

DNA–DNA hybridization experiments among coryneform bacteria from soil, cheese and sea fish were performed and the genome sizes of 60 of these bacteria determined.

According to the D values obtained with hybridization experiments the soil arthrobacters can be divided into an *Arthrobacter simplex* and an *A. globiformis* group. The cheese and sea-fish coryneforms were found to be only remotely related to the soil arthrobacters of the *A. globiformis* type.

The greater part of the orange cheese coryneforms are homologous to a high degree and appear to be of the *Brevibacterium linens* type. Low D values between the reference strains indicate that the orange cheese coryneforms are only remotely related to the non-orange ones.

In spite of the morphological resemblance only a minority of the orange sea-fish coryneforms hybridized significantly with *B. linens*. The % GC of the majority of these coryneforms are in the range of 63 to 64%.

INTRODUCTION

Several authors studied the taxonomy of the coryneform bacteria, in particular the soil arthrobacters (Cummins and Harris, 1958, 1959; Davis and Newton, 1969; Skyring and Quadling, 1970; Crombach, 1972). So far, pleomorphism has been the principal criterion for classifying soil isolates into the genus *Arthrobacter*. However, cell transformation is not restricted to this genus. In other genera it is less conspicuous. Keddie, Leask and Grainger (1966) pointed out that a genus should not be defined solely on morphological characters. The more pleomorphic soil isolates have been studied, the less distinct the boundaries between *Arthrobacter* and other genera of the coryneform bacteria have become. According to Jensen (1966) it is difficult to differentiate *Arthrobacter*

from *Nocardia* on a morphological basis. This was confirmed by Bousfield (1972). On the other hand, the pattern of major fatty acids of *Arthrobacter* species differs markedly from that of *Nocardia* species (Bowie et al., 1972). The latter authors stated that the genus *Arthrobacter* occupies a central position between the less pleomorphic bacteria of the *Corynebacterium* type and the mycelial *Nocardia* species. Further, they concluded from the results of Mulder and Antheunisse (1963), Mulder et al. (1966) and Kaneko, Kitamura and Yamamoto (1969), that the genus *Arthrobacter* cannot be restricted to soil bacteria. However, Mulder et al. (1966) had shown that the coryneform bacteria from cheese and from sea fish differed, mainly in physiological characters, from the arthrobacters isolated from soil. It was also found that the DNA base composition of the soil arthrobacters differed from those of the orange cheese and sea-fish coryneforms. However, the % GC values of the non-orange cheese strains (grey-white strains of Mulder et al., 1966) and those of the soil arthrobacters are in the same range (Crombach, 1972).

The occurrence of coryneforms on the skin of fish has already been reported by Georgala (1957), Mulder et al. (1966), and Bousfield (1972). Shewan, Hobbs and Hodgkiss (1960) found 20–48% coryneforms on the skin of fresh fish (see also Shewan, 1971). These results were in agreement with those obtained in this laboratory by J. Obdam (unpublished data). Spencer (1959) found that coryneforms comprised 50% of the total flora on wooden fish boxes. The composition of the microbial flora on different species of fresh fish appeared to be very similar and it was found to be a reflection of the microflora of the water in which the fish was caught (Shewan, 1971).

Orange sea-fish coryneform strains were found to resemble physiologically the *Brevibacterium linens* type from cheese (Mulder et al., 1966). Their DNA base composition was found to be similar to that of *B. linens* (Crombach, 1972). In the present study a greater number of strains of orange sea-fish coryneforms were investigated, and the genetic relationship among the latter bacteria, the cheese coryneforms and the soil arthrobacters was determined.

MATERIALS AND METHODS

Microorganisms. The soil arthrobacters and the cheese coryneforms were from our collection (Crombach, 1972). The sea-fish coryneforms in this collection are isolates from different fish samples and wooden fish boxes (Table 3). The pleomorphic coryneform morphology was the sole criterion for classifying the fish isolates as coryneform bacteria.

Media. The soil arthrobacters and the non-orange cheese coryneforms were

grown in the slightly modified medium of Yamada and Komagata (1970) as described by Crombach (1974*a*). The orange cheese coryneforms, the sea-fish coryneforms, *Pseudomonas putida* CCEB 520 (CCEB: Culture Collection Entomogenous Bacteria, Prague) and *Xanthomonas pelargonii* ICPB P 121 (ICPB: International Collection of Phytopathogenic Bacteria, Davis, Cal.) were grown in a medium containing yeast extract, 0.7% and glucose, 1.0%. The bacteria of the genera *Mycobacterium*, *Corynebacterium* and *Nocardia* were grown in a medium containing nutrient broth, 1.3%, and glycerol, 7%. *Corynebacterium bovis* strain C 28 was grown in a medium composed of soja tryptone, 3% and Tween 80, 1%.

DNA preparation. DNA was purified as described by Crombach (1973).

DNA base composition. The DNA base composition was calculated from the thermal denaturation curves (Crombach, 1972).

Hybridization. The renaturation-rate method of De Ley, Cattoir and Reynaerts (1970) was used in DNA – DNA hybridization experiments carried out in quadruplicate.

Molecular weight. The molecular weight (M.W.) of the genomes of all the strains, except ten, were calculated from the apparent renaturation rate using the equation of Gillis, De Ley and De Cleene (1970):

$$\frac{M_u}{M_r} = \frac{k'_r}{k'_u} \qquad (1)$$

M_r and k'_r stand for the M.W. of the genome and the apparent renaturation rate, respectively, of reference strain DNA; M_u and k'_u are the same symbols for the DNA of the strain under study.

Reference strains. Pseudomonas putida CCEB 520 was used as a reference for the calculation of the genome size of *B.linens* B 42. Subsequently, the latter was employed as a reference for the calculation of the genome sizes of the sea-fish and orange cheese coryneforms. *Xanthomonas pelargonii* ICPB P 121 was used as a reference for the calculation of the genome sizes of AC 405 and AC 256; the latter strains were employed as references for the calculation of the genome sizes of the soil arthrobacters and the non-orange cheese coryneforms.

The genome sizes of strains AC 501, AC 506, EC 14, EC 20, AC 253, C 33, C 34, C 37, AC 86 and AC 555 were calculated using the formula of Gillis et al. (1970):

$$M_u \cdot k'_u = 98.37 - 0.91 \times \%\,GC \qquad (2)$$

This equation was used when the DNAs which were hybridized differed by more than 2% GC.

RESULTS AND DISCUSSION

Beside the DNA base composition of a bacterium, its genome size is an important parameter in taxonomy, as the genetic potential is proportional to the M.W. of DNA. Consequently, the genome sizes of closely related bacteria will cover a rather small range. Therefore, the genome sizes have to be considered when interpreting the hybridization data. When this is neglected, hybridization experiments between DNAs with distinctly different genome sizes may abut in erroneous conclusions, in particular if determinations with the membrane-filter method have not been completed by reciprocal hybridizations (Park and De Ley, 1967).

The thermal stability of the heterologous duplexes has to be taken into consideration when the hybridization data are to be interpreted, because mismatching and unpairing of bases result in a decreased thermal stability. In a previous paper (Crombach, 1974*a*) it was shown that, with De Ley's method, no more than a few % mismatching occurred in duplexes of closely related bacteria, while in duplexes of moderately and remotely related bacteria mismatching never exceeded 10%. It is clear that D values less than 70–75% have to be considered more qualitatively than quantitatively. The average deviation of all the mean D values obtained in the study now reported equals 3.5%.

It should be stressed that in interpreting the results of hybridization experiments, a low D value has only a limited value and utmost care is needed in drawing conclusions about evolutionary relationship unless other parameters (*viz.* % GC, physiological and morphological differences) are taken into consideration. Cistrons differing by more than 20 % in base pairs outside their active centre do not hybridize at T_{or}, although homologous enzymes may be produced (De Ley et al., 1973).

Since the determination of the apparent reaction-rate constant is accompanied by a high standard deviation (Gillis et al., 1970; Crombach, 1973), it is advisable to calculate the genome size according to equation (1) from experiments where the DNA of the reference strain and that of the strain under study are hybridized in the same experiment. Calculating the genome sizes of the reference strains B 42, AC 405 and AC 256 from a large number of different hybridization experiments – about 40 – both equations given by Gillis et al. yielded similar values (Table 2).

Soil arthrobacters

Though the soil arthrobacters have similar % GC values (Crombach, 1972), two groups could be distinguished when they were hybridized with *Arthrobacter globiformis* AC 405 (Table 1): (1) "*Arthrobacter simplex*" strains and (2) "*A.*

globiformis" strains. The strains of the *simplex* group hybridized only slightly with the reference strain AC 405, the degree of binding (D) being lower than 30 %. In contrast, the strains of the *globiformis* group except AC 8 were partly moderately, and for the rest closely related to *A. globiformis* AC 405 (D = 30–75 %, and > 75 %, respectively).

These hybridization data supported the division of the soil arthrobacters into two groups on a physiological basis (J. Antheunisse, personal communication; Crombach, 1974*b*), and they were also in agreement with the different fatty acid composition of *A. globiformis* and *A. simplex* species (Bowie et al., 1972). The eccentric position of *A. simplex* within the genus *Arthrobacter* had already been shown by cell-wall analysis (Cummins and Harris, 1959) and by numerical taxonomy (Davis and Newton, 1969). Bousfield (1972) found that *A. globiformis* clustered with *A. simplex* at a similarity level S of 55 % and with AC 4 and AC 11 at S-levels of 65 % and 62 %, respectively. However, some microorganisms differing by about 10 % GC also occurred in clusters based on an S-level of 55 %. Such a difference in % GC implies that the number of complementary nucleotide sequences cannot be large and the clusters at an S-level of 55 % were

Table 1. Degree of binding (D) among the DNA samples of a number of soil arthrobacters, and their genome sizes

Microorganism	Strain number	Genome size (daltons)	Degree of binding, calculated in relation to			
			AC 405 (%)	AC 1 (%)	AC 166 (%)	AC 206 (%)
Arthrobacter simplex	AC 4	2.0×10^9	26			
A. simplex	AC 11	2.1×10^9	27	23		
A. simplex	AC 16	2.9×10^9	30		35	30
A. simplex	AC 29	1.7×10^9	22			
A. simplex	AC 157	2.0×10^9	24			
Arthrobacter spec.	AC 1	2.1×10^9	34			
A. globiformis	AC 8	3.6×10^9	36			
A. globiformis	AC 158	1.8×10^9	54			
A. globiformis	AC 166	2.1×10^9	51			64
A. globiformis	AC 206	2.0×10^9	55			
A. globiformis	AC 403[1]	2.0×10^9	102			
A. globiformis	AC 405[2]	2.1×10^9	100			
Corynebacterium flaccumfaciens	C 33	1.5×10^9	30			
Mycobacterium smegmatis	M 25	4.0×10^9	44			
Nocardia convoluta	N 2	4.9×10^9	52			

[1] American Type Culture Collection ATCC 8602, Rockville, Maryland U.S.A.
[2] American Type Culture Collection ATCC 8010, Rockville, Maryland U.S.A.

genetically heterogeneous; therefore numerical taxonomy should not be overestimated.

The fair homogeneity of the *globiformis* group minus AC 8 is in agreement with the occurrence of AC 166 and *A. globiformis* in one cluster at an S-level of 68% (Bousfield, 1972). This homogeneity was supported by the fact that the M.W. of the genomes ranged from 1.8 to 2.1 $\times$ 10^9 dalton (Table 1). This may be compared with results of Brenner et al. (1972) who found a relative binding of 70% between two *E. coli* strains differing at least 23% in genome size.

The genome size of AC 8 fell distinctly outside the above-mentioned range, implying an only remote relatedness with AC 405. The D value of 36% was relatively high because it was calculated in relation to the small genome of AC 405; D calculated in relation to the large genome of AC 8 would be 18 % (see De Ley et al., 1970).

Strain AC 1 occupied an intermediate position as regards physiological characters, between *A. simplex* and *A. globiformis* and as to morphology it differed slightly from the other soil arthrobacters. This intermediate position was corroborated by a D value of 34% with AC 405 (Table 1).

The hybridization data between strains of each of the two groups were in agreement with the division in a *simplex* and a *globiformis* group. For instance, the D value between AC 206 and AC 166 was 64%, whereas both strains only slightly hybridized with AC 16.

In order to obtain an impression as to the genetic relationship between soil arthrobacters and named cultures of coryneform bacteria, hybridizations were carried out between *A. globiformis* AC 405 and *Corynebacterium flaccumfaciens* C 33 (D = 30%), *Mycobacterium smegmatis* M 25 (D = 44%) and *Nocardia convoluta* N 2 (D = 52%). However, the latter two values are somewhat exaggerated as the genome sizes of M 25 and N 2 are markedly larger than that of AC 405. Therefore, it is unlikely that these strains are evolutionary closely related. The genome size of M 25 is in agreement with the results of Bradley (1973) who reported *Mycobacterium smegmatis* strains to have genome sizes in a range of 4.2 to 4.5 $\times$ 10^9 dalton.

Orange cheese coryneforms

The degree of binding between the DNA of the orange cheese coryneforms, including *B. linens* B 41 and *B. linens* B 42, the latter of which was used as reference strain, together with the genome sizes of these strains are recorded in Table 2. According to the hybridization data, the % GC values (Crombach, 1972), and the genome sizes, the orange cheese coryneforms, except strain B 3, are closely related to strain B 42 of the type species.

The deviating M. W. of the genome of strain B 41 is in agreement with the less close genetic relationship of this strain with B 42, as compared to *B. linens*' B 42 relationship with the majority of the orange cheese coryneforms.

Notwithstanding the morphological resemblance, B 3 appeared to be only remotely related to *B. linens* B 42. This was shown by the poor hybridization (D = 25 %) and the difference between both strains in % GC, in requirement of B vitamins and amino acids as nitrogen source (Schonewille, personal communication), and in the utilization of lactose and acetate as carbon sources (Crombach, 1974*b*).

The degree of binding of 91 % between AC 275 and AC 448 (not shown in Table 2) is in accordance with their close relatedness to B 42.

From the hybridization data one may conclude that the great majority of orange cheese coryneforms are identical with *B. linens*, as suggested by Mulder et al. (1966). Strain B 3 is an exception.

Table 2. Degree of binding (D) between the DNA of *Brevibacterium linens* strain B 42 and that of a number of orange cheese coryneforms and some other coryneform bacteria, together with their genome sizes

Microorganism	Strain number	Genome size (daltons)	Degree of binding calculated in relation to *B. linens* B 42 (%)
Orange cheese coryneforms	B 3	1.6×10^9	25
	B 4	2.2×10^9	74
	AC 251	2.2×10^9	83
	AC 252	2.1×10^9	85
	AC 275	2.1×10^9	86
	AC 423	2.0×10^9	85
	AC 448	2.0×10^9	82
B. linens (orange)	B 41[1]	1.6×10^9	72
B. linens	B 42[2]	2.0×10^9	100
		2.3×10^9*	
Non-orange cheese coryneforms	AC 253	3.5×10^9	47
	AC 256	1.5×10^9	11
		1.6×10^9*	
Arthrobacter globiformis	AC 405	2.1×10^9	19
		2.2×10^9*	
Arthrobacter spec.	AC 1	2.1×10^9	11
Corynebacterium bovis	C 28[3]	4.0×10^9	26
Pseudomonas putida	CCEB 520	2.5×10^9	29

[1] ATCC 9174.
[2] ATCC 9175.
[3] ATCC 7715.
* Genome size determined with equation (2).

It is remarkable that the degree of binding between the DNAs of non-orange cheese coryneform AC 253 and B 42 was 47% though these strains clearly differ morphologically. It should be stressed that the D value if calculated in relation to the genome of AC 253 would be 35% instead of 47%. DNA of B 42 did not significantly hybridize with those of *A. globiformis* AC 405, the intermediate strain AC 1, and the reference strain of the non-orange cheese coryneforms AC 256. These hybridization data, the % GC values (Crombach, 1972) and the morphological and physiological differences among these strains (Crombach, 1974*b*) make a close relationship unlikely. This conclusion is in agreement with the opinion of Mulder et al. (1966) that orange cheese coryneforms differ distinctly from soil arthrobacters and non-orange cheese coryneforms. However, Da Silva and Holt (1965) using numerical taxonomy, and Bowie et al. (1972) who studied the fatty acid composition, supported the opinion of Bousfield (1972), based on numerical taxonomy, that *B. linens*, although differing in several aspects from *A. globiformis*, should be placed into the same genus as the latter.

The % GC values had indicated the improbability of a close relationship of *B. linens* B 42 with the members of the genera *Nocardia*, *Mycobacterium* and the plant-pathogenic corynebacteria (Crombach, 1972). In fact, the degree of binding between the DNAs of B 42 and the *Corynebacterium* strain from animal origin, C 28 was 26%.

Orange sea-fish coryneform bacteria

In another paper (Crombach, 1974*b*) it will be shown that orange sea-fish coryneforms resemble the orange cheese coryneforms mainly morphologically. Both groups consist of bacteria with regular rods, and snapping formation frequently occurs. The % GC of four sea-fish coryneforms and most of the orange cheese coryneforms studied were similar (Crombach, 1972). In the present study the group of orange sea-fish coryneforms was extended to sixteen strains. The % GC values are recorded in Table 3.

The DNA base composition of the sea-fish coryneforms, except AC 501 and AC 506, covered a range of 2.9% GC, which would indicate that this group is rather homogeneous. However, this is at variance with the wide range of 1.4 to 3.3×10^9 dalton for the genome sizes (Table 3), which implies a marked difference in genetic potential. The % GC values of AC 501 and AC 506 allow a moderate genetic relationship with the remaining strains of the group. It is remarkable that the deviating % GC of AC 506 is attended by a slightly aberrant colour (brown-orange), whereas the also aberrant yellow colour of AC 481 is not attended by a deviating % GC. As the % GC values of the majority of the sea-fish coryneforms and those of the orange cheese coryneforms of the

Table 3. DNA base composition and genome size of a number of orange sea-fish coryneforms and degree of binding (D) between DNA of these bacteria and that of *B. linens* strain B 42.

Strain number	Origin	Mean %GC	σ (%GC)	Hyp. effect (%)	Genome size (daltons)	D, calculated in relation to B 42
Group I						
AC 474	salted cod	62.9	3.3	35	2.7×10^9	90
AC 472	plaice	63.6	3.4	37	2.2×10^9	81
AC 473	salted cod	63.4	3.4	36	1.9×10^9	78
AC 484	market fish	63.3	3.2	35	2.6×10^9	78
AC 486	market fish	62.5	3.2	36	2.1×10^9	77
Group II						
AC 490	cod	61.1	4.5	37	3.3×10^9	69
AC 478	market fish	62.4	3.2	33	2.0×10^9	47
AC 501	wooden fish boxes	59.5	5.5	35	2.3×10^9	46
AC 498	wooden fish boxes	63.3	3.3	34	2.3×10^9	38
Group III						
AC 471	plaice	64.0	3.7	35	2.0×10^9	27
AC 470	plaice	64.0	3.1	34	2.1×10^9	26
AC 480	market fish	63.5	3.6	35	1.6×10^9	24
AC 506[1]	wooden fish boxes	66.5	3.6	35	2.2×10^9	25
AC 495	wooden fish boxes	63.3	3.4	36	1.9×10^9	23
AC 481[2]	market fish	63.3	4.6	34	1.4×10^9	20
AC 482	market fish	64.0	2.9	35	2.3×10^9	19
B 42	ATCC 9175	63.7	3.5	34	2.0×10^9	100

[1] Brown-orange
[2] Yellow

B. linens type are in the range of about 63 to 64% (Crombach, 1972; this paper), both groups might be genetically closely related. It will be shown that only a few strains are.

The D values between all of the sea-fish coryneforms available and *B. linens* B 42 are listed in Table 3. According to these D values the sea-fish coryneforms can be divided into three groups.

Group I is closely related to *B. linens* B 42, and consequently, may also be related to the cheese coryneforms of the *B. linens* type. The D value between AC 472 and AC 486 was 77% (not shown in Table 3), which corresponds with the high D values of both strains with B 42 (Table 3). It should be emphasized that the D values between B 42, and AC 474 and AC 484 (D = 90 and 78%, respectively) are somewhat exaggerated as they are calculated in relation to the relatively small genome of B 42; D calculated in relation to the larger genomes of AC 474 and AC 484 would be 64 and 58%, respectively.

Group II. The strains AC 490, AC 478, AC 501 and AC 498 are moderately related to *B. linens* B 42, as D, varying from 38 to 69%, falls within the range 30–75% (Table 3). The DNA of AC 490 hybridized fairly well with that of B 42, but AC 490 has a larger genome, and therefore their evolutionary relatedness can only be slight. As regards % GC, group II is less homogeneous than groups I and III.

As the D values between some strains, one of the pair belonging to group I and the other to group II, viz. AC 486 × AC 501, AC 486 × AC 498, and AC 472 × AC 501 are 51, 42, and 49%, respectively, (not shown in Table 3) the strains of groups I and II may be moderately related.

Group III. The majority are only remotely related to *B. linens* B 42 as both the D values are less than 30% and because they differ from B 42 in some physiological and morphological aspects (Crombach, 1974*b*).

DNA of AC 506 and AC 482 hybridized poorly (D = 25%), in agreement with the differences in % GC and in colour (Table 3). The low degree of binding between AC 471 and two orange cheese coryneforms AC 275 and AC 448 (D = 26 and 25%, respectively) agrees with the D values of the separate strains with B 42 (Tables 2 and 3).

It may be concluded that an orange colour, and a morphology resembling that of *B. linens* do not automatically imply a close genetic relationship to the latter.

Non-orange cheese coryneforms

All of the DNAs of the non-orange cheese coryneforms were hybridized with that of AC 256, because the latter strain's % GC value approximates the average of the group if EC 14, EC 20, and AC 253 are excluded. The hybridization data (Table 4) show that the majority of the strains are closely related, though of different origin (Crombach, 1972).

Because of the deviating % GC values of strains EC 14, EC 20 and AC 253 (Crombach, 1972) less binding between the DNA of these microorganisms and that of AC 256 could be expected and was indeed found (Table 4). These strains also deviate by different physiological and morphological characteristics (Crombach, 1974*b*). Bousfield (1972) found that AC 253 was not included in the cluster which contains other cheese coryneforms.

EC 7 did not hybridize with AC 256 in spite of the agreement in % GC. Morphologically, EC 7 resembles *B. linens* to a certain extent; the DNAs of the latter two strains hybridized fairly well (D = 37%). The D values between EC 7 on the one hand and EC 10 and *Corynebacterium flaccumfaciens* C 33 on the other are 32 and 28%, respectively, which does not indicate a possible relationship.

The low D value between AC 256 and *Arthrobacter globiformis* AC 405

Table 4. Degree of binding between the DNA of AC 256 and that of a number of non-orange cheese coryneforms and some named coryneform bacteria, together with their genome sizes

Microorganism	Strain number	Genome size (daltons)	D, calculated in relation to AC 256 (%)
Non-orange cheese strains	EC 7	1.8×10^9	16
	EC 9	1.6×10^9	98
	EC 10	1.5×10^9	91
	EC 14	1.8×10^9	26
	EC 15	1.4×10^9	88
	EC 16	1.5×10^9	94
	EC 20	2.0×10^9	24
	AC 253	3.5×10^9	21
	AC 256	1.5×10^9	100
	AC 261	1.4×10^9	91
	AC 262	1.5×10^9	100
	AC 263	1.7×10^9	95
	AC 278	1.5×10^9	86
Orange cheese strain	AC 275	2.1×10^9	27
A. globiformis (type culture)	AC 405	2.1×10^9	26
Corynebacterium flaccumfaciens	C 33	1.5×10^9	25
michiganense	C 34	1.5×10^9	29
tritici	C 37	1.2×10^9	30
Nocardia spec.	AC 86	1.8×10^9	32
Nocardia spec.	AC 555	1.4×10^9	29

(Table 4) together with the different physiology and morphology support the conclusion of Mulder et al. (1966) that the grey-white cheese coryneforms do not belong in the genus *Arthrobacter*.

DNA of the reference strain AC 256 did not significantly hybridize with that of some named cultures of *Corynebacterium* and *Nocardia* (D about 30%, Table 4). More isolates classed within the latter genera have to be studied for a possible genetic relationship with the non-orange cheese coryneforms. However, a close relatedness seems improbable because of the % GC values (Crombach, 1972).

Nocardia spec. AC 555 and *Corynebacterium tritici* C 37 appeared to be slightly related (D = 35%) while the DNA of the latter strain hybridized poorly with that of *Corynebacterium michiganense* C 34 (D = 23%).

Owing to the heterogeneity within the various genera of coryneform bacteria (Yamada and Komagata, 1970; Skyring and Quadling, 1970; Bradley, 1973; Bousfield, 1972; Bowie et al., 1972; Crombach, 1972) it is difficult to establish relationships of the bacteria under study with species of these genera. The elucidation of the confused taxonomy of coryneform bacteria requires a sharp delineation of the genera.

Conclusion. A clear difference exists between the soil arthrobacters of the *A. globiformis* type and the cheese coryneforms. The same holds for the non-orange and the orange cheese coryneforms, while a minority of the orange sea-fish coryneforms resemble the latter group.

The author is much indebted to Professor Dr. E. G. Mulder for valuable criticism and helpful discussions during the preparation of the manuscript. He is also grateful to Dr. Maria Deinema and Dr. T. A. Lie for reading the manuscript, and to Mrs. Maja Kokke-Hopstaken and Miss Annie Scholten for technical assistance.

Received 21 *March* 1974

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ERRATUM

Article by W. H. J. Crombach, this volume, p. 142, 6th line from bottom should read:
Taking into account the mismatching in renatured homologous hybrids, the total......

Antonie van Leeuwenhoek **40** (1974) 361–376

Morphology and physiology of coryneform bacteria

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Four groups of coryneform bacteria, viz. soil arthrobacters, orange cheese coryneforms, orange sea-fish coryneforms and non-orange cheese coryneforms, were studied as regards morphological and physiological features.

The soil arthrobacters can be divided into a *simplex* and a *globiformis* group on the basis of their ability of utilizing a number of carbon sources. The group of the orange cheese coryneforms was found to be rather homogeneous, in contrast to the groups of the orange sea-fish coryneforms and the non-orange cheese coryneforms, some strains of which deviated from the others of their group as to the majority of the characteristics tested.

Mainly the physiological characteristics of each of the groups justify the division of the coryneforms into the four chief groups mentioned.

INTRODUCTION

Coryneform bacteria show a cycle of development which is more pronounced in some of the genera than in others. In the stationary phase the cells occur predominantly as cocci which after transfer to an appropriate fresh medium germinate and develop into rods. On ageing, the rods transform more or less completely into cocci, as is particularly evident in the genus *Arthrobacter*. The difference between arthrobacters and other coryneforms with respect to the "life cycle" is of a quantitative rather than a qualitative nature (Keddie, Leask and Grainger, 1966; Veldkamp, 1970).

The growth cycle and some physiological features of soil arthrobacters, and of coryneform bacteria isolated from dairy-waste activated sludge, the surface of soft cheese, or the skin of sea fish, have been investigated by Mulder and Antheunisse (1963) and Mulder et al. (1966). Their statement that soil arthrobacters and activated-sludge coryneforms differ mainly physiologically from cheese and sea-fish coryneforms was supported by the different ability of

the various groups of organisms for degrading choline, uric acid and pectin. The former two groups are able to utilize choline as sole source of carbon and nitrogen (Kortstee, 1970) and to decompose both uric acid and urea (Antheunisse, 1972), and the latter two groups are not. The ability for degrading pectin was found by Rombouts (1972) in 21 out of 117 strains of soil arthrobacters and in 9 out of 23 strains of coryneforms from activated sludge, but all of 137 strains of coryneforms from cheese and sea fish tested lacked it.

The aim of the present study was to determine the degree of homogeneity of four groups of coryneform bacteria – viz. soil arthrobacters, orange and non-orange cheese coryneforms and orange sea-fish coryneforms – with respect to morphological and physiological characters. This study is part of an investigation into the relationships among these bacteria. The DNA base composition and the results of DNA – DNA hybridization experiments have been recorded in other papers (Crombach, 1972, 1974).

MATERIALS AND METHODS

Strains examined. In this investigation 49 strains of coryneform bacteria divided into four groups based on origin and colour were included (Table 1).

Pigment production. The effects of light and salinity of the medium on pigment production were studied by inoculating each strain in duplicate on a rich agar medium containing yeast extract, 0.7% and glucose, 1%, in either the absence or the presence of 4% NaCl. One of each pair of cultures was exposed to light for four days at 22 C, whereas the other was kept in the dark.

Cycle of development. The growth cycle was followed by incubating the strains on a poor medium consisting of soil extract with yeast extract, 0.1%, and agar, 1.2%, at 25 or 30 C for about two weeks, and then transferring the coccoid cells to a fresh, either poor or rich medium. At appropriate times the cell shapes were observed microscopically and the percentages of rods and cocci estimated. The effects of growth conditions (temperature, solid or liquid medium, and composition of the medium) were checked using at least four strains of each group.

Growth in the coccoid stage. The growth of *Arthrobacter globiformis* AC 166 in the coccoid stage was followed microscopically. The cells were grown at 25 C for twelve days in soil extract with yeast extract, 0.05%. They were collected by centrifugation, washed and transferred onto plates with a medium prepared from the exhausted medium from which the cocci had been collected, and 1.2% agar. The multiplication of individual cocci was followed on a slide culture (Ensign and Wolfe, 1964). In the present study the chamber was not

completely sealed with Vaspar so as to avoid anaerobic conditions. Some water could be added if necessary.

Carbon source. The utilization of several carbon compounds was tested in a basal medium consisting of yeast extract, 0.2%; soil extract, 100 ml; water, 900 ml; carbon compound under study, 0.5%. A culture in the basal medium without any carbon compound added served as control. The pH of the medium was adjusted to 7.4 before sterilization. Fifty ml of the medium was inoculated with two drops of a suspension of young cells. The cultures were incubated at 25 C for four days, and the pH was kept at 7. A 50% higher turbidity as compared to the control was considered a positive result. The following carbon compounds were tested: glucose, lactose, saccharose, glycerol, lactate, acetate, starch and citrate. Of the latter, only 0.25% was added to the basal medium.

Hydrolysis of gelatin was tested on a medium containing nutrient broth, 1.3%; gelatin, 0.5%; agar, 1.2%. The cultures were incubated at 25 C for six days. The presence of gelatin was detected by flooding the plates with a 15% $HgCl_2$ solution in 5 N HCl.

Hydrolysis of casein was tested on a medium containing casein, 0.2%; nutrient broth, 0.3%; $MgSO_4 \cdot 7H_2O$, 0.1%; K_2HPO_4, 0.1%; $(NH_4)_2SO_4$, 0.1%, at 25 C for six days. Clarification of the medium around the inoculum was an indication of hydrolysis of casein.

Hydrolysis of starch was tested on the same medium as used for hydrolysis of casein, except that casein was replaced by starch. After six days incubation at 25 C the cell mass was removed and the plate exposed to iodine fumes.

Nitrogen requirement. The ability for utilizing inorganic nitrogen compounds in either the presence or the absence of vitamins was tested in a basal medium with either $(NH_4)_2SO_4$ or NH_4NO_3 as sole N-source. The basal medium contained: K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; $FeCl_3 \cdot 6H_2O$, 0.1 g; $CuSO_4 \cdot 5H_2O$, 0.1 mg; $ZnSO_4 \cdot 7H_2O$, 0.1 mg; $MnSO_4 \cdot 7H_2O$, 0.1 mg; Na_2MoO_4, 0.01 mg; H_3BO_3, 0.01 mg; $CoCl_2$, 0.01 mg; glass-distilled water, 1 liter. This medium was supplied with glucose, 0.5%, and nitrogen, 0.025% as $(NH_4)_2SO_4$ or NH_4NO_3. The vitamin mixture was sterilized by candle filtration prior to the addition of 1 ml to 50 ml of sterilized medium, which resulted in the following final vitamin concentrations (μg/liter): biotin, 10; folic acid, 10; riboflavin, 200; thiamine, 200; pyridoxine, 200; nicotinic acid, 200; calcium pantothenate, 200; *p*-amino-benzoic acid, 200; vitamin B_{12}, 10; inositol, 200. The media, with or without vitamins, were inoculated with two drops of a diluted suspension of young cells and incubated at 25 C for at most twelve days. Growth was compared to that in a medium containing yeast extract, 0.7%, and glucose, 1%. A turbidity of 50 EEL-nephelometer units or more was considered a positive result.

Hugh and Leifson test. The strains were inoculated in a stab medium containing glucose, 1%; peptone, 0.2%; NaCl, 0.5%; K_2HPO_4, 0.03%; bromthymol-blue, 0.001%. The acid or alkaline reaction was checked within five days at 25 C.

Salt tolerance was determined in media containing yeast extract, 0.7%; glucose, 1%; NaCl, 0, 3, 5, 8, 10, 12 or 15%. Before sterilization, the pH was adjusted to 7.5. The media were inoculated with two drops of a diluted suspension of young cells, and incubated at 25 C for six days. A turbidity of 50 EEL-nephelometer units was considered a positive result.

Gram stain. Gram staining was carried out following both the general laboratory method and the modified Hucker method (Adamse, 1970). Soil arthrobacters and cheese coryneforms were tested as one-day-old cells; orange sea-fish strains were tested both as rods and as coccoid cells.

Oxidase and catalase. The oxidase test was carried out according to the modified Kovacs method (Steel, 1962). A purple colour appearing within 20 sec was recorded as positive.

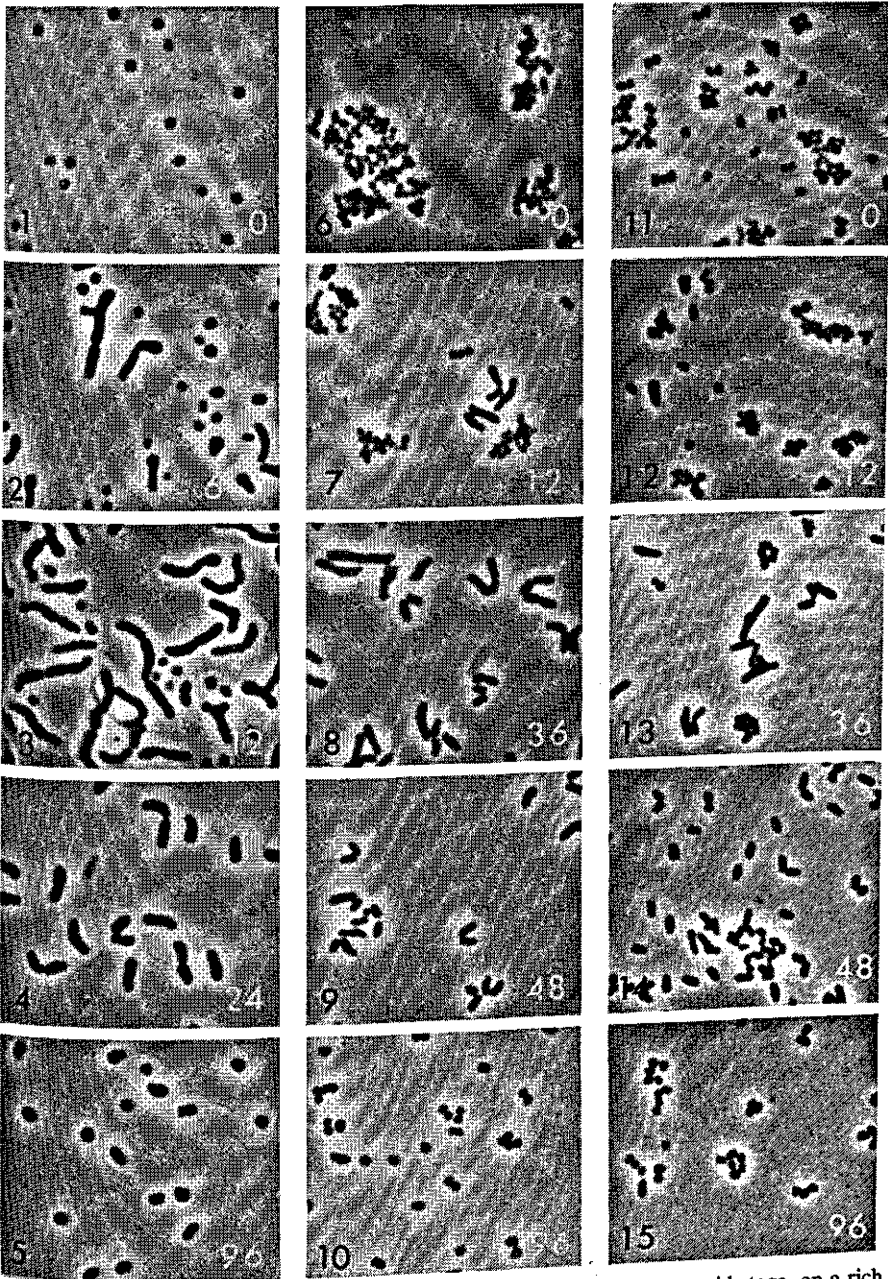
Catalase was tested for by dripping a 3% H_2O_2 solution on the colonies.

RESULTS AND DISCUSSION

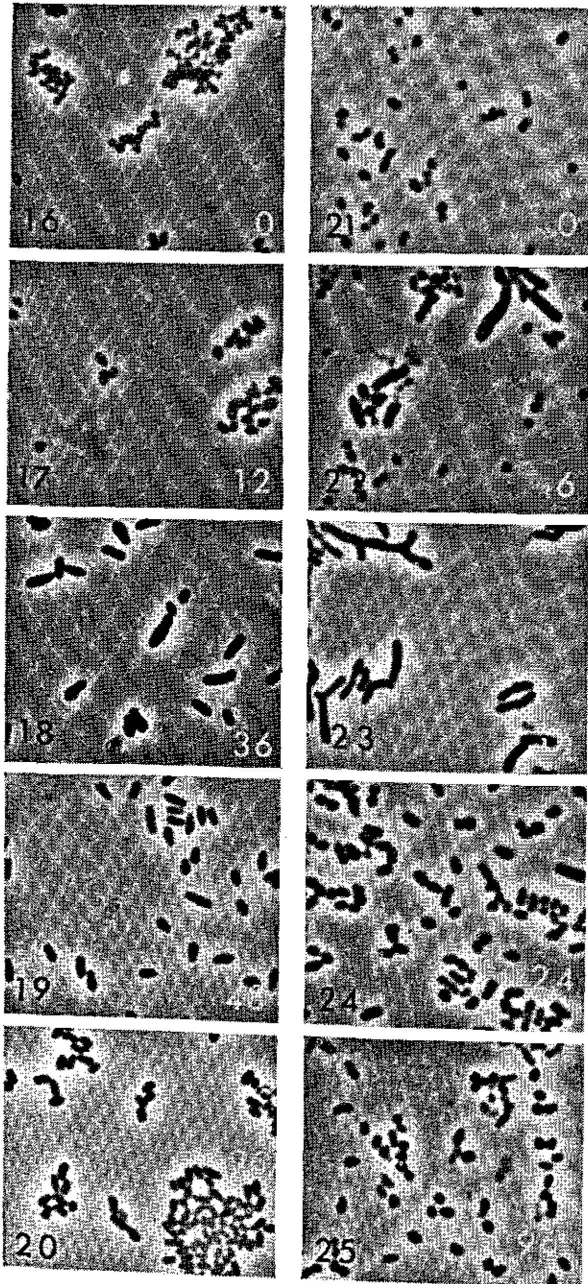
The soil arthrobacters, the orange cheese and sea-fish coryneforms, and the non-orange cheese coryneforms (Mulder et al.'s grey-white strains) differ in colour, in the shape of the rods and in the tendency to form cocci on ageing (Figs. 1–25).

Pigments. Four percent salt did not affect the pigment production of the soil arthrobacters and the cheese strains except for strain B 4 which requires salt to produce an orange pigment (Crombach, 1972). The growth of the soil arthrobacters is retarded by 4% salt as shown by Mulder et al. (1966). Thirty percent of the orange sea-fish strains showed a slightly paler colour on a medium with 4% salt as compared to controls.

Light had no effect on the development of pigment by soil arthrobacters and non-orange cheese coryneforms, except for EC 7 which became red in the dark and orange in the light, but in ageing cells of this strain the orange pigment became completely masked by a red one. The cheese strains of the *B. linens* type produced an orange pigment on exposure to light, but not in the dark, except for strains B 3 and AC 423 which were orange both in the light and in the dark. These results confirm some observations by Mulder et al. (1966); however, these authors found 19 out of 40 strains of the *B. linens* type able to produce the orange pigment both in the light and in the dark. The sea-fish



Figs. 1–15. Growth cycles of coryneform bacteria, starting from the coccoid stage, on a rich medium at 25 C. Figs. 1–5. *Arthrobacter globiformis* AC 166; Figs. 6–10. Orange cheese strain B 4; Figs. 11–15. Orange sea-fish coryneform AC 470. White figures: age of culture in hours. 1625 ×.



Figs. 16–20. Non-orange cheese strain AC 256; Figs. 21–25. Non-orange cheese strain AC 253.

strains AC 482 and AC 501 produced an orange pigment in the light and in the dark, but in the other sea-fish strains the colour was paler in the dark.

Disappearance of the orange colour upon exposure to light for a prolonged period (six weeks, Mulder et al., 1966), was not observed in the present study.

Morphology

Cell shape and germination of cocci. The cocci of the soil arthrobacters (group I of Table 1) form germination tubes on a fresh medium (Figs. 1 and 2). The rods are often irregular, sometimes rudimentarily branched, and snapping division (V-formation) regularly occurs (Mulder et al., 1966; this paper, Fig. 3). The groups II and III consist of orange cheese, and sea-fish coryneforms, respectively. After transfer to fresh media their coccoid cells develop to slender regular rods without forming distinct germination tubes. The rods are often arranged in V-formation; they are only rarely branched (Figs. 8, 9, 14). The rods of the non-orange cheese coryneforms (group IV) are mostly rather regular and thicker than those of the orange strains. In this group, distinct germination tubes (Fig. 18) and V-formation (Fig. 20) have been observed.

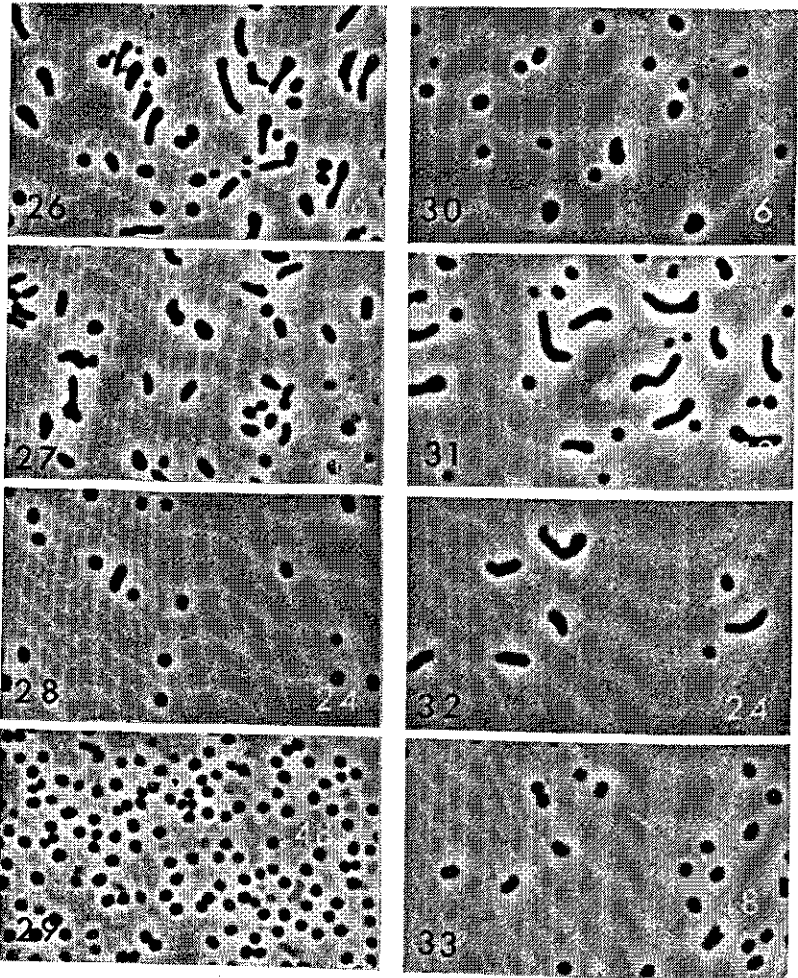
In general, the germination of the coccoid cells is faster at 25 C than at 15 C (Figs. 26 and 30), however, with *globiformis* strains (Table 1) this effect is not very conspicuous. Their coccoid cells germinated always faster than those of the other coryneforms. The cocci of the orange cheese coryneforms when transferred to a rich medium at 15 C germinate slower than do those of the other groups.

Cocci germinate more readily on a poor medium than on a rich one, compare Figs. 2 and 26. This applies in particular to the cheese and sea-fish coryneforms. It is not known whether the difference is due to inhibition by the higher concentration of nutritional compounds.

Transformation of rods into cocci

Effect of temperature. Transformation from rods into cocci is generally faster at 25 C than at 15 C (Figs. 26–33), as already observed by Mulder et al. (1966). Again, this effect is less conspicuous in the *A. globiformis* strains. At 15 C the tendency to form cocci declines in the following sequence: the *A. globiformis* strains, the orange sea-fish and non-orange cheese coryneforms, the *A. simplex* strains, the orange cheese strains of the *B. linens* type. At 25 C the rate of transformation into cocci differs less among the four groups than at 15 C; the non-orange cheese strains tend to transform the most rapidly into cocci, especially the strains EC 20 and AC 253 (Figs. 21–25).

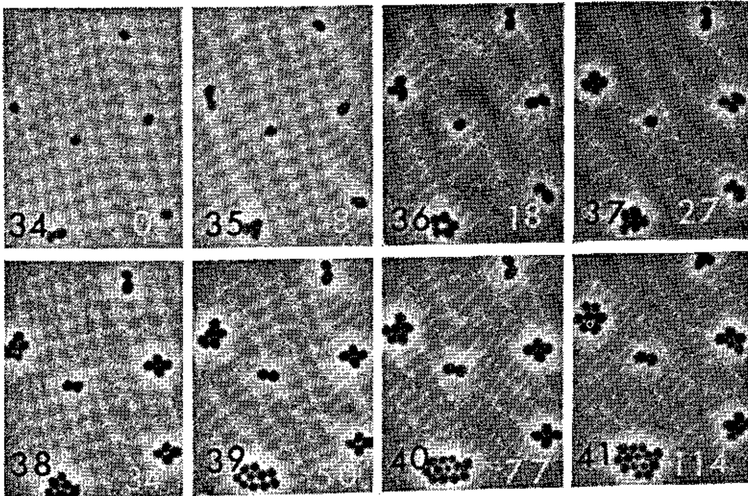
Effect of nutrition. On a rich medium the transformation of rods into cocci is retarded by a great amount of nutrition; the majority of the *simplex* and the non-orange cheese strains do not completely transform into cocci but within 7 days become ovoids.



Figs. 26–33. Effect of temperature on the transformation from rods into cocci. Figs. 26–29. *A. globiformis* AC 166, at zero time in the coccoid stage, grown on a poor medium at 25 C. Figs. 30–33. The same at 15 C. 1625 ×. Fig. 31. Age of culture 12 hr.

Transformation of rods into cocci proceeds more rapidly in an aerated liquid medium than on a solid one, which may be due to faster growth (better supply of substrates) and consequently, a more rapid exhaustion of the medium.

Growth of cocci. On a very poor medium, arthrobacters keep growing in the coccoid stage (Figs. 34–45). The cocci of both series, Figs. 34–41 and Figs. 42–45, represent the fourth or a later generation in cultures aged 114 and 210 hr, respectively, assuming that all of the cocci, including those in the centre of the colony, had taken part in the division process. So the cocci were physiologi-



Figs. 34–41. *A. globiformis* AC 166 growing in the coccoid stage at 25 C. 1625 ×.

cally active, as cell division does not occur without metabolism. The coccoid form is an expression of the condition of reduced growth (Mulder and Antheunisse, 1963) which appears to occur at an appropriate low substrate level. This agrees with the results of Luscombe and Gray (1971): the morphology is related to a specific growth rate and not to specific nutritional compounds, as was reported by Ensign and Wolfe (1964) and Kvasnikov et al. (1972). Mulder and Antheunisse (1963) and Luscombe and Gray (1971) presumed that arthrobacters in soil are in the coccoid stage because of the low energy flow in this environment, and Lowe (1969) confirmed this by direct observation of cells of *Arthrobacter* strains growing in forest soil.

Physiology

Carbon source and hydrolysis of gelatin, casein and starch. The soil arthrobacters can utilize glucose, saccharose, glycerol, acetate and citrate (Table 1). Lactose is utilized by all of the *A. simplex* strains except AC 16, whereas it is not by the majority of the *A. globiformis* strains. Lactate is a good carbon source for the *A. globiformis* strains, but not for the intermediate strain AC 1 and for two of the five *simplex* strains. Also Mulder et al. (1966) found that not all of the soil arthrobacters utilized lactate. AC 8 deviates from the other *globiformis* strains recorded in Table 1. This corresponds with the results of DNA – DNA hybridization experiments (Crombach, 1974).

Gelatin and casein are hydrolyzed by the soil arthrobacters tested, but starch only by the *globiformis* strains (see also Bergey's Manual, Breed, Murray and

Figs. 42–45. *A. globiformis* AC 166 growing in the coccoid stage at 25 C. Two colonies of cocci. 1625 ×.

Table 1. Utilization of several compounds as carbon source by coryneform bacteria, and the hydrolysis of gelatin, casein and starch by these bacteria

Microorganism	Strain number	Utilization of								Hydrolysis of		
		Glucose	Lactose	Saccharose	Glycerol	Lactate	Acetate	Citrate	Starch	Gelatin	Casein	Starch
Group I												
Soil arthrobacters												
Arthrobacter simplex	AC 4	+	+	+	+	−	+	+	−	+	+	−
	AC 11	+	+	+	+	+	+	+	−	+	+	−
	AC 16	+	−	+	+	+	+	+	−	+	+	−
	AC 29	+	+	+	+	+	+	+	−	+	+	−
	AC 157	+	+	+	+	−	+	+	−	+	+	−
Arthrobacter spec. (intermediate)	AC 1	+	+	+	+	−	+	+	−	+	+	±[1]
A. globiformis	AC 8	+	+	+	+	+	+	+	+	+	+	+
	AC 158	+	−	+	+	+	+	+	+	+	+	+
	AC 166	+	−	+	+	+	+	+	+	+	+	+
	AC 206	+	−	+	+	+	+	+	−	+	+	±
	AC 403[2]	+	−	+	+	+	+	+	+	+	+	+
	AC 405[3]	+	−	+	+	+	+	+	+	+	+	+

Table 1, continued

Group II												
Orange cheese coryneforms of the *Brevibacterium linens* type	B 3	+	+	−	+	+	−	nd	−	+	−	−
	B 4[4]	+	−	−	+	+	+	nd	−	+	−	−
	AC 251	+	−	−	+	+	+	−	−	+	−	−
	AC 252	+	−	−	+	+	+	−	−	+	−	−
	AC 275	+	−	−	+	+	+	−	−	+	−	−
	AC 423	+	−	−	+	+	+	−	−	+	−	−
	AC 448	+	−	−	+	+	+	−	−	+	−	−
B. linens (type species)	B 42[5]	+	−	−	+	+	+	−	−	+	−	−
Group III												
Orange sea-fish coryneforms	AC 470	+	−	+	+	+	+	+	−	+	+	−
	AC 471	+	−	−	+	+	+	+	−	+	+	−
	AC 472	+	−	−	+	+	+	−	−	+	−	−
	AC 473	+	−	−	+	+	+	−	−	+	−	−
	AC 474	+	−	−	+	+	+	−	−	+	−	−
	AC 478	+	−	−	+	+	+	+	−	+	+	−
	AC 480	+	−	−	+	+	+	+	−	+	+	−
	AC 481[6]	+	+	+	+	+	+	nd	−	+	+	−
	AC 482	+	−	+	+	+	+	+	−	−	−	−
	AC 484	+	−	−	+	+	+	−	−	+	−	−
	AC 486	+	−	−	+	+	+	−	−	+	−	−
	AC 490	+	−	−	+	+	+	−	−	+	−	−
	AC 495	+	−	−	+	+	+	+	−	+	+	−
	AC 498	+	−	−	+	+	+	+	−	+	+	−
	AC 501	+	−	−	+	+	+	−	−	+	−	−
	AC 506	+	−	+	−	−	+	−	−	−	−	−
Group IV												
Non-orange cheese coryneforms	EC 7	+	−	−	+	+	+	nd	−	+	−	−
	EC 9	+	−	−	+	+	+	nd	−	−	−	−
	EC 10	+	−	−	−	+	−	nd	nd	−	−	−
	EC 14	−	−	−	−	+	+	nd	−	−	−	−
	EC 15	+	−	−	+	+	+	nd	−	−	−	−
	EC 16	+	−	−	+	+	+	nd	−	−	−	−
	EC 20	+	−	+	+	+	+	nd	+	+	+	+
	AC 253	+	−	+	+	+	+	nd	+	+	+	+
	AC 256	+	−	−	+	+	+	−	−	−	−	−
	AC 261	+	−	−	+	+	+	−	−	−	−	−
	AC 262	+	−	+	+	+	+	−	−	−	−	−
	AC 263	+	−	−	+	+	+	−	−	−	−	−
	AC 278	+	−	+	+	+	+	−	−	−	−	−

[1] ± stands for a slight decomposition.
[2] American type culture collection ATCC, 8602, Rockville, Maryland, U.S.A.
[3] American type culture collection ATCC, 8010, Rockville, Maryland, U.S.A.
[4] Requires 4% of added salt in the medium for the production of the orange pigment (Crombach, 1972).
[5] American type culture collection ATCC, 9175, Rockville, Maryland, U.S.A.
[6] Yellow.

Smith, 1957). A slight hydrolysis of starch by strain AC 206 corresponds with an insignificant utilization of starch as carbon source (Table 1).

The difference in physiological characteristics between the *globiformis* strains on the one hand, and the *simplex* strains on the other is in accordance with the results of hybridization experiments (Crombach, 1974). DNA of *simplex* strains hybridized poorly with that of *A. globiformis* AC 405 (degree of binding below 30%); DNA of the *globiformis* strains, however, hybridized for more than 50% with that of AC 405.

The orange cheese coryneforms of the *B. linens* type, except B 3, were found to be a homogeneous group with respect to the utilization of the carbon sources tested, and to the hydrolysis of gelatin, casein and starch (Table 1). B 3 stands somewhat apart of the group in accordance with its aberrant % GC (Crombach, 1972) and its low degree of hybridization with the type culture *B. linens* B 42 (Crombach, 1974).

The orange sea-fish coryneforms are a less homogeneous group than the orange cheese coryneforms, in particular as regards the utilization of saccharose and citrate, and the hydrolysis of gelatin and casein (Table 1). The heterogeneity of the orange sea-fish coryneform group was also shown in DNA – DNA hybridization experiments (Crombach, 1974). The agreement of physiological characters between strains AC 472, AC 473, AC 474, AC 484, and AC 486 on the one hand and B 42 on the other is corroborated by a fairly good hybridization (Crombach, 1974). Strain AC 501 hybridized moderately with B 42, and AC 490 may be only remotely related with B 42 because of different genome size (Crombach, 1974). The strains that can hydrolyze casein and can utilize citrate are either moderately, or only remotely related to B 42 which can neither (Crombach, 1974).

The non-orange cheese coryneform strains EC 14, EC 20, AC 253, and to a lesser extent EC 7 deviate from the other strains of this group (Table 1). EC 7 resembles B 42 both physiologically and morphologically, but the results of the hybridization experiments indicate only a moderate genetic relationship (Crombach, 1974). The deviating physiological characters of strains EC 14, EC 20 and AC 253 – including the ability of EC 20 and AC 253 for hydrolyzing gelatin, casein and starch – together with their aberrant % GC values (Crombach, 1972), suggest that the non-orange cheese strains fall apart into two subgroups, which would agree with Mulder et al.'s (1966) finding that 11 out of 51 strains of grey-white cheese coryneforms were strongly proteolytic. Some deviating physiological characteristics of EC 10 may be due to slow growth; it is genetically closely related with the reference strain of the group: AC 256 (Crombach, 1974).

Nitrogen requirement. The soil arthrobacters utilize inorganic nitrogen; some strains require added vitamins (Mulder et al., 1966).

Twenty-nine out of 31 strains of orange cheese coryneforms of the *B. linens* type tested by Mulder et al. (1966) and El-Erian (1969) required one or more amino acids as N-source. Bousfield (1972) using media solidified with agar found that *B. linens* and orange cheese coryneforms of the *B. linens* type were able to utilize ammonium nitrogen. However, a plate method is probably unsuited for testing the utilization of inorganic nitrogen, as normal agar may provide small amounts of organic nitrogen compounds.

The sea-fish coryneform bacteria, except two, require organic nitrogen compounds as N-source. Strains AC 482 and AC 506 are able to utilize inorganic nitrogen compounds in the presence, but AC 506 probably also in the absence of vitamins. These two strains also deviate as to utilization of carbon sources and hydrolysis of gelatin and casein. Mulder et al. (1966) tested 5 strains of this type of coryneform bacteria and found that 4 grew well with glutamic acid as the only N-source in the presence of a vitamin mixture; the fifth strain required casamino acids plus vitamins.

The majority of the sea-fish coryneforms resemble the orange cheese coryneforms.

Seventeen out of 51 grey-white cheese strains were able to utilize NH_4 salts as sole nitrogen source, and 18 other strains of this group required both NH_4 salt and glutamic acid (Mulder et al., 1966). Strains EC 14 and AC 253 differ from the other non-orange cheese coryneforms by their ability for utilizing $(NH_4)_2SO_4$ as N-source in the presence of vitamins (EC 14) or methionine (AC 253) (El-Erian, 1969; Mulder et al., 1966).

It can be concluded that, in general, the cheese and sea-fish coryneforms require organic nitrogen, whereas the soil arthrobacters do not.

Hugh and Leifson test. Each of the groups appears to be rather homogeneous (Table 2). Nearly all of the orange strains produced an alkaline reaction, in contrast to the non-orange cheese strains and the soil arthrobacters which produced an acid reaction, a few excepted. The strains which in this test did not conform with their group also hybridized poorly with their group's reference strain (Crombach, 1974).

Salt tolerance. Table 2 shows a difference between the soil arthrobacters on the one hand and the cheese and sea-fish coryneforms on the other. The orange sea-fish coryneforms are slightly more salt-tolerant than the cheese strains. Strains AC 482 and AC 506 stand apart of the other strains of their group. The somewhat lower salt-tolerance of the non-orange cheese coryneforms in comparison to the orange ones agrees with the results of Mulder et al. (1966).

It should be emphasized that the pH of a medium containing yeast extract, glucose and a high % of added salt, falls by about 0.5 to 0.6 units during sterilization. Mulder et al. (1966) found that 4% salt inhibited the growth of soil

Table 2. Salt tolerance of soil arthrobacters and coryneform bacteria from cheese and sea fish, and the shift in reaction of these bacteria in the Hugh and Leifson medium

Strain number	Growth in the presence of added salt (%)	Hugh and Leifson
Group I		
Soil arthrobacters		
AC 4	3	acid
AC 11	3	acid
AC 16	3	acid
AC 29	3	acid
AC 157	3	acid
AC 1	5	acid
AC 8	3	acid
AC 158	3	acid
AC 166	5	acid
AC 206	5	acid
AC 403	3	acid
AC 405	5	acid
Group II		
Orange cheese coryneforms		
B 3	15	alkaline
B 4	15	alkaline
AC 251	12	alkaline
AC 252	10	alkaline
AC 275	8	alkaline
AC 423	10	alkaline
AC 448	10	alkaline
B 42	15	alkaline
Group III		
Orange sea-fish coryneforms		
AC 470	15	alkaline
AC 471	15	alkaline
AC 472	15	alkaline
AC 473	15	alkaline
AC 474	15	alkaline
AC 478	12	alkaline
AC 480	10	alkaline
AC 481	12	acid
AC 482	5	alkaline
AC 484	15	alkaline
AC 486	15	alkaline
AC 490	12	alkaline
AC 495	10	alkaline
AC 498	12	alkaline
AC 501	12	alkaline
AC 506	5	acid
Group IV		
Non-orange cheese coryneforms		
EC 7	12	alkaline
EC 9	10	acid
EC 10	10	acid
EC 14	10	acid
EC 15	10	acid
EC 16	10	acid
EC 20	12	acid
AC 253	12	alkaline
AC 256	8	acid
AC 261	8	acid
AC 262	10	acid
AC 263	8	acid
AC 278	5	acid

arthrobacters at $pH < 6$. Consequently, the pH of a medium to be used for the determination of salt tolerance should be adjusted before sterilization to about 7.5. In contrast to soil arthrobacters, cheese coryneforms can grow in media with 4% NaCl at considerably lower pH values than when no salt has been added (Mulder et al., 1966).

Gram staining. No significantly different results were obtained with the gener-

al laboratory, and with the modified Hucker method. The majority of the soil arthrobacters were gram-positive; AC 8 was gram-variable and AC 29 gram-negative. These results are in accordance with Bousfield's (1972), but not with those of Mulder and Antheunisse (1963) who found that the majority of the soil arthrobacters were gram-negative or gram-variable. Cheese, and sea-fish coryneforms were gram-positive except EC 20, AC 253 and AC 490, which were gram-variable. A comparison of rods and cocci, carried out with sea-fish coryneforms, showed no differences in retention of the Gram stain.

More recent investigations by J. Antheunisse have revealed that the results of the Gram staining with soil arthrobacters, in contrast to cheese coryneforms, depend strongly on the way the cells are decolorized.

Oxidase and catalase. The great majority of the soil arthrobacters and the cheese, and sea-fish coryneforms were oxidase-negative. One soil arthrobacter (AC 206), two orange cheese coryneforms (AC 251 and AC 423) and four orange sea-fish coryneforms (AC 478, AC 486, AC 490 and AC 498) were oxidase-positive. This agrees with Steel's (1961) finding that, with few exceptions, gram-positive organisms are oxidase-negative.

All the coryneform strains tested were catalase-positive.

From the present study it can be concluded that strains deviating in one test often also deviate in others. The results of the morphological and physiological studies generally corroborate the results of DNA analysis (Crombach, 1972) and DNA – DNA hybridization experiments (Crombach, 1974).

The author thanks Professor Dr. E. G. Mulder for his advice and criticism during the preparation of the manuscript and Mr. J. Antheunisse for his helpful discussions. The critical reading of the manuscript by Dr. J. L. M. Huntjens and Dr. L. P. T. M. Zevenhuizen is gratefully acknowledged.

Received 25 *March* 1974

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8 Summary and general discussion

8.1 Results of studies at the DNA level and on morphology and physiology

The soil and water arthrobacters, orange cheese coryneforms, orange sea-fish coryneforms and the non-orange cheese coryneforms exhibit more or less clearly the typical pleomorphic arthrobacter morphology: the cells changing from rods into cocci on ageing and developing again to rods after transfer to an appropriate medium. Mulder & Antheunisse (1963) and Mulder et al. (1966) indicated some distinct, mainly physiological, differences among the strains of the different groups.

This study deals with the results of investigations at the DNA level (DNA base composition and DNA-DNA hybridization) and on the homogeneity of the groups with regard to morphological and physiological features.

A comparative study with both native and deep-frozen DNA has shown that freezing at −70 and storing at −21°C for half a year or even one year affect neither thermal denaturation nor hybridization (Tables 4.1, 4.3). The same holds for the molecular weights of the DNA fragments (Table 4.2). With reference to these results some experiments at the DNA level were carried out with deep-frozen DNA, for practical reasons.

When evaluating the hybridization data it should be taken into account that mismatching and unpairing, which may occur in hybrids, reduce the thermal stability of the duplexes. The thermal stability of hybrids obtained with the renaturation-rate method is mainly reduced by mismatching as unpairing is probably present to a minor extent (see Chapter 5).

A comparison of the thermal denaturation of native and renatured DNA revealed that during the initial renaturation phase predominantly stable homologous duplexes were formed and mismatching in heterologous hybrids of closely related DNAs was restricted to a few %. The mismatching in heterologous hybrids of moderately related DNAs was estimated to be restricted to at most 10%.

The suggestion of Brenner & Cowie (1968) that the decreased thermal stability of heterologous duplexes is mainly attributable to a relatively high % AT base pairs in the related nucleotide sequences is unlikely. It also disagrees with D. J. Brenner's unpublished observation (Kingsbury et al., 1969) that no increased interspecies binding between *Escherichia coli* and *Salmonella typhimurium* occurred when *E.coli* DNA fragments rich in A and T were used. De Ley et al. (1973) also demonstrated that preferential hybridization of sequences rich in A and T is very unlikely. These authors prepared homologous *Agrobacterium tumefaciens* hybrids by hybridizing the total denatured DNA with fractionated denatured fragments which were obtained by a preliminary reassociation on membrane filters, followed by releasing the reassociated fragments at high temperature. These homologous hybrids showed exactly the same T_{m_e} value (elution midpoint; see Chapter 5) as that of the original total homologous hybrids.

The narrow range covered by the GC contents of the soil arthrobacters (65.3 – 67.0%, Table 3.2) suggests that this group is homogeneous. However, hybridization experiments revealed two subgroups (Table 6.1). This division closely follows that based on physiological grounds, viz. the *Arthrobacter simplex* and the *Arthrobacter globiformis* types (Table 7.1). DNA of the strains of the former subgroup hybridized to a low degree with that of the type culture *A.globiformis* ATCC 8010 (D⩽30%), whereas DNA of the strains of the latter subgroup except one strain, exhibited a fair hybridization with that of the type culture (D⩾51%). The eccentric position of the deviating strain in its subgroup is also demonstrated by its aberrant genome size (Table 6.1).

The narrow % GC range (63.2 – 63.8, Table 3.2) of the orange cheese coryneforms of the *Brevibacterium linens* type, one strain excepted, and the high degree of hybridization between these strains and the type culture *B.linens* ATCC 9175 (D⩾74%, Table 6.2) clearly show the close genetic relationship among these strains, which is in agreement with their physiological similarity (Table 7.1). The deviation of strain B3 from its group with regard to % GC values and hybridization is also reflected in its aberrant physiological features (see Chapters 3, 6 and 7).

The similarity in GC contents as well as in morphological appearance between the majority of the orange sea-fish coryneforms and the cheese strains of the *B.linens* type (Tables 3.2, 6.3) suggests that both groups of orange strains are closely related. However, the results of hybridization experiments in addition to the distinct difference in some physiologival characteristics (Tables 6.3, 7.1) showed that only the minority of the orange sea-fish coryneforms are closely related to the cheese strains of the *B.linens* type (D⩾77%); consequently, the group is a heterogeneous one.

There was a good correlation between the results of the hybridization experiments with sea-fish strains and those of the physiological studies. All the strains whose DNA hybridized very well with that of the type culture *B.linens* ATCC 9175 showed similar physiological characteristics, the reverse is also true except for two strains that are somewhat aberrant of the other strains of the group in either % GC or in genome size (Tables 6.3, 7.1). DNAs of those strains that can hydrolyze casein and utilize citrate, hybridized either moderately or only poorly with that of *B.linens*, which can do neither.

The majority of the non-orange cheese coryneforms, though of different origin, are closely related as concluded from the data of hybridization experiments and from their resemblance in GC values (65.5 – 66.9%) and genome sizes (Tables 3.2, 6.4). The strains possessing DNAs incapable of a significant hybridization with that of the reference strain of the group, differed distinctly from the other strains in % GC values or morphological and physiological features or both (see Chapters 3, 6 and 7).

The poor hybridization between the DNAs of the reference strains in addition to differences in % GC values or in morphological and physiological characters or both, indicates the low degree of relationship between the soil arthrobacters, the orange cheese and sea-fish coryneforms and the non-orange cheese coryneforms (see Chapters 3, 6 and 7).

Those non-orange cheese strains that deviate from the majority in % GC values (Table 3.2) showed under all conditions tested, a much more pronounced tendency to change from rods into cocci on ageing than all other coryneforms tested. In general, at 15°C the soil arthrobacters of the *A.globiformis* type and at 25°C the non-orange cheese coryneforms exhibited the most pronounced tendency to form cocci. The orange cheese

coryneforms changed the most slowly into cocci at both temperatures (see Chapters 3, 6 and 7).

Preliminary hybridization experiments in addition to comparison of the genome sizes, indicated only low degrees of relationships between the soil arthrobacters of the *A.globiformis* type and the non-orange cheese coryneforms on the one hand and various named cultures of the genera *Corynebacterium, Mycobacterium* and *Nocardia* on the other (Tables 6.1, 6.4). A close relationship of the orange cheese and sea-fish coryneforms with the named cultures is unlikely because of the differences in % GC values or genome sizes, or both (Tables 3.2, 6.1, 6.2, 6.3, 6.4).

8.2 Geno-species and geno-genus

De Ley (1968) and Brenner (1973) defined a geno-species as being a group of micro-organisms exhibiting 70% or more nucleotide-sequence relatedness. This definition has been based on the homology encountered in several well delineated species (e.g. several species of the genera *Agrobacterium, Xanthomonas* and of the Enterobacteriaceae). The former author found some evidence of a minimum border-line of about 45% homology among strains belonging to one geno-genus – *Pseudomonas* was used as model. However, Brenner stated that the definitive level of relatedness for a geno-genus cannot yet be established.

The coryneforms isolated from soil were identified from their morphological and physiological characteristics as either *A.simplex* or *A.globiformis*. In contrast to the soil arthrobacters, the orange cheese coryneforms and the minority of the orange sea-fish coryneforms can be considered as a geno-species, viz. *B.linens*. Half of the orange sea-fish coryneforms may belong to the geno-genus *Brevibacterium*, assuming that their mutual homology approximately equals their separate homology with the type culture *B.linens* ATCC 9175 (D⩾46%). More taxonomic information is needed in search of an appropriate genus for the other half of these strains. The majority of the non-orange cheese coryneforms belong to one geno-species which has not yet been described in the literature. The heterogeneity within the coryneform genera makes a justified classification difficult.

8.3 Correlation between results of studies carried out at different levels

The results obtained from studies at the DNA level and those from morphological and physiological investigations were usually in good agreement. Sometimes, however, they did not agree, perhaps partly because of the limited part of the total bacterial genome involved in morphological and physiological studies. A coryneform-bacterium genome with a molecular weight of e.g. 2×10^9 daltons contains about 3×10^6 base pairs and consequently, $2 - 3 \times 10^3$ cistrons, assuming $1 - 1.5 \times 10^3$ base pairs/cistron (one cistron codes for one polypeptide). If an average of 5–10 cistrons per character is assumed, it can be calculated that such a bacterium has a genetic potential for 200 – 600 different characters.

Homologous genes (one gene codes for a function unit) of which the cistrons differ for more than 20% in nucleotide sequences outside that part that codes for the active centre of the enzymes still give rise to similar phenotypic features. But those cistrons will not hybridize at the optimum renaturation temperature (De Ley et al., 1973). Even within

that part of the cistron coding for the active centre of the enzyme some evolutionary nucleotide divergence may occur – synonymous codons – without affecting the amino acid sequence in the peptides. This is in agreement with the supposition of Bradley et al. (1973) that the DNA nucleotide sequence evolves more rapidly than the amino acid sequence in proteins.

It should be stressed that a low degree of hybridization only indicates a remote genetic relatedness and never gives absolute certainty. Therefore, other parameters, e.g. % GC values, morphological, physiological and chemical characteristics should also be included in taxonomic investigations.

Samenvatting

Genetische, morfologische en fysiologische verwantschap tussen corynebacteriën

Het doel van dit onderzoek was het bestuderen van zowel de genetische als de morfologische en fysiologische verwantschap tussen verschillende groepen van corynebacteriën ('coryneform bacteria'), die naar herkomst en kleur ingedeeld werden in: grond-arthrobacters, oranje corynebacteriën van kaas, oranje corynebacteriën geisoleerd van zeevis en viskisten, en niet-oranje corynebacteriën van kaas.

Mulder en Antheunisse (1963) en Mulder c.s. (1966) vonden dat de genoemde groepen corynebacteriën qua morfologie veel met elkaar gemeen hebben. De bacteriën vertonen alle meer of minder uitgesproken de pleomorfe 'arthrobacter-morfologie', hetgeen inhoudt dat de cellen een groeicyclus doorlopen. De staafvorm gaat bij het ouder worden van de cultuur over in de cocvorm. Deze coccen kiemen en groeien weer uit tot staven nadat zij in een vers medium zijn overgeënt. Binnen deze groepen van corynebacteriën bestaan gradaties in pleomorfie. Bovengenoemde auteurs hebben echter wel een aantal duidelijke fysiologische verschillen aangetoond tussen de stammen van de verschillende groepen: de onderzochte grond-arthrobacters, in tegenstelling tot de kaas- en visstammen, zijn ten aanzien van de voeding weinig eisend en kunnen anorganische stikstof als N-bron gebruiken; de oranje stammen zijn op hun beurt weer meer eisend dan de niet-oranje kaasstammen.

Bij vergelijkende morfologische en fysiologische studies is maar een klein gedeelte van het totale bacterie-genoom betrokken, wat tot verkeerde conclusies ten aanzien van de evolutionaire verwantschap van de betreffende bacteriën kan leiden. De studie op DNA-niveau komt aan dit nadeel tegemoet. Met name de DNA basesamenstelling heeft betrekking op het hele bacterie-chromosoom, en is bovendien een constant gegeven van een organisme. In dit onderzoek werd de DNA basesamenstelling van de bacteriën berekend aan de hand van de smeltcurve van het DNA en uitgedrukt als % GC (moleculair % guanine plus cytosine berekend op de totale hoeveelheid basen van het DNA). Naast de basesamenstelling wordt het bacteriechromosoom verder gekarakteriseerd door de volgorde van de nucleotiden in het DNA en door het moleculairgewicht van het DNA. De overeenkomst in volgorde der nucleotiden van twee verschillende DNAs werd bepaald door middel van hybridisatie-experimenten, die berusten op de spectrofotometrische bepaling van de renaturatiesnelheid van gedenatureerde DNA-fragmenten. De bepaling van het moleculairgewicht van het bacterie-genoom werd uitgevoerd met de methode die in essentie gelijk is aan de laatstgenoemde.

Een vergelijkend onderzoek met diep-gevroren DNA en DNA bewaard als geconcentreerde zoutoplossing bij 4°C, toonde aan dat het invriezen van gezuiverd DNA bij −70°C en bewaren bij −21°C gedurende tenminste een half jaar geen duidelijk effect heeft op de geschiktheid van het DNA voor thermische denaturatie en hybridisatie-experimenten. Ook heeft deze bewerking geen invloed op de lengte van de DNA fragmenten (hoofdstuk 4).

Deze resultaten maakten het mogelijk dat, om praktische redenen, enige experimenten werden uitgevoerd met DNA dat gedurende een bepaalde tijd bewaard was bij −21°C.

In hoofdstuk 5 is een onderzoek naar de stabiliteit van hybriden (DNA-duplexen gevormd uit gedenatureerde DNA fragmenten) beschreven. Bij de interpretatie van hybridisatiegegevens moet men namelijk rekening houden met 'mismatching' (koppelen van niet-complementaire basen) en 'unpairing' (het niet koppelen van basen). Deze verschijnselen verlagen de thermische stabiliteit (smeltpunt) van de hybriden. Uit dit onderzoek is gebleken dat 'unpairing' waarschijnlijk maar in geringe mate voorkomt in hybriden die gevormd worden tijdens de beginfase van de renaturatie. De verlaging van de thermische stabiliteit moet dus hoofdzakelijk worden toegeschreven aan 'mismatching'. In de beginfase van de renaturatie bleef de 'mismatching' in DNA-duplexen van nauw verwante bacteriën beperkt tot enkele procenten. In hybriden van matig verwante stammen bleef de 'mismatching' beperkt tot ten hoogste 10%.

In de hoofdstukken 3 en 6 is de DNA-basesamenstelling van de onderzochte bacteriestammen vermeld. De nauwe grenzen waarbinnen de GC-waarden van de grond-arthrobacters variëren (65,3 – 67,0%, tabel 3.2) doet vermoeden dat deze groep homogeen is. Maar de gegevens van hybridisatie-experimenten, beschreven in hoofdstuk 6, tonen aan dat de onderzochte grond-arthrobacters verdeeld kunnen worden in twee subgroepen. Deze indeling komt nauw overeen met die welke gebaseerd is op fysiologische eigenschappen: de soorten *Arthrobacter simplex* en *Arthrobacter globiformis*. Deze twee subgroepen vertonen maar een geringe genetische verwantschap blijkens de resultaten van de hybridisatie-experimenten.

De oranje kaasstammen, uitgezonderd één stam, vormen een homogene groep, aangezien hun GC-waarden slechts een klein gebied beslaan (63,2 – 63,8%) en hun DNAs goed hybridiseren met het DNA van de 'type culture' *Brevibacterium linens* ATCC 9175 (tabellen 3.2, 6.2). De stammen vertonen verder grote gelijkenis in morfologie en fysiologie (tabel 7.1). Deze stammen behoren tot één genetische soort ('geno-species'), namelijk *B.linens*, omdat zij een overeenkomst in nucleotiden-volgorde vertonen van meer dan 70% (hoofdstukken 6 en 8).

Zowel de morfologie van de oranje corynebacteriën, geïsoleerd van zeevis en viskisten, als de GC-waarden van het grootste gedeelte van deze bacteriën, suggereren een nauwe genetische verwantschap met de oranje kaasbacteriën. De resultaten van de hybridisatie-experimenten en van een vergelijkend fysiologisch onderzoek tonen echter aan dat slechts een klein gedeelte van de oranje visstammen nauw verwant is met de oranje kaasstammen. Deze kleine groep kan ook tot de 'geno-species' *B.linens* gerekend worden. De helft van de oranje visstammen behoort tot het genetisch geslacht ('geno-genus') *Brevibacterium* omdat deze stammen waarschijnlijk een overeenkomst van meer dan 45% in hun nucleotiden-volgorde vertonen (hoofdstukken 6 en 8).

Ondanks het verschil in herkomst bleek het grootste gedeelte van de niet-oranje kaasstammen toch tot één genetische soort te behoren. De DNAs van enkele stammen hybridiseerden slechts in geringe mate met het DNA van de referentiestam. Hun geringe genetische verwantschap met de rest van de groep werd bevestigd door hun duidelijk afwijkende GC-waarden en eveneens afwijkende morfologische en fysiologische eigenschappen (hoofdstukken 3, 6 en 7).

De DNAs van de referentie-stammen van de grond-arthrobacters, de oranje stammen van kaas, vis en viskisten, en van de niet-oranje kaasstammen, hybridiseerden onderling maar zeer beperkt. Bovendien bleken de stammen van deze groepen te verschillen in % GC-waarden of in morfologische en fysiologische eigenschappen of in beide (hoofdstukken 3, 6 en 7). Aan de hand van deze gegevens mag men concluderen dat er tussen de grond-arthrobacters, de oranje stammen van kaas, vis en viskisten en de niet-oranje kaasstammen slechts een geringe verwantschap bestaat.

De gegevens van hybridisatie-experimenten met DNA van de referentie-stammen en DNA van enkele vertegenwoordigers van de geslachten *Corynebacterium, Mycobacterium* en *Nocardia* duidden niet op een nauwe verwantschap tussen de grond-arthrobacters van het *A.globiformis* type en de niet-oranje kaasstammen enerzijds en de vertegenwoordigers van de genoemde geslachten anderzijds (tabellen 6.1, 6.4). Een nauwe verwantschap van de oranje kaas- en visstammen met de stammen van deze genoemde geslachten is onwaarschijnlijk vanwege de verschillen in % GC of in genoom-grootte (tabellen 3.2, 6.1, 6.2, 6.3, 6.4).

Resumerend kan gezegd worden dat de onderzochte grond-arthrobacters tot de twee soorten *A.simplex* en *A.globiformis* behoren. De oranje kaasstammen en een kleine groep van de oranje visstammen behoren tot de genetische soort *B.linens*, terwijl de ene helft van de oranje visstammen kan worden ingedeeld in het genetische geslacht *Brevibacterium*. Verder onderzoek is vereist om de andere helft van de laatste groep, alsmede de niet-oranje kaasstammen op verantwoorde wijze te classificeren. Het grootste gedeelte van de niet-oranje kaasstammen behoort evenwel tot één genetische soort die in de literatuur nog niet is beschreven.

In hoofdstuk 7 zijn resultaten van proeven vermeld betreffende de kleurontwikkeling door de onderzochte corynebacteriën in licht en donker bij aan- of afwezigheid van 4% zout in het groeimedium, de vorm van de cellen en de snelheid waarmee de verschillende stammen de groeicyclus coc-staaf-coc onder verschillende omstandigheden doorlopen. Het bleek dat op een arm agarmedium de coccen sneller kiemden en de staven sneller transformeerden in coccen dan op een rijk medium; hoge temperatuur (25°C ten opzichte van 15°C) bevorderde een snellere voltooiing van de cyclus. De niet-oranje kaasstammen die in % GC duidelijk verschillen van de rest van de groep toonden onder alle onderzochte omstandigheden een snellere overgang van staaf in coc dan alle andere geteste coryne-bacteriën. In het algemeen echter, kan gesteld worden dat bij 15°C de *A.globiformis* stammen en bij 25°C de niet-oranje kaasstammen het snelst transformeerden in coccen; de oranje kaasstammen toonden de geringste tendens om van staaf in coc over te gaan.

In dit hoofdstuk is ook duidelijk aangetoond dat de grond-arthrobacters in de cocvorm fysiologisch actief zijn en dat ze kunnen blijven doorgroeien in deze vorm indien het medium maar een voldoend laag substraatniveau heeft.

In de hier besproken studie is in het algemeen een goede correlatie gevonden tussen de resultaten van het DNA-onderzoek en die van de morfologische en fysiologische proeven. In enkele gevallen echter, geldt dit niet. Een mogelijke verklaring hiervoor wordt gegeven in hoofdstuk 8:

1. bij de gebruikelijke morfologische en fysiologische testen is slechts een klein gedeelte van het totale bacteriechromosoom betrokken,

2. homologe cistronen (een cistron codeert voor een polypeptide) kunnen buiten het gedeelte dat codeert voor het actieve centrum van een enzym, zodanig in nucleotiden-volgorde verschillen dat ze niet meer hybridiseren.
3. de nucleotiden-volgorde zou sneller evolueren dan de aminozuur-volgorde in de peptiden (Bradley et al., 1973).

Tenslotte, het classificeren van nieuw geïsoleerde specifieke groepen van coryne-bacteriën ('coryneform bacteria') wordt bemoeilijkt doordat de bestaande geslachten *Corynebacterium*, *Arthrobacter*, *Brevibacterium*, *Mycobacterium* en *Nocardia* niet scherp zijn afgebakend en vaak in elkaar overlopen.

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