INSTITUT FÜR MIKROBIOLOGIE
FORSCHUNGSZENTRUM FÜR MILCH UND LEBENSMITTEL WEIHENSTEPHAN
TECHNISCHE UNIVERSITÄT MÜNCHEN

FT-IR spectroscopic identification and infraspecific diversity of coryneform bacteria in relation to 16S rDNA sequence analysis

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Vollständiger Abdruck der von der Fakultät
Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt
der Technischen Universität München
zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften
(Dr. rer. nat.)

genehmigten Dissertation.

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	2.	Univ.-Prof. Dr. K.-H. Schleifer

Die Dissertation wurde am 29.01.2001 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 23.02.2001 angenommen.

Preface

The main chapters 2 to 4 of this thesis are published, accepted for publication or submitted. Each main chapter has its own introduction which may overlap to a certain extent since each is part of a separate publication. Additionally, a general introduction was added describing the current taxonomy and habitats of coryneform bacteria, explaining their role in the ripening process of smeared cheeses and introducing FT-IR spectroscopy in microbial analysis (chapter 1). A general discussion and general conclusions have been included as well (chapters 5 and 6). The appendix lists the strains comprising the spectral reference library which forms the basis of the research reported in chapters 2 and 3.

Chapter 2, the identification of coryneform bacteria and related taxa by FT-IR spectroscopy, constitutes the main part of my research. It was written by Helene Oberreuter, Herbert Seiler and Siegfried Scherer (2001) and has been accepted for publication in *International Journal of Systematic and Evolutionary Microbiology*. I performed or supervised the experimental work and and wrote the major part of the publication.

Chapter 3 deals with the infraspecific diversity of three coryneform species. It has been submitted by Helene Oberreuter, Joachim Charzinski and Siegfried Scherer (2001). This chapter represents a detailed investigation of aspects that arose out of the work presented in chapter 2. Again, the experimental work was performed or supervised by myself. Joachim Charzinski provided computer programs used for the statistical distribution analyses. I wrote the main part of the publication.

Chapter 4 has been published as "Quantification of microorganisms in binary mixed populations by Fourier transform infrared (FT-IR) spectroscopy" by Helene Oberreuter, Frank Mertens, Herbert Seiler and Siegfried Scherer (2000) in *Letters in Applied Microbiology* 30, pp. 85-89. The work presented in this chapter originated from a separate project. The experimental work was carried out by myself while Frank Mertens contributed the Principle Component Analyses. The major part of the publication was written by myself.

Summary

Fourier transform infrared (FT-IR) spectroscopy is not only an efficient method for the quantitative and qualitative analysis of chemical substances but has also been introduced as a tool for classification and identification of microorganisms. In this thesis, the application of FT-IR spectroscopy to microbial analysis is illuminated under three aspects: (i) establishment and validation of a spectral database for the identification of coryneform bacteria and related taxa, (ii) assessment of the infraspecific diversity of three coryneform species, and (iii) the quantitative analysis of mixed microbial populations.

(i) In order to allow the rapid, simple and cost-efficient identification of coryneform bacteria and related taxa from different habitats, an extensive FT-IR reference database was established, comprising bacteria from the two suborders *Micrococcineae* and *Corynebacterineae* (*Actinomycetales, Actinobacteria*) as well as other morphologically similar genera. The spectral library consists of averaged infrared spectra from 730 reference strains, covering 220 different species out of 46 genera. 192 species are represented by type strains. The identity of 352 reference strains was determined by comparative 16S rDNA sequence analysis and, if necessary, strains were reclassified accordingly. FT-IR frequency ranges, weights and reproducibility levels were optimized for this section of high G+C gram positive bacteria. In an internal validation, 98.1 % of the strains were correctly identified at the species level. An external validation which was carried out using 544 strains from 54 species out of 16 genera resulted in a correct identification of 87.3 % at the species level and 95.4 % at the genus level. The performance of this identification system is well within the range of those having been reported in the literature for the identification of coryneform bacteria by phenotypical methods. Apparently, coryneform bacteria and related taxa display a certain degree of overlap between different taxonomical markers, leading to a limited differentiation capacity of non-genotypical identification methods in general. However, easy handling, rapid identification within 25 h starting from a single colony, a satisfactory differentiation capacity and low cost render FT-IR technology clearly superior over other routine methods for the identification of coryneform bacteria and related taxa.

(ii) The infraspecific diversity of 23 strains of *Brevibacterium linens*, 25 strains of *Corynebacterium glutamicum* and 26 strains of *Rhodococcus erythropolis* was determined by pairwise partial 16S rDNA sequence analysis and FT-IR spectroscopy in parallel. The infraspecific comparison of FT-IR spectral distances suggested that *R. erythropolis* and *C. glutamicum* strains are less diverse than *B. linens*, whereas at the level of pairwise 16S rDNA

similarity, *R. erythropolis* strains had a very low diversity, *B. linens* displayed a high variability and *C. glutamicum* might have an intermediate position. Therefore, no correlation of FT-IR spectral similarity and 16S rDNA sequence similarity below the species level was observed, i.e. strains belonging to the same species which have only a low spectral distance between each other might not display an equivalent sequence similarity in their 16S rDNA and vice versa. Hence, diversification of 16S rDNA sequences and microevolutionary change of the cellular components detected by FT-IR spectroscopy appear to be de-coupled. In total, 27 strains derived from international culture collections carried invalidly described or wrong species denotations and had to be reclassified in accordance with polyphasic taxonomical analyses. It was found that FT-IR spectroscopy is a rapid and reliable method to screen for similar isolates.

(iii) Furthermore, FT-IR spectroscopy was used for the first time to determine the ratios of different microorganisms in mixtures. Exemplarily, systems composed of two food-associated yeast species (*Saccharomyces cerevisiae* / *Hanseniaspora uvarum*) and two yoghurt lactic acid bacteria (*Lactobacillus acidophilus* / *Streptococcus salivarius* ssp. *thermophilus*) were investigated. Determination of the cell number ratio in the lactic acid bacteria system was possible with a minimal prediction accuracy of ± 16 ratio percentage points while the minimum accuracy of prediction in the yeast two-component system was ± 4 % (both at a 95 % confidence level). These results show that FT-IR spectroscopy is potentially a rapid method for the quantification of cell ratios in mixtures of two different microorganisms, provided that the cell ratio does not drop below a certain, system-specific threshold.

Zusammenfassung

Fourier-Transformations-Infrarot (FT-IR) - Spektroskopie stellt nicht nur eine bewährte Methode zur quantiativen und qualitativen Analyse chemischer Verbindungen dar, sondern ist auch bereits als Verfahren zur Klassifizierung und Identifizierung von Mikroorganismen eingeführt worden. In dieser Dissertation wird die Anwendung der FT-IR-Spektroskopie in der mikrobiellen Analyse unter drei Aspekten betrachtet: (i) Aufbau und Validierung einer Spektrendatenbank zur Identifizierung coryneformer Bakterien und verwandter Taxa, (ii) Einschätzung der infraspezifischen Diversität dreier coryneformer Arten und (iii) quantitative Analyse gemischter mikrobieller Populationen.

(i) Um eine schnelle, einfache und kostengünstige Identifizierung coryneformer Bakterien und verwandter Taxa aus verschiedenen Habitaten zu ermöglichen, wurde eine umfassende FT-IR-Referenzdatenbank aufgebaut, die Bakterien der beiden Unterordnungen *Micrococcineae* und *Corynebacterineae* (*Actinomycetales, Actinobacteria*) zusammen mit anderen morphologisch ähnlichen Gattungen umfasst. Die Spektrenbibliothek besteht aus Mittelwertspektren von 730 Referenzstämmen aus 220 verschiedenen Arten und 46 Gattungen. 192 Arten sind durch Typstämme vertreten. Die Identität von 352 Referenzstämmen wurde über vergleichende 16S rDNA Sequenzanalyse bestimmt und die Stämme, falls nötig, entsprechend reklassifiziert. Die für die Identifizierung dieser G+C-reichen Gram-positiven Bakterien relevanten FT-IR-Parameter wie Wellenzahlbereiche, Gewichtungen und Reproduktionsniveaus wurden ermittelt. In einer internen Validierung wurden 98.1 % der Stämme auf Speziesebene korrekt identifiziert. Desweiteren wurde eine externe Validierung mit 544 Stämmen aus 54 Arten und 16 Gattungen durchgeführt, die in 87.3 % der Fälle eine korrekte Identifizierung auf Artebene und in 95.4 % eine richtige Identifizierung auf Gattungsebene ergab. Die Leistungsfähigkeit dieses Identifizierungsverfahrens liegt damit genau im Rahmen der in der Literatur angegebenen Leistung von Identifizierungmethoden für coryneforme Bakterien anhand phänotypischer Merkmale. Anscheinend weisen coryneforme Bakterien und verwandte Taxa einen bestimmten Grad an Überlappung verschiedener taxonomischer Charakteristika auf, die insgesamt zu einer eingeschränkten Differenzierungsfähigkeit bei nicht-genotypischen Methoden führen. Dennoch machen Eigenschaften wie eine einfache Handhabung zusammen mit einer schnellen Identifizierung innerhalb von 25 h (ausgehend von einer Einzelkolonie) bei einer guten Differenzierungsfähigkeit und geringen Kosten das FT-IR-Verfahren anderen

Routinemethoden zur Identifizierung coryneformer Bakterien und verwandter Taxa deutlich überlegen.

(ii) Die infraspezifische Diversität von 23 *Brevibacterium linens*-, 25 *Corynebacterium glutamicum*- und 26 *Rhodococcus erythropolis*-Stämmen wurde im Vergleich paarweise betrachteter partieller 16S rDNA Sequenzanalyse sowie FT-IR-Spektroskopie ermittelt. Der Vergleich der infraspezifischen spektralen Distanzen deutete darauf hin, dass die Spezies *R. erythropolis* und *C. glutamicum* weniger divers sind als *B. linens.* Dagegen wiesen in Bezug auf die Ähnlichkeit innerhalb der 16S rDNA *R. erthropolis*-Stämme eine geringe und *B. linens*-Stämme eine hohe Diversität auf; *C. glutamicum*-Stämme nahmen eine Mittelstellung ein. Eine Korrelation zwischen FT-IR-Spektralähnlichkeit und 16S rDNA Sequenzähnlichkeit wurde also nicht beobachtet, d.h., Stämme derselben Art, die untereinander eine hohe spektrale Ähnlichkeit aufweisen, ähneln sich nicht unbedingt in gleicher Weise in ihren 16S rDNA Gensequenzen und umgekehrt. Die Diversifizierung der ribosomalen 16S Gensequenzen und die durch die FT-IR-Spektroskopie detektierten mikroevolutiven Veränderungen der Zellkomponenten sind anscheinend nicht miteinander gekoppelt. Insgesamt trugen 27 Stämme aus internationalen Stammsammlungen nicht gültig beschriebene oder falsche Artbezeichnungen und mussten in Übereinstimmung mit polyphasisch-taxonomischen Untersuchungen reklassifiziert werden. Es wurde festgestellt, dass die FT-IR-Spektroskopie eine schnelle und zuverlässige Methode zum Screenen ähnlicher Isolate darstellt.

(iii) Desweiteren wurde die FT-IR-Spektroskopie zum ersten Mal verwendet, um die Mengenverhältnisse zwischen verschiedenen in Mischungen vorliegenden Mikroorganismen zu ermitteln. Als Beispiele wurden zwei Mischungssysteme untersucht, von denen eines aus zwei in Lebensmitteln vorkommenden Hefearten (*Saccharomyces cerevisiae* / *Hanseniaspora uvarum*) und das andere aus zwei Joghurtmilchsäurebakterien (*Lactobacillus acidophilus* / *Streptococcus salivarius* ssp. *thermophilus*) bestand. Die Ermittlung des Zellzahlverhältnisses im Milchsäurebakteriensystem war mit einer minimalen Vorhersagegenauigkeit von ± 16 Prozentpunkten möglich, wohingegen die minimale Vorhersagegenauigkeit im Hefe-Zweikomponentensystem ± 4 % betrug (jeweils im 95 % Vertrauensintervall). Die Ergebnisse zeigen, dass die FT-IR-Spektroskopie im Prinzip eine schnelle Methode zur Quantifizierung von Zellzahlverhältnissen in mikrobiellen Zweikomponentenmischungen darstellen kann, sofern die Voraussetzung gegeben ist, dass die Zellzahlverhältnisse nicht unter einen systemspezifischen Grenzwert fallen.

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1 General Introduction

1.1 Taxonomy of the coryneform bacteria

The term "coryneform bacteria" was introduced into the general literature by Jensen (1952) and at that time meant only those bacteria which had then been classified to belong to the genus *Corynebacterium.* This genus was defined mainly on the basis of morphological characteristics and staining reactions, features which were considered very characteristic of these organisms. Due to the importance attached to morphology at that time, it was believed that the coryneform bacteria were highly related (Keddie, 1978). In time, the concept was expanded to include other genera as well and in 1974, Bergey's Manual of Determinative Bacteriology (Buchanan *et al.,* 1974) listed the genera *Corynebacterium, Arthrobacter, Brevibacterium, Microbacterium, Cellulomonas* and *Kurthia* as comprising the "coryneform group of bacteria". However, with the growing influence of more sophisticated taxonomical methods and an increasing awareness of these bacteria's phylogenetic diversity, the term was eventually abandoned by taxonomists and dropped in the subsequent Bergey's Manual of Systematic Bacteriology of 1986 (Sneath *et al.,* 1986). Nevertheless, to simplify matters while not intending to imply close phylogenetic relationships between the genera, this handy term is still used by clinical and food microbiologists. It comprises aerobically growing, asporogenous, non-partially acid fast, irregular shaped Gram-positive rods with a high G+C content in their DNA (Funke *et al.,* 1997b).

Coryneform genera are characterized by a rich spectrum of mainly chemotaxonomic properties such as different profiles of fatty acids (Suzuki & Komagata, 1983, Kämpfer & Kroppenstedt, 1996), polar lipids (Minnikin *et al.*, 1976), amino acids of peptidoglycan (Schleifer & Kandler, 1972, Sasaki *et al.*, 1998), teichoic acids (Schleifer & Stackebrandt, 1983, Sutcliffe, 1994), isoprenoid quinones (Collins & Jones, 1981) and polyamines (Altenburger *et al.*, 1997, Busse & Schumann, 1999). Attempts to link phylogenetically neighboring genera into a higher taxon on the basis of either of these patterns or of morphological and physiological traits have often failed, as no common non-molecular denominator can be identified that would circumscribe this taxon (Stackebrandt & Schumann, 2000). The problem of existing "border-line" genera which are difficult to assign satisfactorily to higher taxa has been pointed out as early as in 1976 (Kroppenstedt & Kutzner, 1976). The results of numerical taxonomic identification studies on the basis of mainly physiological characteristics were likewise not unequivocal (Seiler, 1983, Kämpfer *et al.*, 1993).

Throughout the past decade, large areas of bacterial taxonomy have been revised on the grounds of comparative ribosomal RNA gene sequence analyses of mainly the 16S rRNA, confirmed by the 23S rRNA (Woese, 1987, Ludwig & Schleifer, 1994). In 1997, Stackebrandt *et al.* (1997) proposed a new hierarchic classification system for organisms with Gram-positive staining reaction and a DNA G+C content of at least 50 %: the class *Actinobacteria* (Fig 1.1). The delineation is based solely on 16S rDNA/rRNA sequence data. Membership to

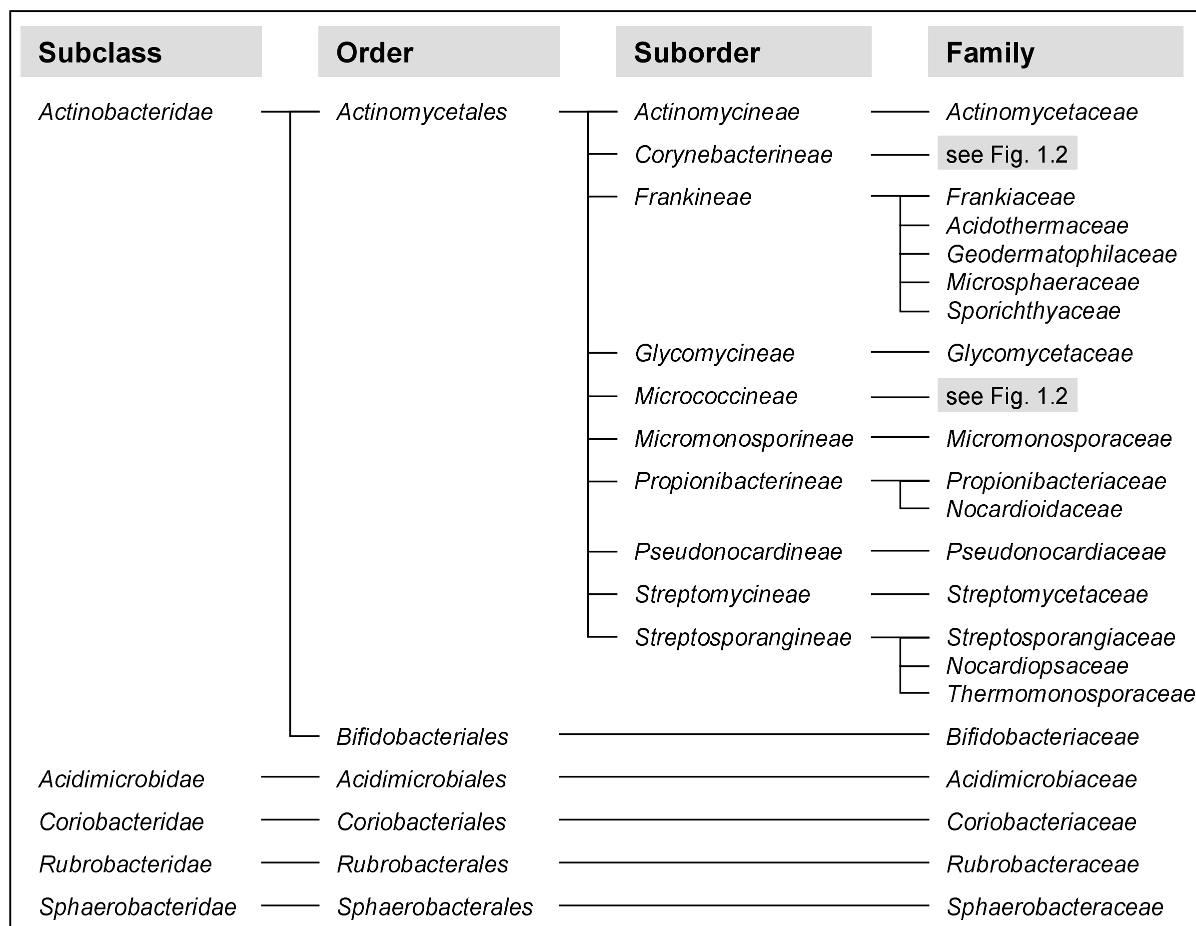

Fig. 1.1: The class *Actinobacteria* according to Stackebrandt *et al.,* 1997.

the class *Actinobacteria* is indicated by 16S rDNA sequence similarity values above 80 % and the presence of signature nucleotides which were chosen for their presence in more than 95 % of the members of the respective taxon. The taxonomy was recently updated to accomodate new taxa of this quickly expanding group of bacteria (Stackebrandt & Schumann, 2000).

Within this scheme, the classical coryneform genera are grouped into the suborders *Micrococcineae* and *Corynebacterineae* (Fig. 1.2). Chapter 2 of this thesis describes the establishment of an FT-IR spectral reference database for the routine identification of coryneform bacteria and related taxa within 25 hours. The genera and species selected for inclusion in this reference library were chosen with the objective to include as many taxa out

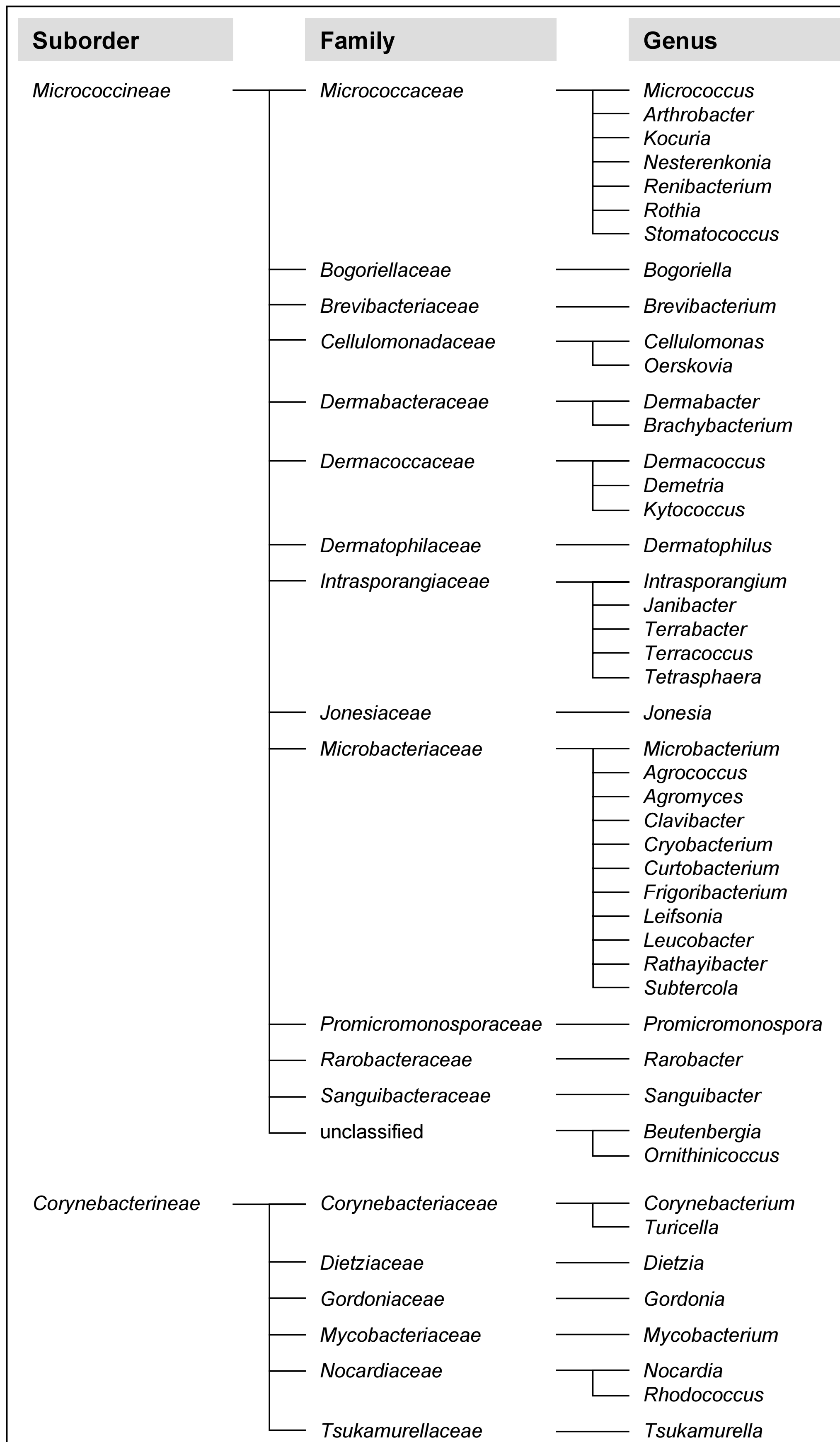

Fig. 1.2: The suborders *Micrococcineae* and *Corynebacterineae* according to Stackebrandt *et al.,* 1997 and Stackebrandt & Schumann, 2000, updated with Groth *et al.,* 1999, Evtushenko *et al.,* 2000, Kämpfer *et al.,* 2000, Männistö *et al.,* 2000, Maszenan *et al.,* 2000.

of this classification system as possible in order to achieve a maximum identification capacity.

1.2 Habitats of coryneform bacteria

Coryneform bacteria and related taxa are widely disseminated in nature. They occur in a broad variety of habitats including soil from different geographical regions, feces, fresh water, sea water, sewage and activated sludge (Liebl, 1994, Stackebrandt & Prauser, 1994). Recently, they have also been isolated from subsurface sediments (Crocker *et al.,* 2000) and antarctic samples (Junge *et al.,* 1998, Tindall *et al.,* 2000). Reasons for this ubiquity are their extreme resistance to drying, starvation or high salt concentrations and their widespread nutritional versatility (Collins, 1994, Hartmans & De Bont, 1994, Jones & Keddie, 1994). In soils, they play important roles as saprophytes in organic decomposition and crumb structure (Goodfellow, 1994, Logan, 1994). Members of the genus *Arthrobacter* have been found to be the most numerous single bacterial group recorded in aerobic plate counts from soil samples (Jones & Keddie, 1994) and *Nocardiae* have been isolated at concentrations of $7 \cdot 10^4$/g dry weight from soil out of tropical and temperate regions (Goodfellow, 1994). Furthermore, coryneform bacteria have been found on plant material where they may dwell as harmless commensals or, as species from the genera *Curtobacterium* and *Clavibacter*, occur as plant pathogens (Collins & Bradbury, 1994). Fish and mammals are common habitats for coryneform bacteria as well (Collins, 1994, Jones & Keddie, 1994). On humans, they play any part from innocuous commensals to opportunistic or true pathogens (for review see Funke *et al.,* 1997b). For instance, the primary habitat of *Micrococci* is mammalian skin: on human skin, these commensals form a proportion of 1-20 % of the total aerobic bacteria isolated (Kocur *et al.,* 1994). On the other hand, some of the most severe human infectious diseases are caused by members of the genera *Corynebacterium* such as *C. diphtheriae* (von Graevenitz & Krech, 1994) or members of the *Mycobacteria* such as *M. tuberculosis* or *M. leprae* (Good, 1994). Identification of these high G+C Gram-positive bacteria is therefore not only of ecological but also of medical relevance.

Finally, coryneform bacteria represent the main proportion of the microflora of bacterial surface ripened cheeses. Since the primary application of the established coryneform reference database is the identification of these bacteria from cheese smears, their role in cheesemaking will be presented in more detail in the following section.

1.3 Coryneform bacteria dominate the surface flora of smeared cheeses

The ripening process of a cheese is one of the most significant factors for its quality development. For a certain group of cheeses, the formation of a viscous, red-orange surface layer ("smear") is essential for the desired organoleptic properties. Several cheeses such as Tilsit, Appenzeller, Gruyère, Bergkäse, Vacherin, Beaufort, Limburger, Romadur, Münster and Weinkäse are often called "red smear cheeses". This term is referring to the significant content of orange-colored *Brevibacterium linens* bacteria among the microorganisms which thrive on the surface of the cheese during the ripening process. In the course of ripening, first, lactic acid produced by lactic acid bacteria is metabolized by yeasts, which leads to an increase of the pH. Meanwhile, the young cheeses are washed several times with a brine that has been inoculated either with the microflora of ripe cheeses ("old-young smearing") or *Brevibacterium linens* as the sole culture organism (Eliskases-Lechner & Ginzinger, 1995, Valdés-Stauber *et al.* 1997.) When the pH of the curd rises up to pH 6.5-7, the growth of mainly coryneform bacteria is promoted (Reps, 1987). These bacteria are highly salt tolerant up to 8 % or even 15 % NaCl (Eliskases-Lechner & Ginzinger, 1995) and are adapted to these conditions by means of compatible solutes which have been identified, e.g., in the genera *Brevibacterium* and *Corynebacterium* (Bernard *et al.*, 1993, Frings *et al.*, 1993, Peter *et al.*, 1998).

The traditional method of old-young-smearing promotes the establishment and maintenance of a factory-specific "house flora". However, it also bears the risk of transferring unwanted, possibly pathogenic bacteria such as *Listeria monocytogenes, Staphylococcus aureus* or enteropathogenic *Escherichia coli* (Busse, 1989, Eppert *et al.*, 1997, Rudolf & Scherer, 2001). Attempts have been made to reduce this risk by using defined starter cultures instead. However, apart from the experiments using miniature model cheeses reported by Bockelmann *et al.* (1997b), the resulting cheeses were of low quality or showed a prolonged ripening period (Eliskases-Lechner & Ginzinger, 1994, Valdés-Stauber *et al.*, 1997, Carminati *et al.*, 1999).

One of the reasons for these unsatisfactory results is certainly that even though some studies on the identification of the species present on the surface of smeared cheeses have been published (Tab. 1.1), the surface flora is still largely undefined (Valdés-Stauber *et al.*, 1997) and not well understood in its complexity. Consequently, the defined starter cultures applied so far have only consisted of a very limited number of strains. Since FT-IR spectroscopy provides a means for rapid, simple and routine-suitable identification of microorganisms, the establishment of a comprehensive identification database for coryneform

bacteria from all kinds of habitats including smeared cheeses would enable researchers and dairy companies alike to identifiy at once hundreds of bacterial isolates from cheeses or to monitor their house-flora, respectively. More thorough knowledge about the composition of the smear flora at different stages of ripening would allow the design of a more complex surface starter culture which could both ensure proper ripening and, at the same time, reduce the risk of transferring pathogenic microorganisms.

Tab. 1.1: Actinobacterial and staphylococcal species isolated from smeared cheeses. **References:** **A** Bockelmann *et al.,* 1997a, **B** Carnio *et al.,* 1999, **C** Carnio *et al.,* 2000, **D** Eliskases-Lechner & Ginzinger, 1995, **E** Irlinger *et al.,* 1997, **F** Schubert *et al.,* 1996, **G** Seiler, 1986, **H** Valdés-Stauber *et al.,* 1991, **I** Valdés-Stauber *et al.,* 1997. **Notes:** Species marked by (*) are classified as risk group 2 organisms. References B, D, G, H and I have used the identification database by Seiler, 1983.

Genus	Species	References
Agromyces	*mediolanus*	A
Arthrobacter	*aurescens*	B
	citreus	B, D, H, I
	crystallopoites	B
	globiformis	A, B, D
	ilicis	B
	nicotianae	B, G, H, I
	protophormiae	B
	spp.	A, B, D, G, H, I
Brachybacterium	*alimentarium*	F
	tyrofermentans	F
Brevibacterium	*casei*	B
"Brevibacterium	*helvolum"*	D
	iodinum	B
	linens	A, B, C, D, G, H, I
	spp.	D, H
Cellulomonas	*cellulans*	H, I
Clavibacter	*michiganense*	D, H
Corynebacterium	*ammoniagenes*	B, D, G, I
	bovis (*)	B
	callunae	B
	flavescens	B
	glutamicum	B
	pseudodipheriticum (*)	B
	renale (*)	B
	striatum (*)	B
	variabilis	B, D, G, I
	xerosis (*)	A, B
	spp.	B, D, G, H, I
Kocuria	*varians*	B
Leifsonia	*aquatica*	B
Curtobacterium	*flaccumfaciens*	D
Microbacterium	*imperiale*	D, H, I
	lacticum	B
	oxidans	D, I
	schleiferi	B
	spp.	B, I
Micrococcus	*luteus*	A, B
Mycobacterium	*celatum* (*)	A
	fortuitum (*)	A
	nonchromogenicum	A
	spp.	A, H
Propionibacterium	spp.	A
Rhodococcus	*fascians*	C, H, I
	spp.	G
Staphylococcus	*capitis*	E
	cohnii	E
	equorum	A, C, E
	lentus	E
	saprophyticus (*)	A, E
	sciuri	A, E
	vitulinus	E
	xylosus	E

1.4 FT-IR spectroscopy of microorganisms

The use of infrared spectroscopy to differentiate and identify bacteria was reported as early as in the 1950's (e.g. Thomas & Greenstreet, 1954, Riddle *et al.*, 1956, Kenner *et al.* 1958). It was demonstrated that (i) infrared spectra of intact bacteria may reproducibly be measured provided that growing conditions, sample preparation, and instrumental parameters are controlled rigidly and that (ii) intact bacteria exhibit infrared spectra that are unique for individual strains. However, the necessity of a detailed evaluation of the complex spectral data obtained prevented the method to be used for routine analyses at that time. Naumann and coworkers reintroduced the technique in the 1980's and developped the basic instrumental and sample parameters for today's microbial analyses by FT-IR (Helm *et al.* 1991b, Naumann *et al.*, 1991b). By this method, unknown microorganisms can be identified very easily and quickly once an extensive database (spectral reference library) is available. For identification, the infrared spectrum of an unknown isolate is compared with all spectra present in the reference library and matched to the library strain whose spectrum is most similar. The advantages of this technique clearly lie in its high discriminatory power down to species or even strain level together with simple applicatory steps without the need for extensive user training, furthermore rapidity, low per-sample effort and low per-sample costs. These features make the method suitable to be used for routine identification in both industrial, clinical or food laboratories as well as research institutes.

Infrared spectroscopy takes advantage of the fact that the atoms of a molecule can be envisioned to perpetually oscillate about their equilibrium position. The frequency of this oscillation depends on their mass, the type and nature of the chemical bonds as well as other effects such as hydrogen bonds, hydrophobic and electrostatic interactions. Furthermore, molecular bonds can be excited to vibration via the absorption of energy quanta of impinging infrared radiation (Günzler & Heise, 1996). The infrared spectrum of a sample shows the attenuation of the infrared light due to absorption as a function of the wavenumber, which is the reciprocal of the wavelength (example in Fig. 4.1). In FT-IR spectroscopy, the unit wavenumber is commonly used instead of the wavelength since the former is directly proportional to the radiation energy. The absorption spectrum is computed by a Fourier transform from an interferogram originally collected by the measurement equipment. Infrared radiation is divided into the near, mid and far infrared region. The mid-infrared region covers wavelengths 4000-500 cm^{-1} and is used in this study. An extensive overview over the basics of the technique with special emphasis on its application in microbial analyses can be found in Kümmerle (1999).

FT-IR spectra of microorganisms show broad and complex contours rather than distinct peaks. Since intact cells are tested, the spectra represent complex images of the total chemical composition (proteins, membranes, cell wall, nucleic acids etc) of the cell. Owing to the multitude of cellular compounds, broad and superimposed absorbance bands are observed throughout the entire spectral range (Helm *et al.* 1991b) and most of the structural information is hidden beneath the shape of the spectrum. Some of the bands can be assigned to distinct functional groups or chemical substructures such as poly-*β*-hydroxybutyric acid granules or endospores by a marker band for dipicolinic acid (Helm & Naumann, 1995) but the information content of the spectra is not yet fully understood as a whole. Naumann *et al.* (1991b) identified five spectral frequency ranges which contain significant spectral information. Determination of the selection of frequency ranges relevant for the differentiation of the group of microorganisms under study is an important task in the course of the establishment of a validated reference library (Helm *et al.* 1991a) and has been carried out in this study as well (see Chapter 2).

The application of FT-IR spectroscopy for identification and classification of microorganisms has been reported for some species of the genera *Bacillus* (Beattie *et al.* 1998, Lin *et al.* 1998), *Lactobacillus* (Curk *et al.* 1994), *Listeria* (Holt *et al.* 1995, Lefier *et al.* 1997), *Streptococcus* (Goodacre *et al.* 1996), and for urinary tract infection bacteria (Goodacre *et al.* 1998). However, these studies have dealt with only a limited number of organisms. A comprehensive validated FT-IR library for the identification of fermentative yeasts has been established at this institute which was demonstrated to be well suited for the routine identification of a wide variety of yeast isolates from different habitats (Kümmerle *et al.*, 1998, Kümmerle, 1999). Additionally, FT-IR spectroscopy is a valuable tool for rapid screening of environmental isolates (Tindall *et al.*, 2000). Two studies have dealt with actinomycetes previously. Haag *et al.* (1996) have placed their emphasis on strains from the families *Thermomonosporaceae, Streptosporangiaceae, Micromonosporaceae* and *Streptomycetaceae* while including a few strains from different families from the suborder *Corynebacterineae* as well. Klatte (1995) has studied numerous species from the two genera *Corynebacterium* and *Rhodococcus* in detail. However, these investigations have not resulted in comprehensive databases and did not allow to conclude on the capacity of this method to identify unknown strains. Therefore, it remained to be demonstrated that this simple and cost-efficient method is also suitable for the identification of a broad range of coryneform bacteria.

2 Identification of coryneform bacteria and related taxa by Fourier-transform infrared (FT-IR) spectroscopy

2.1 Abstract

An extensive Fourier-transformed infrared (FT-IR) spectroscopy database for the identification of bacteria from the two suborders *Micrococcineae* and *Corynebacterineae* (*Actinomycetales, Actinobacteria*) as well as other morphologically similar genera was established. The database consists of averaged infrared spectra from 730 reference strains, covering 220 different species out of 46 genera. 192 species are represented by type strains. The identity of 352 reference strains was determined by comparative 16S rDNA sequence analysis and, if necessary, strains were reclassified accordingly. FT-IR frequency ranges, weights and reproducibility levels were optimized for this section of high G+C gram positive bacteria. In an internal validation, 98.1 % of the strains were correctly identified at the species level. An external validation which was carried out using 544 strains from 54 species out of 16 genera resulted in a correct identification of 87.3 % at the species level and 95.4 % at the genus level. The performance of this identification system is well within the range of those having been reported in the literature for the identification of coryneform bacteria by phenotypical methods. Apparently, coryneform and related taxa display a certain degree of overlap between different taxonomical markers, leading to a limited differentiation capacity of non-genotypical identification methods in general. However, easy handling, rapid identification within 25 h starting from a single colony, a satisfactory differentiation capacity and low cost render FT-IR technology clearly superior over other routine methods for the identification of coryneform bacteria and related taxa.

2.1 Introduction

Coryneform bacteria and related taxa occur almost everywhere on living and non-living matter in the environment, in soil, on cellulosic plant fibres (Lednická *et al.,* 2000), on mammals, on smeared cheeses (e.g. Bockelmann *et al.,* 1997a, Carnio *et al.*, 1999, Kollöffel *et al.,* 1997, Seiler 1986, Schubert *et al.,* 1996, Valdés-Stauber *et al.*, 1997), even in subsurface sediments (Crocker *et al.,* 2000), and in antarctic samples (Junge *et al.,* 1998, Tindall *et al.,* 2000). Several strains or species are classified as opportunistic or obligate human pathogens (for review see Funke *et al.,* 1997b) as well as animal and plant pathogenic

species (Holt *et al.*, 1994). Identification of these high GC gram positive bacteria is therefore not only of ecological and technological, but also of medical relevance.

A variety of methods have been applied to classify coryneform bacteria and related genera such as partial analysis of 16S rRNA sequences by temperature-gradient-gel-electrophoresis (TGGE) (Felske *et al.,* 1999), analysis of polyamine patterns (Altenburger *et al.,* 1997, Busse & Schumann 1999), fatty acid analysis (Kämpfer & Kroppenstedt 1996) and numerical taxonomic analysis (Seiler, 1983). These methods have proven to be valuable but somewhat limited tools for characterization and differentiation of these organisms studied. Other methods have also been reported which placed more emphasis on the identification capability for coryneform genera: Analysis of physiological characteristics by the Biolog Identification System (Lindenmann *et al.,* 1995), the API (RAPID) Coryne Database (Funke *et al.,* 1997a) or the RapID CB Plus system (Funke *et al.,* 1998), comparative 16S rDNA sequence analysis (Bockelmann *et al.,* 1997a), the use of genus-specific oligonucleotide probes (Kollöffel *et al.,* 1997) and fluorescence in situ hybridization (FISH) as well as colony hybridization for the analysis of cheese surface bacteria (Kollöffel *et al.,* 1999). Not all of these methods have provided satisfactory results and they are either too laborious and time-consuming to be performed on a routine basis with respect to the molecular methods, or the costs per sample are high which is the case with commercially available identification systems for the analysis of physiological properties.

Originally introduced by Naumann and coworkers (Helm *et al.,* 1991b, Naumann *et al.,* 1994), Fourier-transformed infrared (FT-IR) spectroscopy is a fast technique for classification and identification of microorganisms (e.g. Goodacre *et al.,* 1998, Holt *et al.,* 1995, Kümmerle *et al*. 1998, Timmins *et al.,* 1998). Moreover, it is a valuable tool for rapid screening of environmental isolates (Tindall *et al.*, 2000). The method would gain more attraction if an extensive database were available for the identification of unknown strains, since FT-IR is a very cost-efficient technique which allows a rapid and simple identification of microorganisms within 25 h (Kümmerle *et al.*, 1998).

Actinomycetes have been investigated by FT-IR previously. Haag *et al.* (1996) have placed their emphasis on strains from the families *Thermomonosporaceae, Streptosporangiaceae, Micromonosporaceae* and *Streptomycetaceae* while including a few strains from different families from the *Corynebacterineae* as well. Klatte (1995) has studied numerous species from the two genera *Corynebacterium* and *Rhodococcus* in detail. However, these investigations have not resulted in comprehensive databases and did not allow to conclude on the capacity of this method to identify unknown strains.

In 1997, Stackebrandt *et al.* established a novel hierarchic classification system for actinomycetes, the class of the *Actinobacteria* (high G+C gram-positive bacteria) (Stackebrandt *et al.,* 1997). This taxonomy was recently updated to accomodate new taxa of this quickly expanding group of bacteria (Stackebrandt & Schumann, 2000). Based on this classification system, a spectral database consisting of reference strains from 46 genera out of the two suborders *Micrococcineae* and *Corynebacterineae* (order *Actinomycetales*) was established which allows the rapid and simple identification of isolates of this taxonomic group. Morphologically similar taxa such as strains from the genus *Staphylococcus* were included in the database for practical reasons. In order to check the identities of reference strains, comparative 16S rDNA sequence analysis was carried out for more than half of the reference strains.

2.2 Material and methods

2.2.1 Strains

730 strains from international culture collections, commercial starter culture companies and other laboratories provided the reference material. Strains with questionable classification were identified by comparative 16S rDNA sequence analysis. The reference strains represent 220 species out of 46 genera from the two suborders *Micrococcineae* and *Corynebacterineae* (*Actinomycetales, Actinobacteria)* as well as morphologically similar genera (Tab. 2.1).

2.2.2 Sample preparation

Sample preparation was performed according to Kümmerle *et al.* (1998). The cells were incubated at 30 °C for 24 h (tryptone soya agar, containing 15.0 g tryptone, 5.0 g soya peptone, 5.0 g sodium chloride and 15.0 g agar per liter, Oxoid). Few strains yielding a clumpy suspension were subjected to ultrasonification for 2 x 10 seconds at 25 % power with a Bandelin Sonopuls HD2200, probe MS72 (Bandelin Electronic) in order to improve spectral quality.

Tab. 2.1: Alphabetical list of coryneform and related or morphologically similar species contained in the FT-IR spectral identification database. #: Number of reference strains per species. **T**: Presence of the corresponding type strain. **S**: Number of strains identified by comparative 16S rDNA sequence analysis. **R**: Number of strains reclassified due to sequence analysis.

Genus	Species	#	T	S	R
Agrococcus	*jenensis*	1	T		
Agromyces	*cerinus* subsp. *cerinus*	1	T		
	cerinus subsp. *nitratus*	1	T		
	fucosus subsp. *fucosus*	1	T		
	fucosus subsp. *hippuratus*	1	T		
	mediolanus	2	T	1	0
Arthrobacter	*agilis*	2	T	2	1
	aurescens	3		3	2
	chlorophenolicus	1		1	1
	citreus	2	T	1	1
	crystallopoietes	3	T	2	2
	cumminsii	1	T		
	globiformis	9	T	5	3
	histidinolovorans	4	T	1	1
	ilicis	1	T		
	nicotianae	17	T	14	11
	nicotinovorans	3	T	2	2
	oxidans	13	T	11	11
	polychromogenes	1	T	1	0
	protophormiae	6	T	3	3
	ramosus/pascens	1		1	1
	sp.	3		3	3
	sulfureus	4	T	2	2
	uratoxidans	2	T		
	ureafaciens	4	T	3	3
	woluwensis	1	T		
Beutenbergia	*cavernae*	2	T		
Brevibacterium	*casei*	8	T	1	0
	epidermidis	1	T		
"Brevibacterium	*helvolum"*	1		1	1
	iodinum	1	T		
	linens	57	T	42	6
	mcbrellneri	1	T		
	otitidis	1	T		
Brachybacterium	*alimentarium*	2	T	2	0
	conglomeratum	7	T	2	2
	faecium	1	T		
	nesterenkovii	2	T	1	1
	paraconglomeratum	1	T		
	rhamnosum	1	T		
	tyrofermentans	2	T	2	0
Cellulomonas	*biazotea*	1		1	0
	cellulans	7		1	1
	cellasea	1	T	1	0
	fermentans	1	T		
	fimi	1	T	1	0
	flavigena	3	T	3	1
	gelida	2	T	2	1
	turbata	2	T	1	0
	uda	2	T	1	0
Clavibacter	*michiganensis*	4		1	0
	michiganensis subsp. *insidiosus*	13	T	1	0
	michiganensis subsp. *michiganensis*	15			
	michiganensis subsp. *nebraskensis*	1	T		
	michiganensis subsp. *sepedonicus*	6	T		
Corynebacterium	*accolens*	1	T		
	afermentans subsp. *afermentans*	1	T		
	afermentans subsp. *lipophilum*	1	T		
	ammoniagenes	11	T	9	7
	amycolatum	2	T	1	0
	asperum	4		4	4
	argentoratense	1	T		
	auris	1	T		
	callunae	4	T	3	3
	coyleiae	1	T		
	diphteriae	1	T		
	flavescens	1	T		
	glucuronolyticum	1	T		
	glutamicum	47	T	40	21
	hoagii	1	T		
	jeikeium	4	T	1	0
	kutscheri	1			
	macginleyi	2		2	0
	minutissimum	1	T		
	mycetoides	2	T	1	0
	pilosum	1	T		
	propinquum	1	T		
	pseudodiphtheriticum	4	T	3	0
	pseudotuberculosis	1	T		
	renale	2	T		
	seminale	1	T		
	striatum	5	T	1	0
	ulcerans	1	T		
Corynebacterium	*urealyticum*	1	T		
	variabilis	11	T	8	8
	vitaeruminis	1	T		
	xerosis	3	T	2	1
Curtobacterium	*albidum*	1	T		
	citreum	5	T	3	3
	flaccumfaciens	22	T		
	flaccumfaciens pvar. *betae*	7			
	flaccumfaciens pvar. *oortii*	1	T		
	flaccumfaciens pvar. *poinsettiae*	10			
	flaccumfaciens pvar. *violaceum*	1	T		
	luteum	2	T	1	1
	pusillum	2	T		
Dermabacter	*hominis*	1	T		
Dermacoccus	*nishinomiyaensis*	1	T		
Deinococcus	*radiodurans*	1	T		
Demetria	*terragena*	1	T		
Dietzia	*maris*	6	T	5	5
Dermatophilus	*congolensis*	1	T		
Gordonia	*aichiensis*	1	T		
	amarae	1	T		
	bronchialis	1	T		
	hirsuta	1	T		
	hydrophobica	2	T	1	1
	rhizosphera	1		1	1
	rubropertincta	8	T	7	5
	sputi	2	T	1	1
	terrae	1	T		
Intrasporangium	*calvum*	1	T		
Janibacter	*limosus*	2		2	2
Jonesia	*denitrificans*	1	T		
Kocuria	*kristinae*	1	T		
	rhizophila	4		4	4
	rosea	6	T	3	1
	varians	3	T		
Kurthia	*zopfii*	1		1	0
Kytococcus	*sedentarius*	1	T		
Lactobacillus	*coryniformis* subsp. *coryniformis*	1	T		
Leucobacter	*komagatae*	1	T		
Leifsonia	*aquatica*	1		1	0
	poae	1	T		
Macrococcus	*caseolyticus*	1	T		
Microbacterium	*arabinogalactanolyticum*	1	T		
	arborescens	3	T	2	2
	aurum	1	T		
	barkeri	1	T		
	dextranolyticum	3	T	2	2
	esteraromaticum	6	T	5	3
	flavescens	1	T		
	imperiale	2	T		
	keratanolyticum	1	T		
	lacticum	26	T	17	17
	laevaniformans	1	T		
	liquefaciens	8	T	4	4
	oxidans	10	T	6	6
	schleiferi	12	T	4	2
	sp.	1		1	1
	terrae	2	T		
	testaceum	3	T	1	1
	trichothecenolyticum	1	T		
Micrococcus	*luteus*	3	T	3	0
	lylae	1	T		
Nesterenkonia	*halobia*	1	T		
Nocardia	*asteroides*	1			
	brasiliensis	1	T		
	farcinica	1	T		
	otidiscaviarum	1		1	1
	seriolae	1	T		
Nocardioides	*simplex*	3	T	3	1
Ornithinicoccus	*hortensis*	2	T		
Promicromonospora	*citrea*	1	T		
	enterophila	1	T		
	sukumoe	1	T		
Rathayibacter	*iranicus*	1	T	1	0
	rathayi	4	T	3	3
	toxicus	1	T		
	tritici	5	T	1	1
Rhodococcus	*coprophilus*	1	T		
	equi	14	T	6	4
	erythropolis	35	T	32	23
	fascians	12	T	2	0
	globerulus	4	T	3	2
	opacus	4	T	3	3

(Tab. 2.1 continued)

Genus	Species	#	T	S	R
Rhodococcus	*percolatus*	1	T		
	rhodnii	1	T	1	0
	rhodochrous	6		2	1
	roseus	2		2	2
	ruber	12	T	7	7
	sp.	2			
	zopfii	1	T		
Rothia	*dentocariosa*	2	T	1	0
Sanguibacter	*keddieii*	1	T		
	suarezii	1	T		
Stomatococcus	*mucilaginosus*	1	T		
Staphylococcus	*arlettae*	1	T		
	auricularis	1	T		
	aureus subsp. *aureus*	1	T		
	caprae	1	T		
	capitis subsp. *capitis*	1	T		
	capitis subsp. *ureolyticus*	1	T		
	carnosus	1	T		
	chromogenes	1	T		
	cohnii subsp. *cohnii*	1	T		
	cohnii subsp. *urealyticum*	1	T		
	delphini	1	T		
	epidermidis	3	T	1	1
	equorum	2	T	1	0
	gallinarum	1	T		
	haemolyticus	2	T		
	hominis subsp. *hominis*	1	T		
Staphylococcus	*hyicus*	1	T		
	intermedius	1	T		
	kloosii	2	T		
	lentus	1	T		
	ludgunensis	1	T		
	lutrae	1	T		
	muscae	1	T		
	pasteurii	1	T		
	piscifermentans	1	T		
	pulvereri	4	T	3	3
	saprophyticus subsp. *saprophyticus*	2	T	1	1
	schleiferi subsp. *coagulans*	1	T		
	schleiferi subsp. *schleiferi*	1	T		
	sciuri	1	T		
	simulans	1	T		
	succinus	2		2	2
	vitulinus/pulvereri	3		2	2
	warneri	1	T		
	xylosus	2		2	1
Terracoccus	*luteus*	1	T		
Terrabacter	*tumescens*	2	T		
Tsukamurella	*inchonensis*	1	T		
	paurometabola	2	T	2	0
	pulmonis	1	T		
	tyrosinosolvens	1	T		
	wratislaviensis	1	T		
Turicella	*otitidis*	1	T		
46	**220**	**730**	**192**	**352**	**224**

2.2.3 FT-IR spectroscopy

All spectra were recorded and evaluated according to Kümmerle *et al.* (1998) using an IFS-28B FT-IR spectrometer (Bruker). To diminish the difficulties arising from unavoidable baseline shifts and to improve the resolution of complex bands, the first derivation of the digitized original spectra was used.

The adjustment of FT-IR parameters such as selection of spectral frequency ranges, weights and reproducibility levels was done based on Bruker (1998) but was extended in this work. First, the reproducibility level was determined for each of the five relevant frequency ranges (spectral windows) W1 (3000-2800 cm^{-1}), W2 (1800-1500 cm^{-1}), W3 (1500-1200 cm^{-1}), W4 (1200-900 cm^{-1}) and W5 (900-700 cm^{-1}). In each window, the reproducibility level was adjusted such that a valid identification in the identity test could be expected up to a normalized spectral distance of approximately 1.0 to 1.5 for the first hit. The distance values of all frequency ranges are therefore “normalized“ according to the reproducibility of the FT-IR spectra. The reproducibility level was determined separately for each frequency range for eight species, those species being represented by 5 to 42 strains each. Subsequently, the selection of spectral windows and weights was done according to the results from the internal validation.

2.2.4 Comparative 16S rDNA sequence analysis

352 reference strains of questionable identity were subjected to comparative 16S rDNA sequence analysis. The strains were lysed and an amplification PCR of the almost

complete 16S rDNA molecule was performed according to von Stetten *et al.* (1998). Two universal 16S rDNA binding primers were used for the amplification PCR: 5‘f [5‘-AGAGTTTGATCCTGGCTCA-3‘] (position 8-26 in the *E. coli* numbering system [Brosius *et al.*, 1978]) and 3‘r [5‘-CGGCTACCTTGTTACGAC-3‘] (position 1511-1493 in the *E. coli* numbering system). The PCR protocol started with a denaturation step for 5 min at 95 °C, followed by 30 cycles of denaturation for 20 s at 95 °C, primer annealing for 40 s at 55 °C and elongation for 2 min at 72 °C each. A final elongation step was added at 72 °C for 5 min. After PCR amplification, the DNA was purified using the QIAquick PCR purification kit (Qiagen) according to the instructions of the manufacturer, followed by a PEG precipitation of the purified product according to Facius *et al.* (1999). After purification, the samples were subjected to a cycle-sequencing PCR according to Facius *et al.* (1999), using the ThermoSequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech). 11 % (v/v) DMSO and 7 % (v/v) Formamide were added to facilitate the cycle sequencing PCR of these high G+C gram positive bacteria. Fluorescently labeled primers 5‘f, 3‘r, 699R [5‘-GGGTTG(AGT)GCTCGTT-3‘, *E. coli* numbering 1114-1100] or 609V [5‘-TTAGATACCCT(AG)GTAGT-3‘, *E. coli* numbering 788-804] were used for the cycle-sequencing PCR (primers 699R and 609V: Ludwig & Strunk). Independently of the primer applied, the protocol for the cycle-sequencing PCR started with a first denaturation step for 5 min at 88 °C, followed by 25 cycles of denaturation for 30 s at 88 °C, 25 s at the primer specific annealing temperature and elongation for 3 min at 50 °C. A final elongation step at 50 °C for 5 min was added. Annealing temperatures for the different cycle sequencing primers used were as follows: 42 °C for 5‘f, 45 °C for 3‘r, 37 °C for 699R and 35 °C for 609V. Sequencing was performed on a LI-COR sequencer (MWG Biotech), typically yielding sequence lengths of approximately 800 to 1000 bases per run.

Identification of the strains was accomplished by comparison of the partial sequences with sequences in databases such as Genbank, RDP and ARB. According to Stackebrandt & Goebel (1994), a homology of more than 97 % strongly points to species identity between query sequence and retrieved database sequences, although there are exceptions to this rule (e.g. Fox *et al.,* 1992, Lechner *et al.*, 1998). Identification of a sequence in the ARB software environment was accomplished by addition of the aligned query sequence into a validated and optimized tree based on aligned 16S rDNA sequences by maximum parsimony analysis while keeping its topology constant (Ludwig *et al.,* 1998).

2.2.5 Internal validation

Performing an internal validation means that newly recorded single spectra from strains already available in the spectral library are tested against the database. Randomly selected single spectra from 208 strains from 208 species out of 41 genera provided the test set for this internal validation. The reference set consisted of average spectra of the complete spectral library of 730 strains. The average spectra from the 208 test strains were included in the reference set which did not comprise the single spectra of the test set. The test set spectra were then identified by the reference set and the result was determined at the strain, species and genus level by evaluating the relationship between the the test spectrum and the first hit in the identification hit list. The result was counted as a correct identification at the strain level if the test spectrum was identified with a spectral distance value $D < 1.5$ by its corresponding average spectrum. In case of an identificaton of the test spectrum by an average spectrum of a strain belonging to the same species, the result corresponded to a correct identification at the species level. Finally, if at least the genus matched, the result was counted as a correct identification at the genus level. The results of identification of all test spectra were averaged to obtain the so-termed percental correctness of identification. This parameter was determined for each frequency range W1 to W5 separately and subsequently also for combinations of the five ranges altogether or only spectral windows W2 to W5. For comparison, the correctness of identification was also determined for the frequency ranges and parameters used by Kümmerle *et al.,* (1998).

2.2.6 External validation

In contrast to an internal validation, the strains tested against the database are not comprised in the spectral library in this case. 544 strains from 54 species with at least three strains per species from 16 genera were used for the external validation for proof of principle. The average spectrum of one strain was excluded from the spectral library and this strain was then tested against the remaining database. If the first hit belonged to an average spectrum of a strain from the same species and the spectral distance value D was below 1.5, the result was counted as a correct identification at the species level. If the identification corresponded to a different species from the same genus, the result represented a correct identification at the genus level. In case of a spectral distance value $D > 1.5$, the strain was counted as not-identified. A misidentification was noted if a strain was identified incorrectly. The results of the identification of all test strains belonging to the same species was averaged to obtain the mean correctness of identification for each species considered.

2.3 Results

2.3.1 Composition of the identification database

The established database consists of infrared average spectra from 730 reference strains belonging to 220 different coryneform species and phylogenetically or morphologically related taxa out of 46 genera, 40 of them from the two suborders *Micrococcineae* and *Corynebacterineae* (*Actinomycetales, Actinobacteria*) (Tab. 2.1). 192 species are represented by type strains (87 %). Each average spectrum was calculated from at least three matching single spectra.

Several genera were not included in the database. These are the single-species genera *Frigoribacterium* (Kämpfer *et al.,* 2000), *Cryobacterium* (Suzuki *et al.,* 1997) and *Renibacterium* (Sanders & Fryer, 1980) due to their obligate psychrophily. Furthermore, the genus *Bogoriella* (Groth *et al.,* 1997) and the genus *Rarobacter* (Yamamoto *et al.*, 1988) were excluded for obligate alkalophily and for requirement of catalase, hemoglobin or hemin for growth in air, respectively. The genera *Tetrasphaera* (Maszenan *et al.,* 2000) and *Mycobacterium* Lehmann & Neumann, 1896 are not represented due to their extremely slow growing species. All of these genera did not allow cultivation under the required culture conditions.

On the other hand, strains from morphologically similar genera such as *Staphylococcus* were included to enable safe identification results of gram positive cocci independent of their G+C-content, in particular, since staphyloccci are widely distributed in the environment and have frequently been isolated from cheeses (Bockelmann *et al.,* 1997a, Carnio *et al.,* 2000, Irlinger *et al.,* 1997).

Not considering species from the above mentioned excluded genera, there are approximately 210 species from the two suborders *Micrococcineae* and *Corynebacterineae* validly described so far, but this number is growing continuously. 180 of these species are included in the database which corresponds to a coryneform coverage of 85 %.

2.3.2 Comparative 16S rDNA sequence analysis

After having included several hundred strains in the database, it became apparent from FT-IR cluster analyses that, sometimes, strains carrying completely different species and/or genus names grouped together at a very high level of spectral similarity. In order to check the identity of these questionable strains, comparative 16S rDNA sequence analysis was carried out for 352 strains. Partial sequences of typical sequence lengths between 800 and 1000

nucleotides were determined and identified using Genbank's BLAST (Altschul *et al.*, 1997), the RDP database (Maidak *et al.*, 2000) and the ARB database (Ludwig & Strunk). Generally, there was good agreement between the results obtained from the hitlists of Genbank and the RDP database. As a result, 224 (64 %) of these strains were reclassified according to the sequence analysis. Fig 2.1a shows an example for a number of strains clustering at a very high level of spectral similarity while carrying many different genus and species denotations, some of which have never been validly described (names with quotation marks). After comparative 16S rDNA sequence analysis, each of these strains was identified as *Rhodococcus erythropolis* (Fig 2.1b).

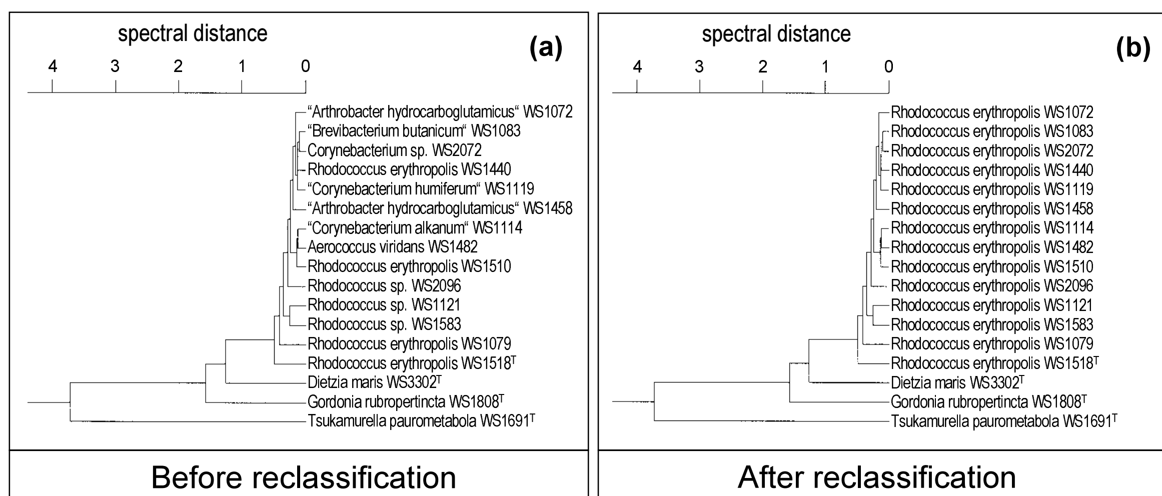

Fig. 2.1: Dendrogram of spectrometrically similar reference strains. Average linkage, correlation with normalized to reproducibility level. Frequency ranges with weights and reproducibility levels: 3000-2800 cm^{-1} / 0.8 / 3.3; 1800-1500 cm^{-1} / 0.8 / 5; 1500-1200 cm^{-1} / 0.9 / 20; 1200-900 cm^{-1} / 0.9 / 33; 900-700 cm^{-1} / 0.9 / 116. Unweighted pair group method algorithm (UPGMA). **(a)** Classification of reference strains before comparative 16S rDNA sequence analysis. **(b)** Partial reclassification after sequence analysis.

According to Stackebrandt & Goebel (1994), homologies of more than 97 % strongly point to species identity between query sequence and database sequences, although exceptions to this rule have been reported (e.g. Fox *et al.,* 1992). However, a more thorough examination of the strains including DNA-DNA hybridization and determination of physiological and biochemical properties was not possible due to the vast number of strains dealt with. For this reason, further efforts to differentiate strains reclassified as *Arthrobacter ramosus/pascens* or *Staphylococcus vitulinus/pulvereri* which are indistinguishable by means of comparative 16S rDNA analysis have not been undertaken (Tab. 2.1).

The need to carefully check the identities of reference strains on a regular basis has also been noted by Felske *et al.*, (1999) in the course of assessing the heterogeneity of partial 16S rRNA sequences of coryneform strains by TGGE. These authors explained the aberrant

behavior of some strains by the so-called "human factor" which refers to the handling of strains in laboratories over long periods of time. Moreover, coryneform bacteria have often been misclassified in the past (Altenburger *et al.*, 1997). Kollöffel *et al.* (1997) have also reported irregularities after investigating reference strains.

2.3.3 Internal validation

In order to calibrate the spectral distance values obtained in the identification hitlists, so-called reproducibility levels were determined (given in the legend of Fig. 2.1). With adjusted reproducibility levels, the threshold value for a valid identification of an isolate at the species level can be set at a spectral distance of 1.0 to 1.5, approximately. This implies that, if the spectral distance between an isolate and the first hit of the identification hitlist is greater than 1.5, the isolate cannot be identified. The reproducibility levels differ greatly between different frequency ranges, corresponding to the different degree of spectrum variation within the spectral windows.

On the basis of this calibration, the different frequency ranges' capacity for correct identification was determined by running identifications of a spectra test set against the complete database separately for each of the five spectral windows. The overall correctness of identification obtained for each frequency range (data not shown) was used for a permutation of different spectral window combinations in order to obtain those yielding highest values for correct identification. According to the theoretical investigations, combinations of all five spectral ranges or, alternatively, windows W2-W5 were supposed to be equally optimal. In the course of the following internal validation, all test strains were tested against both frequency range combinations. The combination comprising all five spectral windows was found to be optimal. In this internal validation, 93.9 % and 98.1 % of the test strains were correctly identified at the strain and species level, respectively (Fig. 2.2a). These values are optimized, meaning that when in four cases a single spectrum was not correctly identified by its corresponding average spectrum, several further single spectra were recorded and included to form a new average spectrum in order to increase the representation of the spectral variety of the same strain in the database.

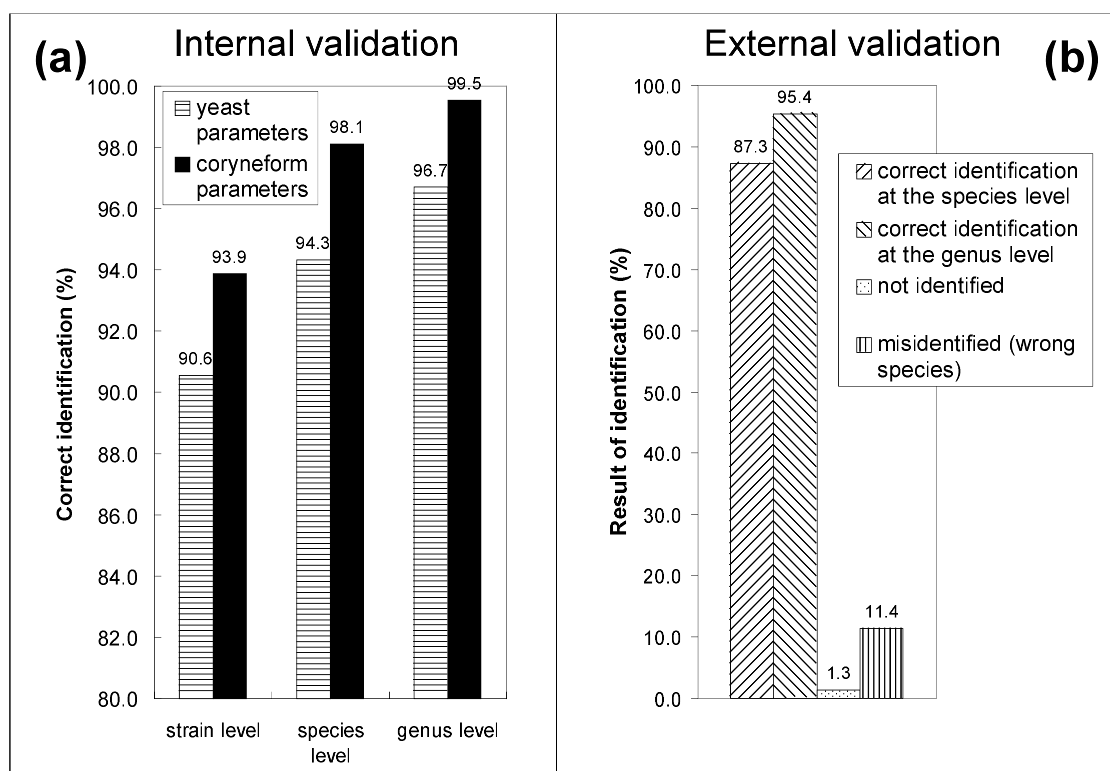

Fig. 2.2: **(a)** Internal validation based on 208 strains belonging to 208 species from 41 genera. Correctness of identification was determined at the strain, species and genus level for both "yeast parameters" (Kümmerle *et al.*, 1998) and "coryneform parameters". **(b)** External validation with 544 strains belonging to 54 species from 16 genera; the species included were represented by a minimum of three strains per species in the database. Correctness of identification was determined at the species and at the genus level. A few strains were either not identified (spectral distance D > 1.5) or misidentified.

The comparison between "yeast parameters" (Kümmerle *et al.*, 1998) and "coryneform parameters" clearly shows that it is worthwhile determining the optimal settings for each group of organisms separately and that adjustments proven to be optimal for one group do not necessarily represent the optimum for another group. This has been known previously for different microorganisms (e.g. Helm *et al.*, 1991a).

2.3.4 External validation

The quality of the database was further assessed by carrying out an external validation. One strain was singled out and the library was built up without the average spectrum of this strain. Subsequently, the strain was identified by the spectral library and the correct identification was checked at the species level. While some species yield 100 % correctness of identification even if being represented only by a low number of strains per species, the general trend shows a correlation between the number of strains per species in the library and the percentage of correct identification obtained (Fig. 2.3, shading added for illustrating

reasons only). As expected, the more strains per species are stored in the identification database, the more likely is a correct identification at the species level. Likewise, the probability of a misidentification decreases with an increasing number of strains (data not shown). This observation can be explained with an increased coverage of the intraspecies variety in case of a high number of strains per species. The number of non-identified strains, i.e. spectra that were identified with a spectral distance D > 1.5, equals zero for most of the species under investigation. Seven species out of 54, however, contained strains that could not be identified. This indicates that the variability range of some species is better represented in the reference library than that of others, independent of the number of strains available in the spectral database. It is sensible to retain the non-identified strains in the database for future identification of isolates whose subsequent addition as reference spectra might provide the “missing link“ between now non-identified spectra and their corresponding species spectra from other strains.

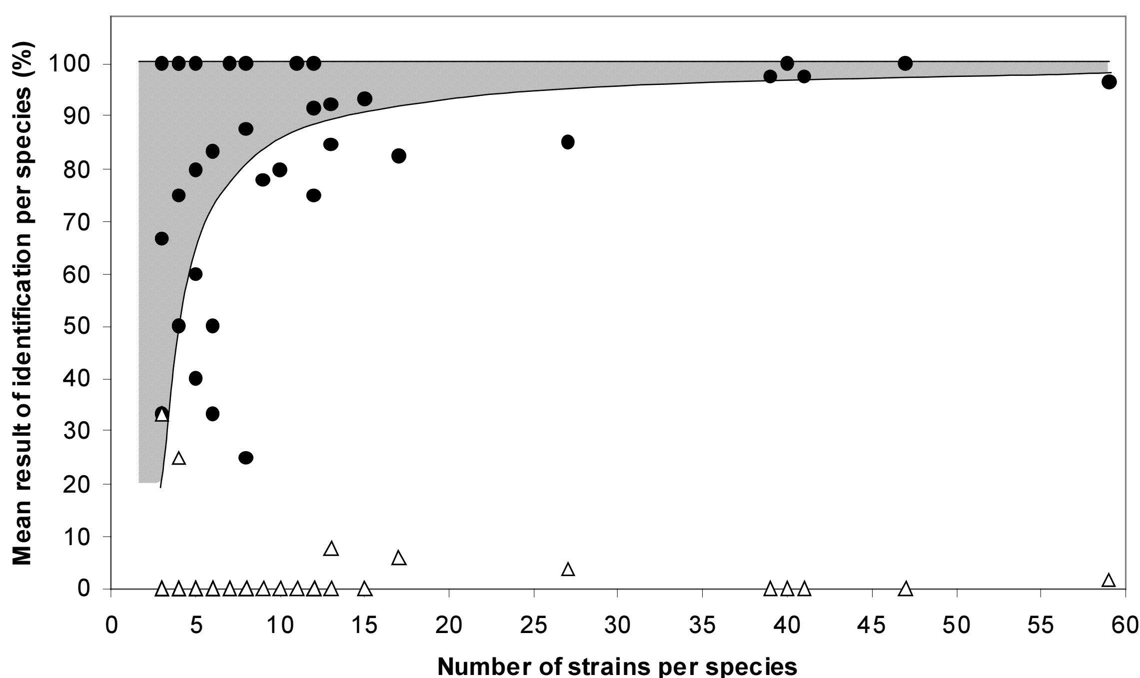

Fig. 2.3: External validation using 544 strains belonging to 54 species from 16 genera. One datapoint displays the mean result of identification over all strains per considered species vs. number of strains per species in the spectral library. ●: Correct identification at the species level. △: Non-identified (spectral distance D > 1.5). Shading added for illustrating reasons only.

Fig. 2.2b displays the mean values of identification for the 544 strains considered. Taking all species with a minimum number of three strains per species into account, 87.3 % of the strains were correctly identified at the species level and 95.4 % at the genus level.

12.7 % did not result in a correct identification at the species level, 1.3 % of which were not identified while 11.4 % were misidentified.

2.4 Discussion

Several publications report on the identification capacity of various methods dealing with different groups of microorganisms (Tab. 2.2). The identification of coryneform bacteria and related genera has been evaluated by using the Biolog Identification System (Biolog), API Coryne Database (bioMérieux) and the RapID CB Plus system (Remel), three commercially available identification kits based on the analysis of physiological properties. The latter two identification databases contain only a limited number of species, most of which are clinically relevant coryneform bacteria. The percentage values of correct identification achieved in external validations differ greatly between these systems, yielding best results for the API Coryne Database (Tab. 2.2).

Several studies analyzed the capacity for identification of microorganisms by FT-IR. Most studies have only performed internal validations where the percentage of correct identification is generally higher than in the case of an external validation. Moreover, the size of the spectral libraries under investigation has been rather limited except for the databases reported by Kümmerle *et al*., (1998) and by this study.

When comparing published identification results, it is therefore necessary to compare the size and composition of the databases evaluated as well as the size and composition of the set of test organisms used for validation. Moreover, the kind of validation performed (internal vs. external) must be noted carefully since the results of correct identification will largely be influenced by the presence or absence of the tested strains in the library. The effects of the composition and size of both database and test set on the identification quality on the one hand is hardly separable from the effect of the kind of validation performed on the other hand with the limited data available. Generally, an internal validation performed with a small heterogeneous database (i.e. only few strains per genus and genera from several different phylums) (Tab. 2.2, Helm *et al.*, 1991a) will yield much higher values for correct identification than an external validation performed on a rather homogeneous database (i.e. closely related taxa) due to a higher percentage of misidentified strains in the latter (e.g. Funke *et al.*, 1998). Misidentification occurs if a test strain is less similar to other strains of its own species than to a strain of a different species.

Tab. 2.2: Literature overview: Identification capacity of various methods used for identifying different groups of microorganisms. st: strain(s), sp: species, gen: genus/genera, lev: level, non-id: non-identified (FT-IR: spectral distance $D > 1.5$), misid: misidentified at the species level.

Method	Organisms studied	Composition of identification database (average distribution of st/sp and st/gen)	Validation performed	Composition of test organisms for validation	Result of validation	Reference
Biolog Identification System 3.50 (Biolog)	Asporogenous, aerobic gram positive rods	approx. 250 sp, 35 genera (7.1 sp/gen), 96 sp and 21 gen of which are high G+C gram positive bacteria (4.6 sp/gen)	External	174 st, 42 sp, 12 gen (4.2 st/sp, 14.5 st/gen)	34 % correct at sp lev, 7 % non-id, 58 % misid	Lindenmann *et al.*, 1995, Biolog, 2000
API Coryne Database 2.0 (bioMérieux)	Clinically relevant coryneform bacteria and related taxa as well as *Listeria* spp.	approx. 48 sp, 15 genera (3.2 sp/gen), 44 sp and 13 gen of which are high G+C gram positive bacteria (3.4 sp/gen)	External	407 st, 54 sp, 20 gen (8 st/sp, 20 st/gen)	90.5 % correct at sp lev, 5.6 % non-id, 3.8 % misid	Funke *et al.*, 1997a, bioMérieux, 2000
RapID CB Plus system (Remel)	Clinically relevant coryneform bacteria and related taxa as well as *Listeria* spp.	approx. 52 sp, 14 genera (3.7 sp/gen), 44 sp and 12 gen of which are high G+C gram positive bacteria (3.7 sp/gen)	External	378 st, 49 sp 16 gen (8 st/sp, 23 st/gen)	80.9 % correct at sp lev, 3.2 % non-id, 15.7 % misid	Funke *et al.*, 1998, Remel, 2000
FT-IR	Actinomycetes	46 st, 41 sp, 14 gen (1.1 st/sp, 3.3 st/gen)	Internal	39 st out of the identification database	89 % correct at st lev, no further information provided	Haag *et al.*, 1996
FT-IR	Low G+C gram positive bacteria (*Staphylococcus* spp., *Clostridium* spp., *Streptococcus* spp.), γ-subdivision of *Proteobacteria* (*Aeromonas* spp., *Pseudomonas* spp. and *Enterobacteriaceae*)	97 st, 42 sp, 17 gen (2.3 st/sp, 5.7 st/gen)	Internal	72 st out of the identification database	83.3 % correct at st lev, 94.4 % correct at sp lev, 97.2 % correct at gen lev	Helm *et al.*, 1991a
FT-IR	Yeasts	332 st, 74 sp, 18 gen (4.5 st/sp, 18 st/gen)	External	717 st, 36 sp, 11 gen (20 st/sp, 65 st/gen)	97.5 % correct at sp lev, 0.8 % non-id, 1.7 % tentatively identified	Kümmerle *et al.*, 1998
FT-IR	Coryneform bacteria and phylogenetically or morphologically related genera	730 st, 220 sp, 46 gen (3.3 st/sp, 16 st/gen)	Internal	208 st, 208 sp, 41 gen out of the identification database (1.0 st/sp, 5.1 st/gen)	93.9 % correct at st lev, 98.1 % correct at sp lev, 99.5 % correct at gen lev	this study
			External	544 st, 54 sp, 16 gen (10 st/sp, 34 st/gen	87.3 % correct at sp lev, 95.4 % correct at gen lev, 1.3 % non-id, 11.4 % misid	

Results are also influenced by the intrataxal separation of the particular group of organisms studied: In case of comparable heterogeneity of databases and similar heterogeneity of the test sets (i.e. numbers of strains per species or genus in the same order of magnitude), a highly different result of correct species allocation in an external validation points to a different intrataxal separapability between the organisms analyzed, e.g. between yeasts and coryneform bacteria (Kümmerle *et al*., 1998 vs. this study). Our results seem to indicate that yeast taxa can be considered more distinctive than coryneform taxa since the percentage of misidentified isolates was found to be much lower. It appears that, in constrast to yeast taxa, coryneform taxa seem to be rather contiguous, exhibiting a certain amount of overlap of different taxonomical characteristics. Taxa merging into each other lacking rather discrete boundaries at the species or genus level will easily be confused, resulting in high percentages of misidentified isolates. This hypothesis, drawn from comparison between identification results of two phylogenetically well separated kinds of microorganisms by the same technique (FT-IR) is further supported by the observation that a high percentage of misidentification has also been noted for the identification of coryneform bacteria by physiological properties (cf. Tab. 2.2). Reference to the literature reveals that even though a wide spectrum of chemotaxonomical differentiation methods have been applied to the classification of actinobacterial taxa, differentiation down to the species level could not be achieved satisfactorily in all cases, sometimes not even to the genus level. Each of these techniques allows differentiation between some taxa while they fail to separate others (Altenburger *et al.,* 1997, Busse & Schumann 1999, Felske *et al.,* 1999, Kämpfer & Kroppenstedt 1996). Stackebrandt *et al.* (1997) have noted that the rich chemical, morphological and physiological diversity of phylogenetically closely related genera makes the description of families and higher taxa so broad that they become meaningless for the description of the enclosed taxa. Most main actinobacterial lines of descent, described as orders, suborders and families, are not well separated and the statistical significance of branching points is low. Because of the lack of common properties of phylogenetic significance shared by most taxa of a higher taxon, their delineation from each other is somewhat arbitrary and artificial (Stackebrandt & Schumann, 2000). Therefore, even though actinobacterial taxa form a systematically well derivable group, due to their overlapping (or even contradictory) distribution of taxonomical characters, the possibilities for their differentiation by non-genotypical methods remain somewhat limited.

Examples for a certain degree of interference between different taxonomical characteristics and, as a result, diverging taxonomical opinions can be taken from the ongoing

discussion as to the separation or unification of different genera such as *Microbacterium*/*Aureobacterium* (Rainey *et al.*, 1994, Takeuchi & Hatano, 1998), *Cellulomonas*/*Oerskovia* (Stackebrandt *et al.*, 1982, Stackebrandt & Prauser, 1994), or *Corynebacterium*/*Turicella* (Funke *et al.*, 1994, Pascual *et al.*, 1995, Ruimy *et al.*, 1995). These genera are or appear to be phylogenetically intermixed but can be differentiated by means of other taxonomic properties.

Taxonomically overlapping reference species are likely to result in an FT-IR hit list confusion and misidentification of test strains. Thus, an extensive representation of intraspecies and intragenus variety by a substantial number of reference strains is indeed critical for a high percentage value of correct species identification, but will not decrease confusion within a highly contiguous group of organisms. On the other hand, with regard to the behavior of a single strain, e.g., in epidemiological analyses, a good coverage of intraspecies variety by a large number of reference strains will decrease the probability for differentiation of this particular strain from the others, (i.e., yield low values of correct identification at the strain level in general), especially if the strains are very similar, but will yield high values of correct species identification. From the data presented in Fig. 2.3, we expect that, on the average, approximately five to ten different strains per species in a coryneform FT-IR database are appropriate in order to achieve a reasonable identification capacity. A considerable advantage of working with an FT-IR spectral library for identification is the users' possibility to influence the size and composition of the database themselves, i.e., by continuously adding strains in order to keep the reference library up to date or to include own sets of microorganisms.

2.5 Conclusion

The FT-IR spectral database is a valuable tool for the rapid, simple and cost-efficient identification of coryneform strains from a variety of sources. Comparison of identification results reported in the literature showed that an overall correct identification of more than 95 % at the species level has not been achieved by any method so far. Therefore, it can be concluded that coryneform bacteria are a group of organisms whose taxa are not well separated by phenotypical characteristics and, therefore, are difficult to be identified properly. The identification values obtained in this work are well within the range of those having been reported in the literature for identification of these high G+C gram-positive bacteria. In order to obtain an identification at the species level with a reasonable probability, each species should be represented by an approximate number of five to ten strains in the database. For

improvement of the library's capability for correct identification of coryneform isolates, the number of strains should be increased constantly both for intraspecific representation and for coverage of relevant taxa in general.

2.6 Acknowledgments

The authors wish to thank Wolfgang Ludwig for providing the ARB software environment and the ARB 16S rDNA database and for giving valuable information whenever necessary. We are grateful to Lyudmila Evtushenko (VKM, Russia), Ingrid Groth (Hans-Knöll-Institut für Naturstoff-Forschung e.V., Jena, Germany), Klaus P. Schaal (Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany), and Karin Schubert (Ludwig Maximilians Universität, Munich, Germany) for supplying us with strains from their collections and to the companies Chr. Hansen GmbH, Rhodia GmbH, SKW Biosystems GmbH, and Wisby GmbH & Co. KG (all Germany) for supplying us with starter culture strains. Thanks are due to Joachim Charzinski for helping us with the LINUX operating system. The technical assistance of Sven Illgner, Patrizia Hägele and Louise Arnold is greatly appreciated. Special thanks are due to Cornelia Fischer for excellent technical assistance.

This work was supported by the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn), the AiF (Arbeitskreis für industrielle Forschung) and the Ministry of Economics and Technology, Project Nr. 11627N.

3 Infraspecific diversity of *Brevibacterium linens*, *Corynebacterium glutamicum* and *Rhodococcus erythropolis* as assessed by partial 16S rDNA sequence analysis and Fourier-transform infrared (FT-IR) spectroscopy

3.1 Abstract

The infraspecific diversity of 23 strains of *Brevibacterium linens*, 25 strains of *Corynebacterium glutamicum* and 26 strains of *Rhodococcus erythropolis* was determined by partial 16S rDNA sequence analysis and Fourier-transform infrared (FT-IR) spectroscopy. In total, 27 strains derived from culture collections carried invalidly described or wrong species denotations and had to be reclassified in accordance with polyphasic taxonomical analyses. Three main conclusions emerged from the observations: (i) FT-IR spectroscopy is a rapid and reliable method to screen for similar isolates and to identify these actinomycetes at the species level. (ii) Comparison of infraspecific 16S rDNA similarities suggested that *R. erythropolis* strains have a very low diversity , *B. linens* displays high diversity and *C. glutamicum* may have an intermediate position. (iii) No correlation of FT-IR spectral similarity and 16S rDNA sequence similarity below the species level (i.e., between strains of one species) was observed. Therefore, diversification of 16S rDNA sequences and microevolutionary change of the cellular components detected by FT-IR spectroscopy appears to be de-coupled.

3.2 Introduction

The assessment of infrataxal variability is an important part of adequate taxon delimitation. The infraspecific diversity of several species has been evaluated by different methods. Only a limited number of in-depth studies have been performed recently, employing comparative 16S rDNA sequence analysis by using a larger number of strains (Chatellier *et al.*, 1998, Harrington *et al.*, 1999, Chen *et al.*, 2000). Other methods such as DNA-DNA hybridization (Christensen *et al.,* 1997, Szállás *et al.,* 1997), multilocus enzyme electrophoresis profiling (Farfán *et al.*, 2000), PCR - denaturing gradient gel electrophoretic analysis of protein-coding gene sequences (Dahllöf *et al.*, 2000), random amplified

polymorphic DNA fingerprinting (Ridell *et al.*, 1995, Zavaleta *et al.*, 1997, Cibik *et al.,* 2000), and temperature gradient gel electrophoresis pattern analysis (Nübel *et al.*, 1996) have been used separately or in combination with comparative 16S rDNA sequence analysis to qualitatively investigate infraspecific diversity of many isolates.

The determination of infraspecific diversity is also important for a correct identification of isolates belonging to the species contained in any identification database: The more diverse a species is, the more strains must be included in the database in order to represent this species adequately and, consequently, the more reliable is the identification obtained. FT-IR spectroscopy is on its way to become a valuable tool for the rapid identification of microorganisms (e.g. Helm *et al.*, 1991a, Curk *et al.,* 1994, Naumann *et al.,* 1994, Holt *et al.,* 1995, Goodacre *et al.,* 1998, Kümmerle *et al.*, 1998). A validated comprehensive FT-IR spectral reference library has been established by Oberreuter, H., Seiler, H. & Scherer, S. (unpublished results), which allows the rapid identification of coryneform bacteria and related taxa from the two suborders *Micrococcineae* and *Corynebacterineae* (*Actinomycetales, Actinobacteria*) (Stackebrandt *et al.*, 1997).

FT-IR spectroscopy has been used in combination with other techniques for qualitative studies on infraspecific diversity (Seltmann *et al.,* 1994, Seltmann *et al.,* 1995, Irmscher *et al.,* 1999, Stan-Lotter *et al.,* 1999), however, the method has never been applied for quantitative assessment of infraspecific diversity so far. In the present paper, three actinomycete species were chosen which, to our knowledge, have not been subjected to any analysis of infraspecific diversity previously. During the investigation, it became clear that a significant number of strains from official collections was misclassified, in some cases even carrying invalid species names. On the basis of polyphasic taxonomical analyses, we propose reclassifications for these strains.

3.3 Material and methods

3.3.1 Strains

The strains used for this study are listed in Tab. 3.1. Primary depositors and isolation loci are given where available from the official culture collections. A significant proportion of the strains has previously been analyzed in terms of physiological properties (Seiler, 1983, Kämpfer *et al.,* 1993) and fatty acid analyses (Kämpfer & Kroppenstedt, 1996). Several strains carry names not included in the Approved List of Bacterial Names (Skerman *et al.*, 1980) and which have not been validly published since January 1st, 1980. The strains of *B.*

linens were tested for red colony pigmentation upon application of 20 % KOH, a behavior which is specific for *B. linens* (Jones *et al.,* 1973, Kohl *et al.*, 1983).

3.3.2 FT-IR spectroscopy

Sample preparation and measurement of FT-IR spectra was performed as described in Sec. 2.3.2 and 2.3.3. The software Dataopus (Bruker) was used to calculate a matrix listing the spectral distances between the strains of each species in pair-wise comparison. The spectral distance is a measure of the spectra's similarity of two strains and corresponds to the size of non-overlapping areas of both spectra.

3.3.3 16S rDNA sequence analysis

The sequencing of the 16S rDNA was performed as described in Sec. 2.3.4. The GenBank accession numbers for the 16S rDNA gene sequences determined in this study are AY017065 to AY017138.

The sequences were aligned using the ARB alignment editor (Ludwig & Strunk) and trimmed to comprise positions 64-716 in the *E. coli* numbering system. The ARB software environment was employed for calculating a matrix listing pair-wise similarity values between the strains of each species using a separate consensus filter for each species (Ludwig *et al.,* 1998), thereby comparing only those positions at which unambiguous sequence infomation was available for the complete set of reference organisms (600–620 nt).

Identification of the strains for reclassification was accomplished as described in Sec. 2.3.4.

3.3.4 Statistical evaluation

Spectral distance and 16S rDNA similarity distributions were determined by a computer program which grouped the calculated values into classes and determined the relative frequency of occurrence in each class. The class width was set to 0.25 distance units for spectral distances and to 0.5 percentage points for 16S rDNA similarity values. Distance or similarity values coinciding with a class boundary were counted in the lower of the two possible classes. For visualization, the relative frequencies in each class were plotted versus the corresponding class intervals (the sum of all relative frequency values is 100 %). Distance versus similarity correlation graphs were created by plotting the pair-wise spectral distance versus the 16S rDNA sequence similarity for each combination of strains.

Tab. 3.1: Strains used. **Notes:** Binomials in single quotes are not in the Approved List of Bacterial Names (Skerman *et al.*, 1980) and have not been validly published since January 1st, 1980. T: type strain. **Abbreviations of original strain denotation:** AC: I. Antheunisse and W.H. Crombach, University Wageningen, The Netherlands; ATCC: American Type Culture Collection, Manassas, VA, USA; DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; IAM: Institute of Applied Microbiology, Tokyo, Japan; NCDO: National Collection of Dairy Organisms, Reading, UK; NCIB: National Collection of Industrial Bacteria, Aberdeen, UK; Seiler, WS: Weihenstephan Culture Collection, Institute of Microbiology, FML Weihenstephan, Freising, Germany; Splitstoesser, O.F.: Cornell University, NY, USA. Starter culture companies and dairies are encoded by single letters. **Initial depositor or source** and **Source of isolation** according to the official collections or own data. **Numerical studies under comparison: A** Seiler, 1983; **B** Kämpfer *et al.,* 1993; **C** Kämpfer & Kroppenstedt, 1996. Cluster denotations are taken from the respective publication.

Corynebacterium glutamicum				Clusters of numerical studies		
Original strain denotation	**WS No.**	**Initial depositor or source**	**Source of isolation**	**A**	**B**	**C**
ATCC 13059	1493	Kyowa Ferm. Ind. Co., Ltd.	derived from ATCC 13032T	E I	47	I-1
ATCC 13060	1494	Kyowa Ferm. Ind. Co., Ltd.	derived from ATCC 13032T	E I	47	I-1
ATCC 13232	1495	Kyowa Ferm. Ind. Co., Ltd.		E I	47	I-1
ATCC 13286	1496	Kyowa Ferm. Ind. Co., Ltd.	derived from ATCC 13032T	E I	47	
ATCC 13287	1497	Kyowa Ferm. Ind. Co., Ltd.	derived from ATCC 13032T	E I	47	I-1
ATCC 13655	1940	Ajinomoto Co., Inc.		E I	12	
ATCC 13744 ("*Brevibacterium taipei*")	1103	Wei-chuan Foods Corp.	soil	E I	47	I-1
ATCC 13746	1572	Wei-chuan Foods Corp.	soil	E I	47	
ATCC 13747 ("*Brevibacterium glutamigenes*")	1086	Wei-chuan Foods Corp.	soil	E I		
ATCC 14017 ("*Brevibacterium chang-fua*")	1084	Wei-chuan Foods Corp.	rice puddle soil, Formosa	E I	47	I-1
ATCC 14066 ("*Brevibacterium saccharolyticum*")	1096	Ajinomoto Co., Inc.		E I	12	
ATCC 14067	1757	Ajinomoto Co., Inc.		E I	47	I-1
ATCC 14399 ("*Micrococcus maripuniceus*")	1727	C. E. Zobell	marine	E I		
ATCC 14915 ("*Brevibacterium seonmiso*")	1097	Shinhan Flour Mill Co., Ltd.		E I	47	I-1
ATCC 15025	1499	Kyowa Ferm. Ind. Co., Ltd.		E I	47	I-1
ATCC 15283 (*Microbacterium* sp.)	1105	Won Hyung Ind. Company, Ltd.	soil	E I		
ATCC 19165 (*Brevibacterium* sp.)	1100	T. T. Cheng	uncultivated soil	E I		
ATCC 19240 ("*Brevibacterium thiogenitalis*")	1104	Takeda Chem. Ind., Ltd.	soil	E I	47	I-1
ATCC 21179	1582	Kyowa Ferm. Ind. Co., Ltd.		E I	47	
ATCC 21492	2041	Ajinomoto Co., Inc.	mutant derived from ATCC 13032T	E I	47	I-1
ATCC 21517	1575	Kyowa Ferm. Ind. Co., Ltd.		E I	47	I-1
DSM 20137	1795	Int. Mineral and Chemical Corp.		E I	47	I-1
DSM 20163	1756	Wei-chuan Food Corp.	soil	E I		
DSM 20300 T	2049	Kyowa Ferm. Ind. Co., Ltd.	sewage	E I	47	I-1
NCIB 9666 (*Arthrobacter* sp.)	1605	H. L. Kornberg	soil	E I	47	

(Tab. 3.1 continued)

Rhodococcus erythropolis

Original strain denotation	WS No.	Initial depositor or source	Source of isolation	Clusters of numerical studies		
				A	**B**	**C**
ATCC 15108 (*Rhodococcus* sp.)	1121_1	Ajinomoto Co., Inc.		A II	102	
ATCC 15527 ("*Brevibacterium healii*")	1088	Esso Research & Engineering Company	soil	A II	100	I-4
ATCC 15590 ("*Arthrobacter paraffineus*")	1077	Kyowa Ferm. Ind. Co., Ltd.	soil	A II	100	I-4
ATCC 15961 (*Rhodococcus* sp.)	1120	Ajinomoto Co., Inc.	soil	A II	100	
ATCC 19369	1800	M. Brown	chalk soil			
ATCC 21035 (*Rhodococcus* sp.)	1583	Takeda Chem. Ind., Ltd.	soil	A II		
ATCC 21108 ("*Corynebacterium humiferum*")	1119	Takeda Chem. Ind., Ltd.		A II	100	I-4
ATCC 21190 ("*Arthrobacter hydrocarboglutamicus*")	1458	Kyowa Ferm. Ind. Co., Ltd.			100	I-4
ATCC 21194 ("*Corynebacterium alkanum*")	1114_1	Kyowa Ferm. Ind. Co., Ltd.	soil	A rest	103	
ATCC 21195 ("*Brevibacterium paraffinoliticum*")	1094	Kyowa Ferm. Ind. Co., Ltd.	soil		101	
ATCC 21222 ("*Brevibacterium ketoglutamicum*")	1603	Kyowa Ferm. Ind. Co., Ltd.		A II	100	I-4
ATCC 21362 ("*Corynebacterium aurantiacum*")	1115	Kyowa Ferm. Ind. Co., Ltd.		A II	100	I-4
ATCC 21788 ("*Arthrobacter oxamicetus*")	1075	Bristol Labs.	soil, Chiba, Japan	A II	100	I-4
ATCC 21814 ("*Arthrobacter oxamicetus* subsp. *propiophenicolus*")	1076	Bristol Labs.	soil, Japan	A II		
DSM 311	2096	K. Tanaka	soil	A II	100	
DSM 312	1440	K. Taraka & K. Kimura	soil	A II	100	I-4
DSM 313	1944_2	Ayerst Lab., Inc.		A II	100	
DSM 20665	1079	R. L. Tate	soil		100	I-4
DSM 43060	1804	K. A. Bissett	soil			
DSM 43066 ^{T}	1518	P. H. Gray	soil	A II		
exDSM 43065	1510			A II		
IAM 1399	2126	H. Iizuka & K. Komagata	soil in petroleum zone	A II	100	
IAM 1474	2036	H. Iizuka & K. Komagata	soil in petroleum zone	A II		
NCIB 9646 (*Rhodococcus* sp.)	2079	T. Gibson		A II	100	I-4
Splitstoesser 60 (VI) (*Corynebacterium* sp.)	2072	D. F. Splitstoesser		A II	100	I-4
Splitstoesser 66 (VI) (*Corynebacterium* sp.)	2071	D. F. Splitstoesser		A II	100	I-4

(Tab. 3.1 continued)

Brevibacterium linens				Clusters of numerical studies		
Original strain denotation	**WS No.**	**Initial depositor or source**	**Source of isolation**	**A**	**B**	**C**
AC 251	1951	I. Antheunisse & W.H. Crombach		C VII		
AC 252	1949	I. Antheunisse & W.H. Crombach		C VII	90	II-11
AC 474 (*Corynebacterium* sp.)	1504	I. Antheunisse & W.H. Crombach		C VII	90	II-11
AC 478	1950	I. Antheunisse & W.H. Crombach		C VII	90	
AC 578	1952	I. Antheunisse & W.H. Crombach		C VII	90	
AC 825	1939	I. Antheunisse & W.H. Crombach		C VII	90	II-11
AC 831	1975	I. Antheunisse & W.H. Crombach		C VII	90	
ATCC 9174	1694	H. Weigmann	Romadur cheese, Kiel, Germany			
DSM 20425 ^{T}	1782	H. Weigmann	Harzer cheese		90	
DSM 20426	1778	Yale	Camembert cheese		90	
NCDO 1002	1968	P. Kastli		C VII	90	II-11
Seiler 195	1917	H. Seiler			90	II-11
	2903	Starter culture company F				
	2904	Starter culture company F				
	2906	Starter culture company N				
	2909	Starter culture company E				
	2910	Starter culture company E				
	2911	Dairy K	German soft cheese			
	3030	Starter culture company N				
	3033	Starter culture company N				
	3037	Starter culture company F				
	3038	Starter culture company F				
	3459	M. Carnio	French raclette cheese			

3.4 Results and discussion

3.4.1 FT-IR spectroscopy is a reliable and rapid method for species identification

In order to assess infraspecific variability, the classification of all strains under investigation must be unequivocal at the species level. Tab. 3.1 lists the strains used for this study. A significant proportion of the strains has previously been analyzed as regards their

physiological properties (Seiler, 1983, Kämpfer *et al.,* 1993) and by fatty acid analyses (Kämpfer & Kroppenstedt, 1996). Cluster denotations of these analyses are given in the cases where the particular strains were included in the respective study.

All 25 strains of *C. glutamicum,* including the strains which carry invalid species names given in single quotes, are grouped into the same cluster "E I" in the numerical analysis by Seiler (1983). Most of these strains were also analyzed by Kämpfer *et al.* (1993) and Kämpfer & Kroppenstedt (1996). In the latter two publications, all of them are located in the same respective clusters of "*C. glutamicum*" except for strains ATCC 13655 and 14066 which were grouped differently into a small '*Brevibacterium*'/*Corynebacterium*-cluster (Kämpfer *et al.*, 1993), in which, however, a strain denoted as *C. glutamicum* was also included. An FT-IR cluster analysis (Fig. 3.1) depicting the spectral similarities between these strains as well as type strains from other *Corynebacterium* spp. clearly shows that all of these strains exhibit a relatively high level of spectral similarity. They are clearly separated from the type strains of other *Corynebacterium* species. Comparative 16S rDNA sequence analysis of the partial sequences with the programs RDP, ARB and Genbank's BLAST resulted in an overall allocation to the species *C. glutamicum* in all cases. The minimal similarity between the partial sequences of all strains to the type strain's sequence was 97.5 %. Based on this polyphasic approach, we propose reclassification of the strains ATCC 13744, ATCC 13747, ATCC 14017, ATCC 14066, ATCC 14399, ATCC 14915, ATCC 15283, ATCC 19165, ATCC 19240 and NCIB 9666 (Tab. 3.1) as *Corynebacterium glutamicum*.

Likewise, the majority of the *R. erythropolis* strains has been subjected to the same numerical taxonomic analyses. All strains investigated by Kämpfer & Kroppenstedt (1996) fell into the same cluster "I-4", denoted as "*Corynebacterium* sp.". Seiler (1983) grouped all strains into the same cluster "A II" except for ATCC 21194 which was separated only by a small distance. Within a phenogram, all strains form a distinct subbranch (Kämpfer *et al.*, 1993). Again, FT-IR spectroscopy showed a very high spectral similarity between these 26 strains (data not shown) and 16S rDNA sequence analysis revealed a strong similarity to *R. erythropolis*. We therefore propose reclassification of the strains ATCC 15108, ATCC 15527, ATCC 15590, ATCC 15961, ATCC 21035, ATCC 21108, ATCC 21190, ATCC 21194, ATCC 21195, ATCC 21222, ATCC 21362, ATCC 21788, ATCC 21814, NCIB 9646, WS 2071 (Splitstoesser 66 (VI)) and WS 2027 (Splitstoesser 60 (VI)) (Tab. 3.1) as *Rhodococcus erythropolis*.

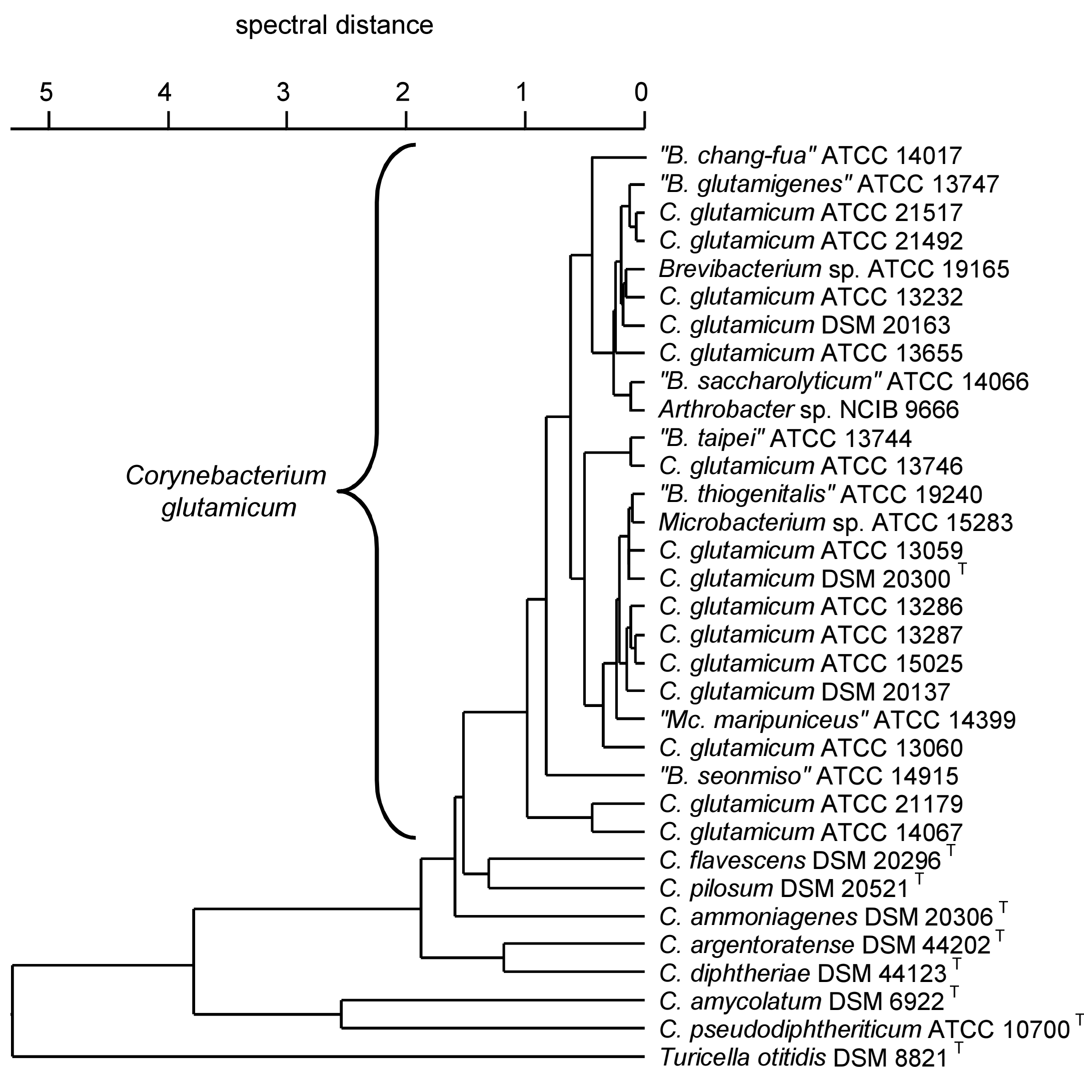

Fig. 3.1: Dendrogram of 25 strains of *C. glutamicum* and the type strains of other *Corynebacterium* spp. and *Turicella otitidis*. Abbreviations: *B. Brevibacterium*, *C. Corynebacterium*, *Mc. Micrococcus*. Single quotes indicate invalid species names. Strains within the *C. glutamicum* cluster not denoted by this name are proposed for reclassification. Average linkage, correlation with normalized to reproducibility level. Frequency ranges with weights and reproducibility levels: 3000-2800 cm^{-1} / 0.8 / 3.3; 1800-1500 cm^{-1} / 0.8 / 5; 1500-1200 cm^{-1} / 0.9 / 20; 1200-900 cm^{-1} / 0.9 / 33; 900-700 cm^{-1} / 0.9 / 116. Unweighted pair group method algorithm (UPGMA).

All 23 strains of *B. linens* displayed an orange colony color after 2 days of incubation at light on TSA. A drop of 20 % KOH resulted in red colony pigmentation, a behavior which is specific for *B. linens* (Jones *et al.,* 1973, Kohl *et al.*, 1983). The strains which have been investigated by Seiler (1983), Kämpfer *et al.* (1993) and Kämpfer & Kroppenstedt (1996) formed the same cluster in each case. The partial 16S rDNA sequences of all strains showed a level of similarity of at least 96.7 % to the type strain's sequence. In the cases of WS 2903, WS 2906 and WS 3025, sequence similarities to the closely related type strains of *B. epidermidis* or *B. iodinum* were noted. However, since these two species are characterised by different colony and pigmentation morphology, the strains may be considered strains of the

species *B. linens*. Therefore, we propose reclassification of AC 474 (Tab. 3.1) as *Brevibacterium linens*.

According to Stackebrandt & Goebel (1994), a 16S rDNA similarity of more than 97 % strongly points to species identity between query sequence and type strain sequence, although there are exceptions to this rule (e.g. Fox *et al.,* 1992, Lechner *et al.,* 1998). Some pair-wise similarity values are somewhat lower than the “cut-off value” of 97 % sequence similarity, but we did decide not to question species identity in these cases. Stackebrandt and Goebel (1994) recommended that their threshold value be lowered in order to maintain flexibility in the phylogenetic definition of a species.

Based on this approach as well as on the polyphasic taxonomic data reported in the literature, we conclude that all strains included in this analysis belong to one of the three species under investigation. This result is a prerequisite to assess infraspecific variability, which will be reported in the following section. Concerning species identification it is important to note that for 71 out of 74 strains investigated, FT-IR spectroscopical analysis arrived at the same result as rDNA sequence analysis. This demonstrates that FT-IR spectroscopy is a fast and reliable screening method to classify unknown actinomycetes at the species level (compare Tindall *et al.,* 2000).

3.4.2 Infraspecific variability of different species

Reliable assessment of infraspecific variability depends on the correct strain allocation to a particular species (see above). Furthermore, it is only possible if the strains under investigation are of independent origin. Tab. 3.1 lists the original depositor or source together with the isolation locus for each strain as far as such information was available from the official collections. These data are not always precise enough to derive firm conclusions as to where the strains originated. It is, however, probable that most of the strains from *C. glutamicum* and *R. erythropolis* were isolated from East Asian environmental sources and that the *B. linens* strains came from Middle-European sources. We also assume that the majority of these strains represent different isolates, except for the five *C. glutamicum* strains ATCC 13059, ATCC 13060, ATCC 13286, ATCC 13287 and ATCC 21492 which appear to be mutants of the type strain.

The 25 strains of *C. glutamicum* exhibited a comparatively high degree of spectral similarity (Fig. 3.2a). The distribution of pair-wise FT-IR spectral distance values ranged from 0.07 to 1.35 (mean at 0.43), focusing around 0.3 and then relatively steeply declining towards higher spectal distance values. On the other hand, the degree of 16S rDNA sequence

similarity between the strains of *C. glutamicum* was lower. The distribution of the pair-wise sequence similarity values ranged from 95.7 to 100 % with a maximum between 99.0 and 99.5 %. This means that even though each of the strains showed a sequence similarity of at least 97.5 % to the type strain sequence, pair-wise sequence comparison between the individual strains revealed similarities of as low as 95.7 %.

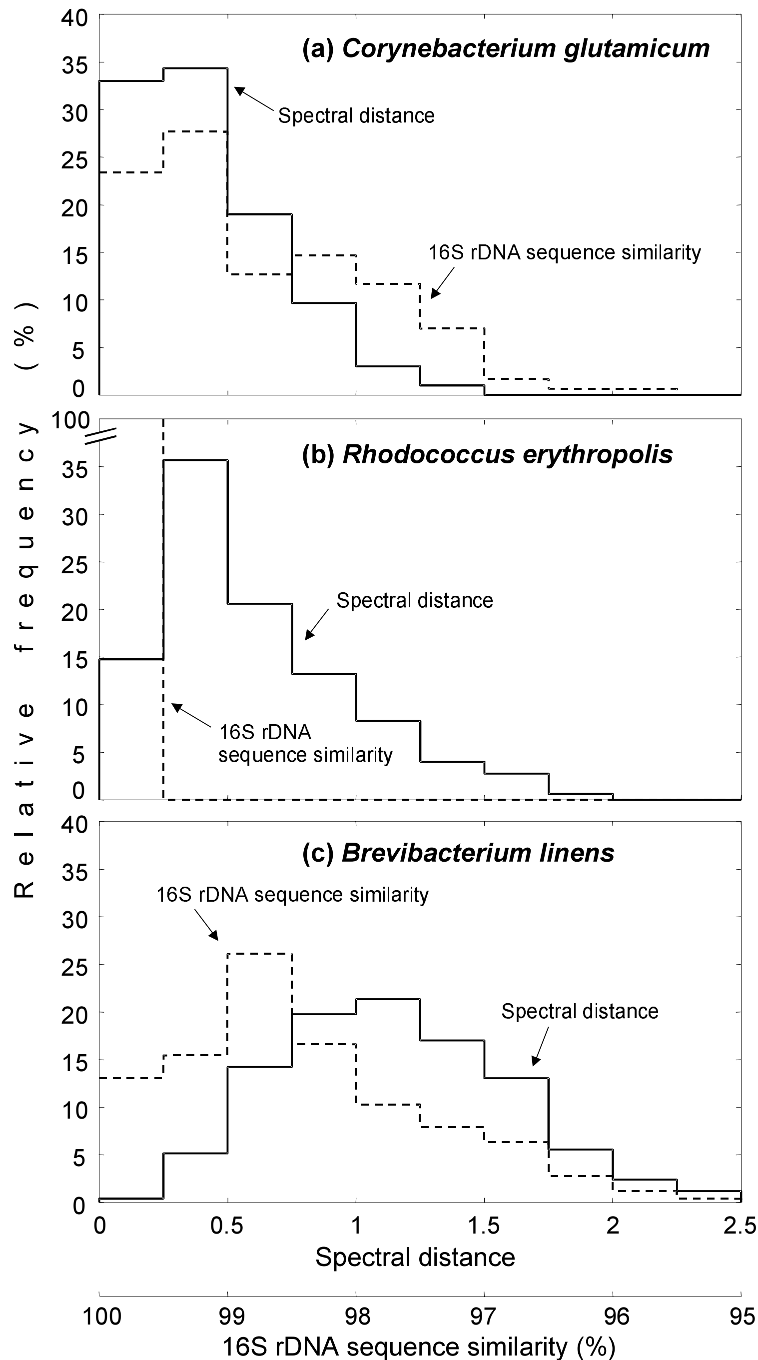

Fig. 3.2: Distributions of spectral distance values and 16S rDNA sequence similarity values: Relative frequency of occurrence in each class versus the corresponding class intervals.

The 26 strains of *R. erythropolis* displayed an extremely high level of 16S rDNA sequence similarity between 99.8 and 100 % with a mean at 99.9 %. For this reason, all strains of *R. erythropolis* were grouped into the same class in Fig. 3.2b. However, in sharp contrast, the variability between their FT-IR spectra was rather high. The distribution of spectral distance values ranged from 0.12-1.78 (mean at 0.60), focusing at a value around 0.3 while gradually declining towards larger distance values. This result is in favour of an independent origin of these isolates in spite of the high similarity of the 16S rDNA sequences.

The highest infraspecific diversity was noted for *B. linens*. Spectral distance values between the 23 strains analyzed ranged from 0.19 to 2.42 with a mean value of 1.2 (Fig. 3.2c). The distance distribution closely resembles a Gaussian bell curve around the mean. For this species, a comparatively wide distribution of spectral distance values between the different strains was observed. The distribution of pair-wise 16S rDNA similarity values remotely resembles a bell curve which is gradually declining towards lower similarity values. The pair-wise levels of similarity ranged from 95.4 to 100 %, with a maximum between 98.5 and 99.0 % (mean at 98.3 %). Similar to *C. glutamicum*, 16S rDNA sequence similarities as low as 95.4 % were noted between different strains in pair-wise comparison, although each of the strains showed a minimal sequence similarity of 96.7 % to the type strain sequence.

Tab. 3.2: Infraspecific diversity of different species as determined by comparative 16S rDNA sequence analysis. * Number of strains sequenced. †: Number of nucleotides considered. ‡: *eaeA*: attachment-effacement gene

Species	*****	**Nt.†**	**Range of 16S rDNA sequence similarity or distance in pairwise comparison (%)**	**Reference**
Campylobacter hyointestinalis	15	1393	Within *C. hyointestinalis* subsp. *lawsonii* 99.0-100, within *C. hyointestinalis* subsp. *hyointestinalis* 96.4-100, between the two subspecies: 95.7-99	Harrington *et al.*, 1999
Hafnia alvei	14	353 (5' end)	92.3 between *eaeA*-positive and *eaeA*-negative strains‡	Ridell *et al.*, 1995
Streptococcus suis	35	1468	93.94-100	Chatellier *et al.*, 1998
Xylella fastidiosa	16	1537	99.0-100	Chen *et al.*, 2000
Brevibacterium linens	23	600 to 620 (5' end)	95.4-100	this study
Corynebacterium glutamicum	25	600 to 620 (5' end)	95.7-100	this study
Rhodococcus erythropolis	26	600 to 620 (5' end)	99.8-100	this study

In-depth studies of infraspecific variability using comparative 16S rDNA sequence analysis of a large number of strains a comparatively rare (Tab. 3.2). 16 strains of *Xylella fastidiosa* exhibited a 16S rDNA sequence similarity of 99.0-100 % over the complete sequence (Chen

et al., 2000), similar to *R. erythropolis*. *Campylobacter hyointestinalis* (Harrington *et al.*, 1999) and *Streptococcus suis* (Chatellier *et al.*, 1998) displayed a high inter-strain sequence variability similar to *C. glutamicum* and especially *B. linens*. Even though the pair-wise similarity values are lower than the cut-off value of 97 % sequence similarity for species delimitation as defined by Stackebrandt and Goebel (1994), none of the authors concluded it sensible to change the then-existing species delimitation of their respective species under study except for Ridell *et al.* (1995) who examined 14 strains of *Hafnia alvei*.

According to Clayton *et al.* (1995), the variability noticed between different published 16S rDNA sequences of the same species in sequence databases such as GenBank may represent interoperon variation within a strain, strain-to-strain variation within a species, inadequate taxon delimitation, sequencing error or other laboratory error. While some actinomycetes such as *Thermobispora bispora* (Wang *et al.,* 1997), *Thermomonospora chromogena* (Yap *et al.,* 1999), *Mycobacterium celatum* (Reischl *et al.*, 1998) and *Mycobacterium terrae* (Ninet *et al.,* 1996) have been found to contain several types of transcriptionally active rRNA operons whose sequences may differ by as much as 6 %, no information was available addressing this question for the three species analyzed in this study. Felske *et al.* (1999) analyzed eight coryneform genera in terms of their 16S rDNA heterogeneity. While neither of the three genera investigated in this study were included, the authors found sequence heterogeneities only in the genus *Curtobacterium* and therefore suggested that the heterogeneity of 16S rRNA genes in the genome is not very common within coryneform bacteria. Sequencing errors cannot be ruled out completely. However, by using consensus filters, regions showing exceptionally high variabilities were excluded from the analyses.

3.4.3 FT-IR spectral similarity and 16S rDNA similarity are not correlated

FT-IR spectroscopy is a whole-cell fingerprint technique that analyzes microbial cells on a completely different basis than techniques comparing a small conserved region of the genome such as the 16S rDNA gene. FT-IR spectroscopy, nevertheless, might reflect the diversity obtained by sequence comparison at least to some degree. In order to determine whether a correlation exists between the pair-wise infrared spectral distance values and 16S rDNA similarity, spectral distances were plotted versus their corresponding 16S rDNA similarity values for each pair of strains (Fig. 3.3). This analysis shows that within *C. glutamicum* and *B. linens* (Fig. 3.3a and c) there are strain pairs which are spectrally very close but have a low 16S rDNA similarity (bottom left of each plot). Others display a highly

similar 16S rDNA but have very different infrared absorption spectra (top right of each plot). In the former cases, the 16S rDNA sequence analysis has a better resolution than FT-IR spectroscopy whereas in the latter cases, different strains can be distinguished via FT-IR spectroscopy which cannot be distinguished by partial 16S rDNA analysis. The strains of *R. erythropolis* (Fig. 3.3b) differed at only two nucleic acid positions and therefore, the entire observed spectral variability is plotted versus sequence similarities of 99.9 and 100 percentage points. However, a considerable number of strain pairs can be distinguished by FT-IR spectroscopy.

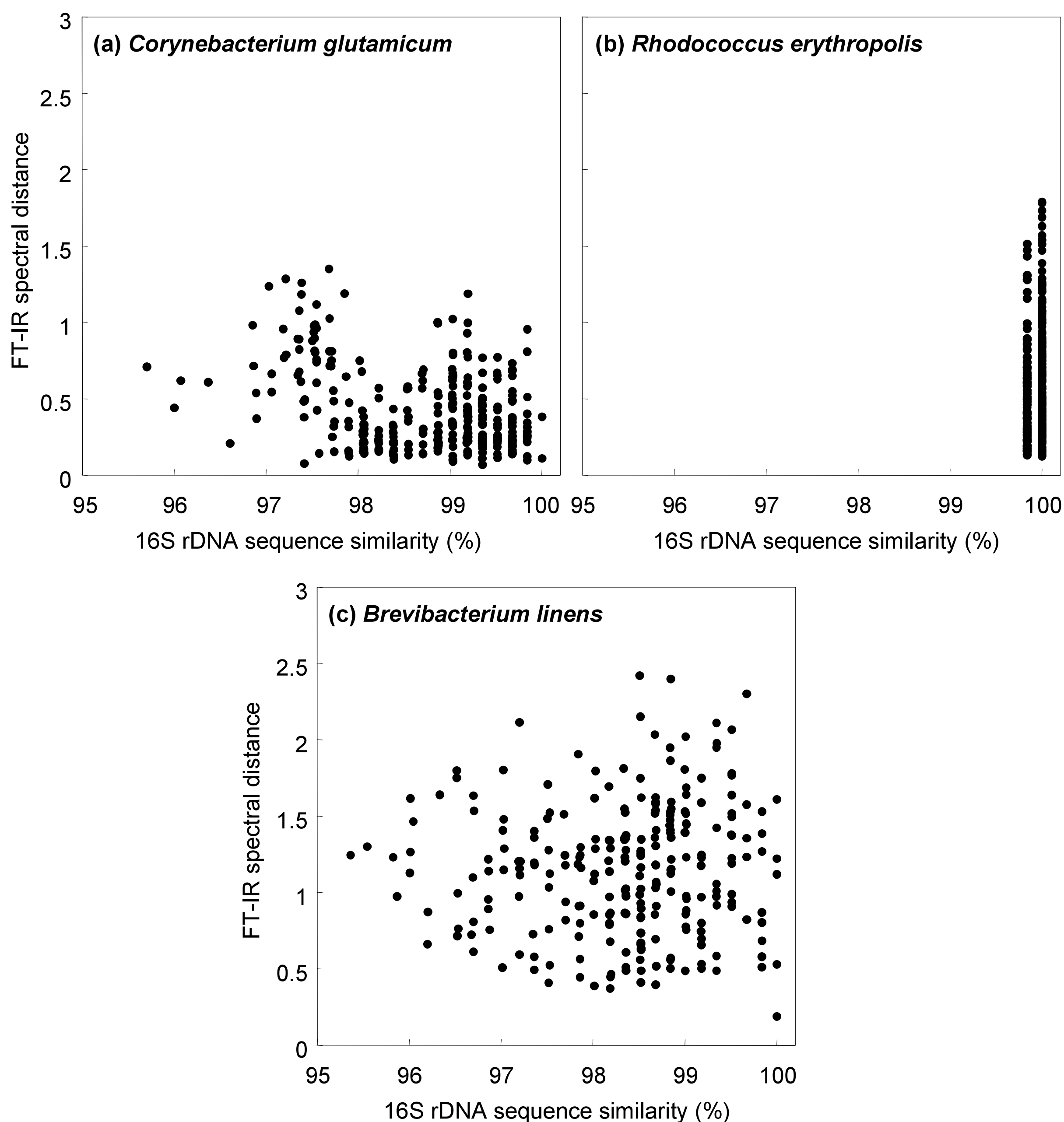

Fig. 3.3: Correlation graphs between corresponding pair-wise spectral distance values and 16S rDNA sequence similarity values.

In contrast to our expectations, no correlation was found between 16S rDNA sequence similarity and FT-IR spectral distance. This means that diversification of 16S rDNA sequences and the microevolutionary change of the cellular overall characters measured by FT-IR spectroscopy is not coupled. For good reasons, 16S rDNA sequence comparison is considered to be the current "gold standard" for elucidating bacterial phylogeny (Amann *et al.,* 1994, Ludwig *et al.*, 1998, Kolbert & Persing, 1999, Ludwig & Schleifer, 1999). Therefore, FT-IR spectroscopy cannot be used to assess the evolutionary relations of strains within these actinomycete species. It has been demonstrated previously that FT-IR spectroscopy is not a reliable parameter to establish taxonomic relationships between different genera of yeasts (Kümmerle *et al.,* 1998).

3.5 Acknowledgments

The authors wish to thank Wolfgang Ludwig for providing the ARB software environment with the ARB 16S rDNA database and for giving valuable information and help whenever necessary. Thanks are due to Michael Kümmerle and Felix von Stetten for introducing H.O. to FT-IR spectroscopy and molecular microbiology, respectively. We are grateful to several starter culture companies for supplying us with strains. The technical assistance of Sven Illgner, Patrizia Hägele and Louise Arnold is greatly appreciated. Cornelia Fischer is acknowledged for excellent technical assistance. This work was supported by the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn), the AiF (Arbeitskreis für industrielle Forschung) and the Ministry of Economics and Technology, Project Nr. 11627N.

4 Quantification of microorganisms in binary mixed populations by Fourier-transform infrared (FT-IR) spectroscopy

4.1 Abstract

Fourier Transform Infrared (FT-IR) spectroscopy was used for the first time to determine the ratios of different microorganisms in mixtures. Exemplarily, systems composed of two food-associated yeast species (*Saccharomyces cerevisiae* / *Hanseniaspora uvarum*) and two yoghurt lactic acid bacteria (*Lactobacillus acidophilus* / *Streptococcus salivarius* ssp. *thermophilus*) were investigated. Determination of the cell number ratio in the lactic acid bacteria system was possible with a minimal prediction accuracy of ± 16 ratio percentage points while the minimum accuracy of prediction in the yeast two-component system was ± 4 % (both at a 95 % confidence level). These results show that FT-IR spectroscopy is potentially a rapid method for the quantification of cell ratios in mixtures of two different microorganisms, provided that the cell ratio does not drop below a certain, system-specific threshold.

4.2 Introduction

Quantification of microorganisms in mixed populations can be achieved by differential plating, nucleic acid techniques including oligonucleotide probes and genotyping (Amann *et al.* 1990, Charteris *et al.* 1997, Erlandson & Batt 1997, Kosse *et al.* 1997) or enzymatic methods (Boquien *et al.* 1989). Techniques using synthetic rRNA-targeted hybridisation probes are particularly promising for detection, enumeration and identification *in situ* or after differential plating since their specificity can be adjusted to fit any taxonomic rank (Charteris *et al.* 1997). However, although highly specific, the majority of these methods still require considerable experience as well as time and are therefore less suitable for use in a routine laboratory.

Here we investigate a method for quantification of microorganisms in mixtures using Fourier transform infrared spectroscopy. The identification of micro-organisms with this technique was originally suggested by Naumann and coworkers (Helm *et al.* 1991b, Naumann *et al.* 1991a and b). With this method, unknown microorganisms can be identified very easily

and quickly once an extensive database (spectral reference library) is available. For identification, the infrared spectrum of an unknown species is compared with all spectra present in the reference library and matched to the library strain whose spectrum is most similar. The application of this technique has been reported for some species of the genera *Bacillus* (Beattie *et al.* 1998, Lin *et al.* 1998), *Lactobacillus* (Curk *et al.* 1994), *Listeria* (Holt *et al.* 1995, Lefier *et al.* 1997), *Streptococcus* (Goodacre *et al.* 1996), for actinomycetes (Haag *et al.* 1996) and for urinary tract infection bacteria (Goodacre *et al.* 1998). An extensive FT-IR library for the identification of fermentative yeasts allowed the correct identification of 97.5 % of 722 field isolates (Kümmerle *et al.* 1998).

The quantitative differentiation of individual species present in a mixed population using FT-IR spectroscopy has not yet been described but would offer the advantage of being comparably fast and easy to perform.

Infrared spectra show only little information that is visible to the eye since the information is hidden behind small differences that affect the entire spectral structure. Over the last years, powerful statistical methods have been introduced to chemometrics. An approach using not only one (or few) peaks but the entire spectral variation for computation is termed multivariate calibration. One of the most important and powerful algorithms is the Partial Least Square (PLS) regression which is used in this study.

4.3 Material and methods

4.3.1 Microorganisms

A two-component yeast system with *Saccharomyces cerevisiae* CBS 1174^T and *Hanseniaspora uvarum* CBS 314^T and a two-component lactic acid bacteria system with *Lactobacillus acidophilus* DSM 20079^T and *Streptococcus salivarius* ssp. *thermophilus* DSM 20617^T were used.

4.3.2 Sample preparation

Yeast strains were grown on yeast-extract-glucose-chloramphenicol-(YGC) agar (Merck) at 27 °C (Kümmerle *et al.* 1998). Bacterial strains were incubated under anaerobic conditions at 37 °C. *S. thermophilus* was cultivated on M17 agar from TERZAGHI (Merck; with the addition of 20 g l^{-1} glucose) while *L. acidophilus* was grown on MRS agar (Oxoid, Wesel, Germany). All strains were grown as lawns of cells on the agar surface (Kümmerle *et al.* 1998). After an incubation time of 24 ± 0.5 h, cells were suspended in distilled water to

yield separate suspensions for each microorganism. Optical densities of the suspensions were adjusted to yield optimal infrared spectral quality. The suspensions of the two species of a particular two-component system were mixed in different cell number ratios based on calibration curves of cell number against optical densities. An aliquot of each mixture was transferred to a ZnSe optical plate (sample holder) where they were dried for 55 min at 42 °C and used directly for FT-IR spectroscopy. The resulting spectra of the mixtures produced in one run of sample preparation comprised one data set.

4.3.3 FT-IR spectroscopy

All spectra were recorded between wave numbers 4,000 and 500 cm^{-1} with an IFS-28B FT-IR spectrometer (Bruker Optik GmbH, Germany). For data processing, the software OPUS (Bruker) was used. The adjustments of instrument parameters were set according to the suggestions of the FT-IR workgroup of the Robert-Koch-Institut, Berlin, Germany (FT-IR workgroup RKI/BIAM, 1992, Kümmerle *et al.* 1998).

4.3.4 PLS model

In order to perform a Partial Least Square (PLS) regression for a particular system, the information of the spectra and the corresponding cell number ratios must be correlated. The variations in the spectra are used to set up a new data point matrix and to determine the so-called Eigenvectors (i.e. the principal components). Subsequently, in lieu of comparing the whole spectra only these Eigenvectors are employed. In the case of a PLS regression, the Eigenvectors are sorted in decreasing order, and only the first few are selected which represent the majority of the relevant variations in the spectra. The number of PLS vectors used to establish a calibration model is termed rank. Generally, the first 15 PLS vectors were calculated from a calibration model based on the spectra of six different data sets. The number of PLS vectors resulting in minimal errors corresponded to the optimum rank of the analytical model. For further information on PLS regression see, e.g., Haaland and Thomas (1988) or Martens and Næs (1989).

All calculations were performed using the OPUS/QUANT-2 software package (Bruker). Instead of the original spectra, their (vector normalized) first or second derivatives were used for analysis of the bacterial and yeast system, respectively. For the bacterial calibration model, a Straight Line Subtraction was performed as a preprocessing procedure of the data and the frequency range from 1800-700 cm^{-1} was selected. For the yeast calibration model, a Multiple Scattering Correction of the data and the selection of an additional

frequency window from 3100-2800 cm^{-1} were found to be optimal. Two normalized absorbance spectra of both components of the bacterial system are shown in Fig. 4.1 as an example.

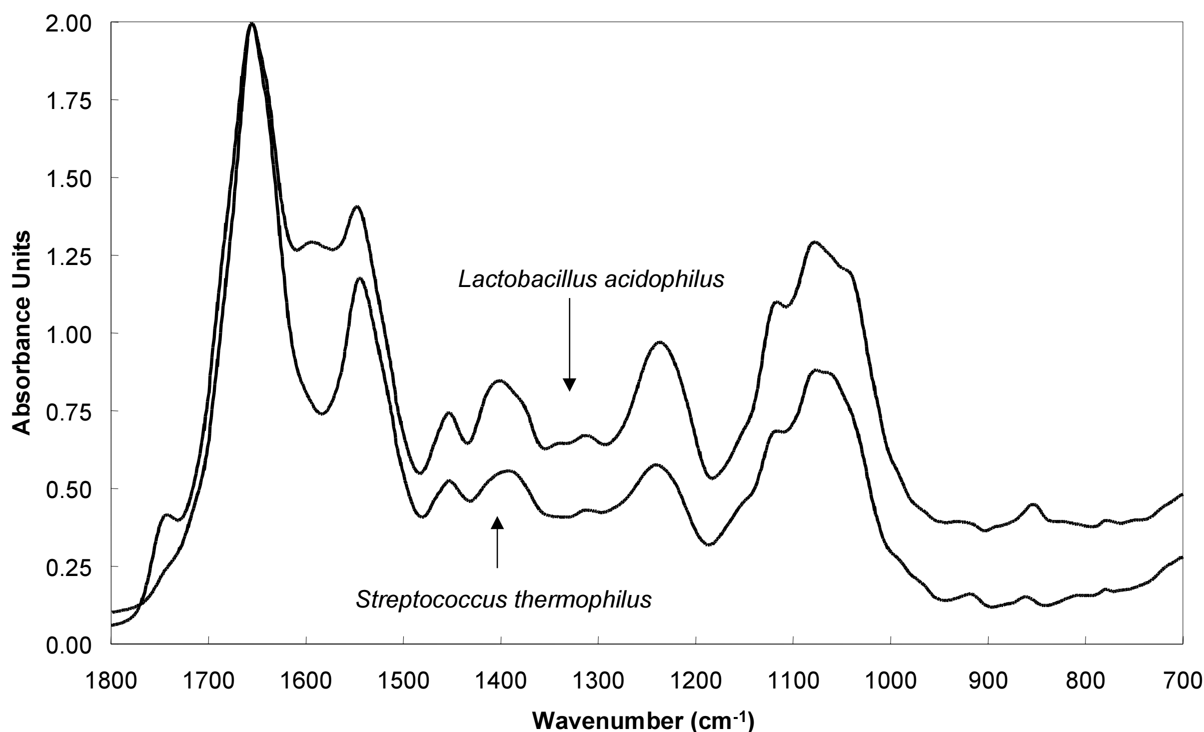

Fig. 4.1: Normalized absorbance spectra of *Lactobacillus acidophilus* and *Streptococcus salivarius* subsp. *thermophilus* between wavenumbers 1800 and 700 cm^{-1}, the frequency range selected to calculate the bacterial calibration model.

4.4 Results and discussion

Two two-component systems with mixtures of lactic acid bacteria (*Streptococcus salivarius* ssp. *thermophilus* / *Lactobacillus acidophilus*) and food-associated yeasts (*Saccharomyces cerevisiae* / *Hanseniaspora uvarum*) were examined. In order to set up calibration models which could subsequently be applied to the determination of the mixture ratios of the two microorganisms under investigation, repeat measurements were carried out. Each organism was grown six times in pure culture. After an incubation time of 24 h, mixtures of the cells were prepared in predefined cell number ratios and their spectra were recorded.

An internal cross validation was performed: all spectra corresponding with the same mixture ratio were first excluded and a model was calculated based on the remaining spectra, which was subsequently used to predict the ratio values of the excluded spectra. This procedure was repeated for all cell ratio values. The error of the model is given as the Root

Mean Square Error of Cross Validation (RMSECV). Since in this case, the model contains the spectra of all data sets available, all possible differences due to variations in the experimental conditions or natural variations are taken into account.

For an external validation, the spectra of all independent data sets were divided into two groups of equal size. One group (the calibration set) provided the basis for the calculation of a calibration model, while the corresponding cell number ratios of the spectra in the other group (comprising the test set) were predicted by this model in order to validate it. The prediction error of the model is given as the Root Mean Square Error of Prediction (RMSEP). Distribution of data between both calibration set and test set was done in order to reduce the RMSEP to the smallest value possible (minimized RMSEP). In this case, ratio values of data sets not contained in the calibration set are predicted. Thus, the RMSEP reflects the model‘s capability to analyse unknown mixtures quantitatively. Fig. 4.2 displays the graphical results of the external validation for the two species pairs.

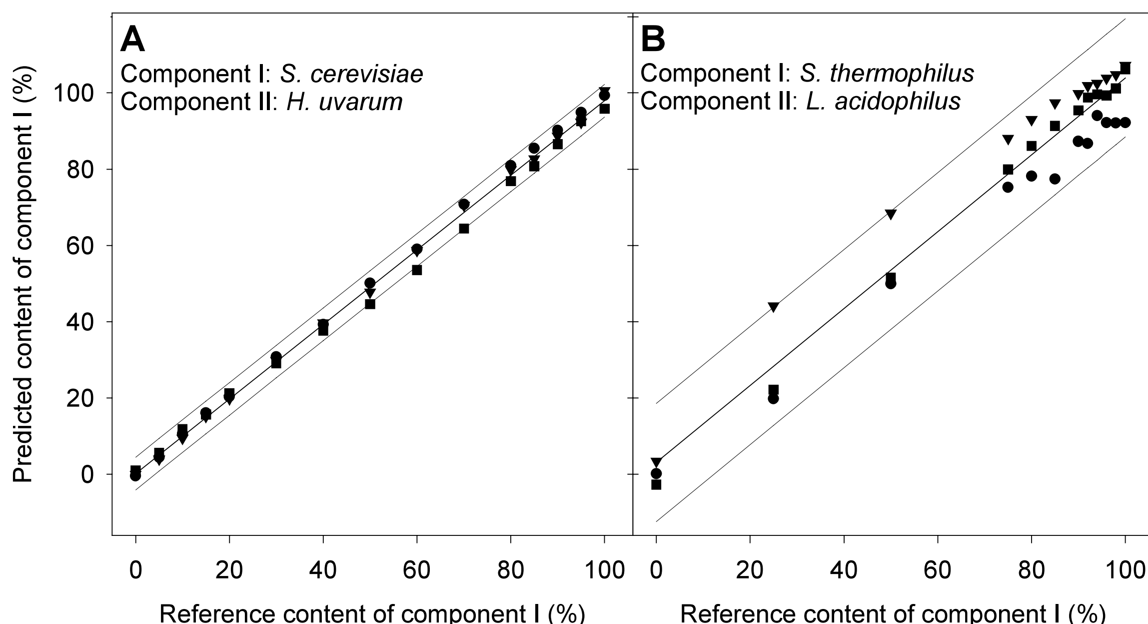

Fig. 4.2: Predicted content versus reference content of component I in a microbial two-component mixture applying an external validation of the calibration model. Linear regression with 95 % confidence bands are calculated from the measurements of three data sets. Symbols represent the test sets‘ content values of component I predicted by the respective calibration models of each system. **A** Component I: *Saccharomyces cerevisiae,* II: *Hanseniaspora uvarum*; R^2 = 0.9972, minimized RMSEP = 2.1. **B** Component I: *Streptococcus salivarius* ssp. *thermophilus* II: *Lactobacillus acidophilus;* R^2 = 0.9541, minimized RMSEP = 7.8.

In Tab. 4.1, the results obtained with both validation methods are compared. For both internal and external validation, the models‘ prediction accuracy is expressed by 95 % confidence intervals which can directly be calculated from the given errors RMSECV and RMSEP, respectively, if a statistically normal distribution of data points is assumed. Fig. 4.2a

shows the predicted content of *S. cerevisiae* as a function of the reference content in a mixture with *H. uvarum* in an external validation of the calibration model. Fig. 4.2b displays the same graph for *S. thermophilus* in a mixture with *L. acidophilus*. In the yeast two-component system, the RMSECV and minimized RMSEP were found to be approximately equal (Tab. 4.1), indicating that the differences between spectra from different sample set preparations caused by preparational variations were about as great as differences within samples of the same set.

Tab. 4.1: Mean errors and 95 % confidence intervals of prediction for optimal calibration models of both two-component microorganism systems investigated, determined by internal and external model validation. For error calculations, an optimum rank of 7 was found. 95 % CI: 95 % confidence interval of prediction, RMSECV: Root Mean Square Error of Cross Validation, RMSEP: Root Mean Square Error of Prediction

	Cross Validation		External Validation	
	RMSECV	95 % CI (% points)	minimized RMSEP	95 % CI (% points)
Saccharomyces cerevisiae / Hanseniaspora uvarum	2.6	5.1	2.1	4.2
Lactobacillus acidophilus / Streptococcus salivarius ssp. *thermophilus*	1.7	3.5	7.8	15.5

With the yeast system, reproducibility was quite high. In contrast, the error in the bacterial two-component system was considerably larger, resulting in wider 95 % confidence bands (compare Fig. 4.2a,b). A relatively high RMSEP reflects large differences between the single independent data sets. One reason for these differences might be the natural biological variability of the bacteria. In addition, the selected concentration values were not evenly distributed over the whole concentration range. Instead, the majority falls in the range between 80 and 100 % *Streptococcus* cells, corresponding with concentration values in probiotic dairy products. For the creation of a more stable model, a higher number of calibration data must be produced.

4.5 Conclusion

When comparing the accuracy of quantification found in this feasibility study to techniques which, at any step, rely on conventional plating methods having an accuracy of approximately ± 30 % (VDLUFA Methodenbuch, 1985), the accuracy obtained was already

much better. However, FT-IR spectroscopy appears to be limited to systems with defined components where the cell number of either component does not drop below certain values. The threshold below which a reliable detection and quantification of one component is not possible anymore, clearly depends on the characteristics of the microorganisms involved. The specificity of the calibration model, for instance, was too low to calculate the content of *L. acidophilus* in the presence of an excess of *S. thermophilus* with the degree of accuracy that would be required for the analysis of probiotic dairy products, while quantification of *S. thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* in yoghurt is well within the range of the method. By using a higher number of repeat measurements, models with higher stabiblity may be generated, which could eventually turn FT-IR spectroscopy into a reliable technique for the quantification of certain mixtures of microorganisms.

4.6 Acknowledgment

This research project was supported by the FEI (Forschungkreis der Ernährungsindustrie e.V., Bonn), the AiF and the Ministry of Economics and Technology, Project No. 11627N.

5 General Discussion

5.1 Identification of coryneform bacteria and related taxa

In order to allow a rapid and simple identification of coryneform bacteria and related taxa, a comprehensive FT-IR spectral reference library was established. The database consists of averaged infrared spectra from 730 reference strains belonging to 220 different coryneform species and phylogenetically related or morphologically similar taxa out of 46 genera, 40 of them from the two suborders *Micrococcineae* and *Corynebacterineae* (*Actinomycetales, Actinobacteria*). Tab. 2.1 lists the different species while a list of the individual strains can be viewed in the appendix. 192 species are represented by type strains (87 %). The identification capacity of the database was assessed by an external validation which was carried out as described in Sec. 2.3.6. 544 strains from 54 species with at least three strains per species out of 16 genera were used for proof of principle. 87.3 % of the strains were correctly identified at the species level and 95.4 % at the genus level. 12.7 % did not result in a correct identification at the species level, 1.3 % of which were not identified while 11.4 % were misidentified (Fig. 2.2b). The unexpectedly high percentage of misidentified strains represented a call to examine the results reported in the literature for the identification of coryneform bacteria by different methods and to search for underlying reasons.

Several publications report on the identification capacity of various methods dealing with different groups of microorganisms (Tab. 2.2). The identification of coryneform bacteria and related genera has been evaluated by using the Biolog Identification System (Biolog), API Coryne Database (bioMérieux) and the RapID CB Plus system (Remel), three commercially available identification kits based on the analysis of physiological properties. The latter two identification databases contain only a limited number of species, most of which are clinically relevant coryneform bacteria. The percentage values of correct identification achieved in external validations differ greatly between these systems, yielding best results for the API Coryne Database (Tab. 2.2).

Furthermore, several studies analyzed the capacity for identification of microorganisms by FT-IR. Most studies have only performed internal validations where the percentage of correct identification is generally higher than in the case of an external validation. Moreover, the size of the spectral libraries under investigation has been rather limited except for the databases reported by Kümmerle *et al*., (1998) and by this study.

When comparing published identification results, it is therefore necessary to compare the size and composition of the databases evaluated as well as the size and composition of the set of test organisms used for validation. Moreover, the kind of validation performed (internal vs. external) must be noted carefully since the results of correct identification will largely be influenced by the presence or absence of the tested strains in the library. The effects of the composition and size of both database and test set on the identification quality on the one hand is hardly separable from the effect of the kind of validation performed on the other hand with the limited data available. Generally, an internal validation performed with a small heterogeneous database (i.e. only few strains per genus and genera from several different phylums) (Tab. 2.2, Helm *et al.*, 1991a) will yield much higher values for correct identification than an external validation performed on a rather homogeneous database (i.e. closely related taxa) due to a higher percentage of misidentified strains in the latter (e.g. Funke *et al.*, 1998). Misidentification occurs if a test strain is less similar to other strains of its own species than to a strain of a different species.

Results are also influenced by the intrataxal separation of the particular group of organisms studied: In case of comparable heterogeneity of databases and similar heterogeneity of the test sets (i.e. numbers of strains per species or genus in the same order of magnitude), a highly different result of correct species allocation in an external validation points to a different intrataxal separapability between the organisms analyzed, e.g. between yeasts and coryneform bacteria (Kümmerle *et al*., 1998 vs. this study). Our results seem to indicate that yeast taxa can be considered more distinctive than coryneform taxa since the percentage of misidentified isolates was found to be much lower. It appears that, in constrast to yeast taxa, coryneform taxa seem to be rather contiguous, exhibiting a certain amount of overlap of different taxonomical characteristics. Taxa merging into each other lacking rather discrete boundaries at the species or genus level will easily be confused, resulting in high percentages of misidentified isolates. This hypothesis, drawn from comparison between identification results of two phylogenetically well separated kinds of microorganisms by the same technique (FT-IR) is further supported by the observation that a high percentage of misidentification has also been noted for the identification of coryneform bacteria by physiological properties (cf. Tab. 2.2). Reference to the literature reveals that even though a wide spectrum of chemotaxonomical differentiation methods have been applied to the classification of actinobacterial taxa, differentiation down to the species level could not be achieved satisfactorily in all cases, sometimes not even to the genus level. Each of these techniques allows differentiation between some taxa while they fail to separate others

(Altenburger *et al.,* 1997, Busse & Schumann 1999, Felske *et al.,* 1999, Kämpfer & Kroppenstedt 1996). Stackebrandt *et al.* (1997) have noted that the rich chemical, morphological and physiological diversity of phylogenetically closely related genera makes the description of families and higher taxa so broad that they become meaningless for the description of the enclosed taxa. Most main actinobacterial lines of descent, described as orders, suborders and families, are not well separated and the statistical significance of branching points is low. Because of the lack of common properties of phylogenetic significance shared by most taxa of a higher taxon, their delineation from each other is somewhat arbitrary and artificial (Stackebrandt & Schumann, 2000). Therefore, even though actinobacterial taxa form a systematically well derivable group, due to their overlapping (or even contradictory) distribution of taxonomical characters, the possibilities for their differentiation by non-genotypical methods remain somewhat limited.

Examples for a certain degree of interference between different taxonomical characteristics and, as a result, diverging taxonomical opinions can be taken from the ongoing discussion as to the separation or unification of different genera such as *Microbacterium*/*Aureobacterium* (Rainey *et al.*, 1994, Takeuchi & Hatano, 1998), *Cellulomonas*/*Oerskovia* (Stackebrandt *et al.*, 1982, Stackebrandt & Prauser, 1994), or *Corynebacterium*/*Turicella* (Funke *et al.*, 1994, Pascual *et al.*, 1995, Ruimy *et al.*, 1995). These genera are or appear to be phylogenetically intermixed but can be differentiated by means of other taxonomic properties.

Taxonomically overlapping reference species are likely to result in an FT-IR hit list confusion and misidentification of test strains. Thus, an extensive representation of intraspecies and intragenus variety by a substantial number of reference strains is indeed critical for a high percentage value of correct species identification, but will not decrease confusion within a highly contiguous group of organisms. On the other hand, with regard to the behavior of a single strain, e.g., in epidemiological analyses, a good coverage of intraspecies variety by a large number of reference strains will decrease the probability for differentiation of this particular strain from the others, (i.e., yield low values of correct identification at the strain level in general), especially if the strains are very similar, but will yield high values of correct species identification. From the data presented in Fig. 2.3, it can be expected that, on the average, approximately five to ten different strains per species in a coryneform FT-IR database are appropriate in order to achieve a reasonable identification capacity. A considerable advantage of working with an FT-IR spectral library for identification is the users' possibility to influence the size and composition of the database

themselves, i.e., by continuously adding strains in order to keep the reference library up to date or to include own sets of microorganisms.

5.2 Infraspecific diversity of *Brevibacterium linens*, *Corynebacterium glutamicum* and *Rhodococcus erythropolis*

In order to assess infraspecific variability, the classification of all strains under investigation must be unequivocal at the species level. 27 of the 74 strains which were used for this investigation were originally misclassified and carried wrong or invalidly described species names (Tab. 3.1). In accordance with their reported numerical taxonomical properties (Tab. 3.1: Seiler, 1983, Kämpfer *et al.,* 1993, Kämpfer & Kroppenstedt, 1996) as well as their infrared spectral data and 16S ribosomal gene sequence data, these strains were reclassified as belonging to one of the three species under investigation (compare Sec. 3.4.1). This demonstrates that FT-IR spectroscopy is a fast and reliable screening method to classify unknown actinomycetes at the species level (compare Tindall *et al.,* 2000).

Reliable assessment of infraspecific variability not only depends on the correct strain allocation to a particular species but is furthermore only possible if the strains under investigation are of independent origin. Tab. 3.1 lists the original depositor or source together with the isolation locus for each strain as far as such information was available from the official collections. These data are not always precise enough to derive firm conclusions as to where the strains originated. It is, however, probable that most of the strains from *C. glutamicum* and *R. erythropolis* were isolated from East Asian environmental sources and that the *B. linens* strains came from Middle-European sources. It can be assumed that the majority of these strains represent different isolates, except for five *C. glutamicum* strains which appear to be mutants of the type strain (Tab. 3.1).

The 25 strains of *C. glutamicum* exhibited a comparatively high degree of spectral similarity (Fig. 3.2a) which stood in sharp contrast to the low degree of 16S rDNA sequence similarity noted between the strains. Even though each of the strains showed a sequence similarity of at least 97.5 % to the type strain sequence, pairwise sequence comparison between the individual strains revealed similarities of as little as 95.7 %.

In contrast to *C. glutamicum*, the 26 strains of *R. erythropolis* displayed an extremely high level of 16S rDNA sequence similarity while showing a rather high variability within their FT-IR spectra (Fig. 3.2b). This variance is in favour of an independent origin of these isolates in spite of the high similarity of the 16S rDNA sequences.

The highest overall infraspecific diversity was noted for *B. linens*. Wide value distributions resembling Gaussian bell curves of both pairwise spectral distance and 16S rDNA similarity were observed between the 23 strains investigated (Fig. 3.2c). Similar to *C. glutamicum*, 16S rDNA sequence similarities as little as 95.4 % were noted between different strains in pairwise comparison, although each of the strains showed a minimal sequence similarity of 96.7 % to the type strain sequence.

In-depth studies of infraspecific variability using comparative 16S rDNA sequence analysis of a large number of strains are comparatively rare (Tab. 3.2). 16 strains of *Xylella fastidiosa* exhibited a 16S rDNA sequence similarity of 99.0-100 % over the complete sequence (Chen *et al.*, 2000), similar to *R. erythropolis*. *Campylobacter hyointestinalis* (Harrington *et al.*, 1999) and *Streptococcus suis* (Chatellier *et al.*, 1998) displayed a high inter-strain sequence variability similar to *C. glutamicum* and especially *B. linens*. Even though the pairwise similarity values are lower than the cut-off value of 97 % sequence similarity for species delimitation as defined by Stackebrandt and Goebel (1994), none of the authors concluded it sensible to change the then-existing species delimitation of their respective species under study except for Ridell *et al.* (1995) who examined 14 strains of *Hafnia alvei*. Stackebrandt & Goebel (1994) also recommended that this threshold value be lowered in order to maintain flexibility in the phylogenetic definition of a species.

According to Clayton *et al.* (1995), the variability noticed between different published 16S rDNA sequences of the same species in sequence databases such as GenBank may represent interoperon variation within a strain, strain-to-strain variation within a species, inadequate taxon delimitation, sequencing error or other laboratory error. While some actinomycetes such as *Thermobispora bispora* (Wang *et al.,* 1997), *Thermomonospora chromogena* (Yap *et al.,* 1999), *Mycobacterium celatum* (Reischl *et al.*, 1998) and *Mycobacterium terrae* (Ninet *et al.,* 1996) have been found to contain several types of transcriptionally active rRNA operons whose sequences may differ by as much as 6 %, no information was available addressing this question for the three species analyzed in this study. Felske *et al.* (1999) analyzed eight coryneform genera in terms of their 16S rDNA heterogeneity. While neither of the three genera investigated in this study were included, the authors found sequence heterogeneities only in the genus *Curtobacterium* and therefore suggested that the heterogeneity of 16S rRNA genes in the genome is not very common within coryneform bacteria. Sequencing errors cannot be ruled out completely. However, by using consensus filters, regions showing exceptionally high variabilities were excluded from the analyses.

FT-IR spectroscopy is a whole-cell fingerprint technique that analyzes microbial cells on a completely different basis than techniques comparing a small conserved region of the genome such as the 16S rDNA gene. FT-IR spectroscopy, nevertheless, might reflect the diversity obtained by sequence comparison at least to some degree. In order to determine whether a correlation exists between the pairwise infrared spectral distance values and 16S rDNA similarity, spectral distances were plotted versus their corresponding 16S rDNA similarity values for each pair of strains (Fig. 3.3). This analysis shows that within *C. glutamicum* and *B. linens* (Fig. 3.3a and c) there are strain pairs which are spectrally very close but have a low 16S rDNA similarity (bottom left of each plot). Others display a highly similar 16S rDNA but have very different infrared absorption spectra (top right of each plot). In the former cases, the 16S rDNA sequence analysis has a better resolution than FT-IR spectroscopy whereas in the latter cases, different strains can be distinguished via FT-IR spectroscopy which cannot be distinguished by partial 16S rDNA analysis. The strains of *R. erythropolis* (Fig. 3b) differed at only two nucleic acid positions and therefore, the entire observed spectral variability is plotted versus sequence similarities of 99.9 and 100 percentage points. However, a considerable number of strain pairs can be distinguished by FT-IR spectroscopy.

5.3 Quantification of microorganisms in binary mixed populations

FT-IR spectroscopy has been applied to the identification and classification for several groups of microorganisms previously but all of these studies have qualitatively dealt with pure cultures. Except for preliminary investigations performed by Kümmerle (1999), the quantitative differentiation of individual species present in a mixed population by FT-IR spectroscopy had not been described. In order to evaluate if this could be a potential application of the technique, two two-component systems with mixtures of lactic acid bacteria (*Streptococcus salivarius* ssp. *thermophilus* / *Lactobacillus acidophilus*) and food-associated yeasts (*Saccharomyces cerevisiae* / *Hanseniaspora uvarum*) were examined. In order to set up calibration models which could subsequently be applied to the determination of the mixture ratios of the two microorganisms under investigation, repeated measurements were carried out. Each organism was grown six times in pure culture. After an incubation time of 24 h, mixtures of the cells were prepared in predefined cell number ratios and their spectra were recorded. Both an internal validation ("cross validation") and an external validation were performed to assess the prediction models' quality (procedures described in Sec. 4.4). In the former case, the error of the model is given as the Root Mean Square Error of Cross

Validation (RMSECV). Since in this case, the model contains the spectra of all data sets available, all possible differences due to variations in the experimental conditions or natural variations are taken into account. In the external validation, the prediction error of the model is given as the Root Mean Square Error of Prediction (RMSEP). In this case, ratio values of data sets not contained in the calibration set are predicted. Thus, the RMSEP reflects the model‘s capability to analyse unknown mixtures quantitatively. Fig. 4.2 displays the graphical results of the external validation for the two species pairs. In Tab. 4.1, the results obtained with both validation methods are compared. For both internal and external validation, the models‘ prediction accuracy is expressed by 95 % confidence intervals which can directly be calculated from the given errors RMSECV and RMSEP, respectively, if a statistically normal distribution of data points is assumed. In the yeast two-component system, the RMSECV and minimized RMSEP were found to be approximately equal (2.6 and 2.1, respectively), indicating that the differences between spectra from different sample set preparations caused by preparational variations were about as great as differences within samples of the same set. In this system, reproducibility was quite high. In contrast, the REMSEP of the bacterial two-component system was considerably larger (7.8), resulting in wider 95 % confidence bands (compare Fig. 4.2a,b). A relatively high RMSEP reflects large differences between the single independent data sets. One reason for these differences might be the natural biological variability of the bacteria. In addition, the selected concentration values were not evenly distributed over the whole concentration range. Instead, the majority falls in the range between 80 and 100 % *Streptococcus* cells, corresponding with concentration values in probiotic dairy products. For the creation of a more stable model, a higher number of calibration data could be produced.

6 General Conclusions

FT-IR spectroscopy is a powerful technique to be used in microbial analysis since it can successfully be applied to a variety of problems, some of which have been investigated in this thesis.

(i) The FT-IR spectral database is a valuable tool for the rapid, simple and cost-efficient identification of coryneform strains from a variety of sources. Comparison of identification results reported in the literature showed that an overall correct identification of more than 95 % at the species level has not been achieved by any method so far. Therefore, it can be concluded that coryneform bacteria are a group of organisms whose taxa are not well separated by phenotypical characteristics and, therefore, are difficult to be identified properly. The identification values obtained in this work are well within the range of those having been reported in the literature for the identification of these high G+C gram-positive bacteria. In order to obtain an identification at the species level with a reasonable probability, each species should be represented by an approximate number of five to ten strains in the database. For improvement of the library's capability for correct identification of coryneform isolates, the number of strains should be increased constantly both for intraspecific representation and for coverage of relevant taxa in general.

(ii) FT-IR spectroscopy is also a valuable method for assessing the infraspecific diversity of coryneform bacteria. Moreover, it was found to be a rapid and reliable tool for screening of similar isolates. Comparison of infraspecific 16S rDNA similarities suggested that *R. erythropolis* strains show a very low diversity, *B. linens* displays a high variability and *C. glutamicum* may have an intermediate position. However, no correlation of FT-IR spectral similarity and 16S rDNA sequence similarity below the species level (i.e., between strains of one species) was observed. This means that diversification of 16S rDNA sequences and the microevolutionary change of the cellular overall characteristics measured by FT-IR spectroscopy is not coupled. For good reasons, 16S rDNA sequence comparison is considered to be the current "gold standard" for elucidating bacterial phylogeny. Therefore, FT-IR spectroscopy cannot be used to assess the evolutionary relations of strains within these actinomycete species.

(iii) Quantitative analysis of mixed populations represents a challenge to FT-IR spectroscopy. When the quantification accuracy found in this feasibility study is compared to techniques which, at any step, rely on conventional plating methods that have an accuracy of approximately ± 30 %, the accuracy obtained here was significantly better. Nevertheless, FT-

IR spectroscopy appears to be limited to systems with defined components where the cell number of either component does not drop below certain values. The threshold below which a reliable detection and quantification of one component is not possible anymore clearly depends on the characteristics of the microorganisms involved. The specificity of the calibration model, for instance, was too low to calculate the content of *L. acidophilus* in the presence of an excess of *S. thermophilus* with the degree of accuracy that would be required for the analysis of probiotic dairy products, while quantification of *S. thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* in yoghurt is well within the range of the method. By using a higher number of repeated measurements, models with higher stabiblity may be generated, which could eventually turn FT-IR spectroscopy into a reliable technique for the quantification of certain mixtures of microorganisms.

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Appendix

List of reference strains for the FT-IR identification database

No.	Species Name or Strain Denotation	Code	T†	WS No.	Glyc. No.‡	Source	Alternative Denotation	Reclassified as *
1.	*"Arthrobacter hydrocarboglutamicus"*			1072	92	ATCC	ATCC 15583	*Rhodococcus erythropolis*
2.	*"Arthrobacter hydrocarboglutamicus"*			1458	112	F. Fiedler	ATCC 21190	*Rhodococcus erythropolis*
3.	*"Arthrobacter oxamicetus* subsp. *propiophenicolus"*			1076	94	ATCC	ATCC 21814	*Rhodococcus erythropolis*
4.	*"Arthrobacter oxamicetus"*			1075	93	ATCC	ATCC 21788	*Rhodococcus erythropolis*
5.	*"Arthrobacter petroleophagus"*			1078	64	ATCC	ATCC 21494	*Gordonia rhizosphera*
6.	*"Arthrobacter pyridinolis"*			2135	257	T. A. Krulwich		*Arthrobacter* sp.
7.	*"Arthrobacter rubellus"*			1081	65	ATCC	ATCC 21495	*Gordonia rubropertinctus*
8.	*"Brevibacterium album"*			1082	161	ATCC	ATCC 15111	*Arthrobacter globiformis*
9.	*"Brevibacterium alkanolyticum"*			1985	89	IFO	ATCC 21033	*Rhodococcus equi*
10.	*"Brevibacterium butanicum"*			1083	20	ATCC	ATCC 21196	*Rhodococcus erythropolis*
11.	*"Brevibacterium chang-fua"*			1084	389	ATCC	ATCC 14017	*Corynebacterium glutamicum*
12.	*"Brevibacterium fulvum"*			1463	299	F. Fiedler	AJ 1485	*Brevibacterium linens*
13.	*"Brevibacterium glutamigenes"*			1086	390	ATCC	ATCC 13747	*Corynebacterium glutamicum*
14.	*"Brevibacterium healii"*			1088	21	ATCC	ATCC 15527	*Rhodococcus erythropolis*
15.	*"Brevibacterium helvolum"*			1753	223	ATCC	ATCC 13715	
16.	*"Brevibacterium insectiphilium"*			1467	208	F. Fiedler	ATCC 21686	*Arthrobacter ureafaciens*
17.	*"Brevibacterium insectiphilium"*			1960	241	UQM	Davis` collection No. 92	*Microbacterium esteraromaticum*
18.	*"Brevibacterium insectiphilum"*			1091	162	ATCC	ATCC 21110	*Arthrobacter nicotinovorans*
19.	*"Brevibacterium minutiferula _2"*			1438_2	700, 709	Rubber Inst.	Rubber 463	*Kocuria rhizophila*
20.	*"Brevibacterium minutiferula _1"*			1438_1	616	Rubber Inst.	Rubber 463	*Microbacterium arborescens*
21.	*"Brevibacterium minutiferula"*			1471	437	F. Fiedler	AJ 1482	*Microbacterium lacticum*
22.	*"Brevibacterium seonmiso"*			1097	394	ATCC	ATCC 14915	*Corynebacterium glutamicum*
23.	*"Brevibacterium sociovivum"*			1963	625	UQM	Davis' collection No. 91	*Corynebacterium xerosis*
24.	*"Brevibacterium sterolicum"*			1102	67	ATCC	ATCC 21387	*Rhodococcus equi*
25.	*"Brevibacterium taipei"*			1103	397	ATCC	ATCC 13744	*Corynebacterium glutamicum*
26.	*"Brevibacterium thiogenitalis"*			1104	398	ATCC	ATCC 19240	*Corynebacterium glutamicum*
27.	*"Corynebacterium acetoacidophilum"*			1111	399	ATCC	ATCC 13870	corresponding to *Corynebacterium glutamicum*
28.	*"Corynebacterium acetoacidophilum"*			1475	438	F. Fiedler	ATCC 21350	correspoding to *Corynebacterium glutamicum*
29.	*"Corynebacterium acetoglutamicum"*			1112	483	ATCC	ATCC 15806	*Corynebacterium glutamicum*
30.	*"Corynebacterium alkanolyticum"*			1113	68	ATCC	ATCC 21511	*Gordonia hydrophobica*
31.	*"Corynebacterium alkanolyticum"*			1870	85	H. Seiler	cluster PM9	*Dietzia maris*
32.	*"Corynebacterium alkanolyticum"*			1871	86	H. Seiler	cluster PM10	*Gordonia rubropertincta*
33.	*"Corynebacterium alkanum _1"*			1114_1	99	ATCC	ATCC 21194	*Rhodococcus erythropolis*
34.	*"Corynebacterium alkanum _2"*			1114_2	98	ATCC	ATCC 21194	*Arthrobacter globiformis*
35.	*"Corynebacterium aurantiacum"*			1115	25	ATCC	ATCC 21362	*Rhodococcus erythropolis*
36.	*"Corynebacterium fujiokense"*			1117	69	ATCC	ATCC 21496	*Gordonia rubropertincta*
37.	*"Corynebacterium humiferum"*			1119	27	ATCC	ATCC 21108	*Rhodococcus erythropolis*
38.	*"Corynebacterium hydrocarbooxidans"*			1122	28	ATCC	ATCC 21767	*Rhodococcus ruber*
39.	*"Corynebacterium melassecola"*			1123	435	ATCC	ATCC 17965	*Corynebacterium glutamicum*
40.	*"Corynebacterium melassecola"*			1480	439	F. Fiedler	ATCC 17966	*Corynebacterium glutamicum*
41.	*"Corynebacterium paraldehydium"*			1127	29	ATCC	ATCC 21361	*Rhodococcus ruber*
42.	*"Corynebacterium petrophilum"*			1128	70	ATCC	ATCC 19080	*Gordonia rubropertincta*
43.	*"Micrococcus maripuniceus"*			1727	452	ATCC	ATCC 14399	*Corynebacterium glutamicum*
44.	*"Micrococcus naucinus"*		T	1728	422	ATCC	ATCC 1728	*Staphylococcus xylosus*
45.	*"Micrococcus ruber"*			2078	202	K. H. Schleifer	CCM 1744	*Arthrobacter agilis*
46.	*"Micrococcus salivarius"*			1734	122	ATCC	ATCC 14344	*Kocuria rosea*
47.	*"Mycobacterium pyogenes"*			1918	88	H. Seiler	Seiler 182	*Dietzia maris*
48.	*Aerococcus viridans*			1482	42	F. Fiedler	NCIB 9642	*Rhodococcus erythropolis*

No.	Species Name or Strain Denotation	Code	T†	WS No.	Glyc. No.‡	Source	Alternative Denotation	Reclassified as *
49.	*Agrococcus jenensis*		T	3265	743	DSM	DSM 9580	
50.	*Agromyces cerinus* subsp. *cerinus*		T	3258	738	DSM	DSM 8595	
51.	*Agromyces cerinus* subsp. *nitratus*		T	3259	739	DSM	DSM 8596	
52.	*Agromyces fucosus* subsp. *fucosus*		T	3260	740	DSM	DSM 8597	
53.	*Agromyces fucosus* subsp. *hippuratus*		T	3261	741	DSM	DSM 8598	
54.	*Agromyces mediolanus*			1784	183	DSM	DSM 20152	
55.	*Agromyces mediolanus*		T	3272	753	DSM	DSM 20152	
56.	*Arthrobacter agilis*		T	1732	121, 539	ATCC	ATCC 966	
57.	*Arthrobacter atrocyaneus*		T	2012	197	DSM	DSM 20127	*Staphylococcus vitulinus/pulvereri*
58.	*Arthrobacter aurescens*		T	2016	10,249	DSM	DSM 20116	
59.	*Arthrobacter citreus*			1450	297	F. Fiedler	AJ 1435	*Arthrobacter protophormiae*
60.	*Arthrobacter citreus*			1451	298	F. Fiedler	AJ 1438	*Arthrobacter sulfureus*
61.	*Arthrobacter citreus*		T	1749	606	DSM	DSM 20133	
62.	*Arthrobacter citreus*			1999	248	AC	AC 34	*Arthrobacter sulfureus*
63.	*Arthrobacter crystallopoietes*		T	1764	178	DSM	DSM 20117	
64.	*Arthrobacter cumminsii*		T	3336	768	DSM	DSM 10493	
65.	*Arthrobacter globiformis*			1457	165	F. Fiedler	NCIB 9759	
66.	*Arthrobacter globiformis*			1991	191	AC	AC 204	
67.	*Arthrobacter globiformis*			1992	192	UQM	UQM 3	
68.	*Arthrobacter globiformis*			1993	193	AC	AC 8	*Arthrobacter oxidans*
69.	*Arthrobacter globiformis*			1996	194	IFO	IFO 3062	*Arthrobacter nicotianae*
70.	*Arthrobacter globiformis*			2000	196	AC	AC 163	
71.	*Arthrobacter globiformis*			2014	198	CCM	ATCC 4336	
72.	*Arthrobacter globiformis*		T	2074	201	DSM	DSM 20124	
73.	*Arthrobacter globiformis*			2107	475	NCDO	NCIB 8605	*Microbacterium lacticum*
74.	*Arthrobacter globiformis*	H9		3070	689	Company H		*Arthrobacter nicotianae*
75.	*Arthrobacter histidinolovorans*		T	1768	182	DSM	DSM 20115	
76.	*Arthrobacter ilicis*		T	1787	542	DSM	DSM 20138	
77.	*Arthrobacter mysorens*			1073	160	ATCC	ATCC 31021	*Arthrobacter nicotianae*
78.	*Arthrobacter nicotianae*			1074	481	ATCC	ATCC 21279	
79.	*Arthrobacter nicotianae*			1093	281	ATCC	ATCC 74929	
80.	*Arthrobacter nicotianae*		T	1765	179	DSM	DSM 20123	
81.	*Arthrobacter nicotianae*			2232	607	H. Seiler	Seiler CS0106	
82.	*Arthrobacter nicotianae*	F3		2905	376	Company F		
83.	*Arthrobacter nicotianae*	F8		3067	686	Company F		
84.	*Arthrobacter nicotianae*	E5		2229	723	Company E	878	
85.	*Arthrobacter nicotinovorans*			2098	204	DSM	DSM 420	*Arthrobacter histidinolovorans*
86.	*Arthrobacter nicotinovorans*		T	3217	749	DSM	DSM 420	
87.	*Arthrobacter oxydans*		T	1762	176	DSM	DSM 20119	
88.	*Arthrobacter oxydans*			1763	177	DSM	DSM 20120	
89.	*Arthrobacter pascens*		T	1766	180	ATCC	ATCC 13346	*Microbacterium lacticum*
90.	*Arthrobacter polychromogenes*		T	1689	172	DSM	DSM 20136	
91.	*Arthrobacter protophormiae*			1453	491	ATCC	ATCC 17775	
92.	*Arthrobacter protophormiae*			1455	492	ATCC	ATCC 21348	
93.	*Arthrobacter protophormiae*		T	1779	541	DSM	DSM 20168	
94.	*Arthrobacter ramosus*		T	1829	185	IFO	DSM 20546	*Arthrobacter nicotinovorans*
95.	*Arthrobacter* sp.			1077	95,482	ATCC	ATCC 15590	*Rhodococcus erythropolis*
96.	*Arthrobacter* sp.			1448	164	F. Fiedler	ATCC 21375	*Arthrobacter globiformis*
97.	*Arthrobacter* sp.			1477	494	NCIB	DSM 20125	*Arthrobacter oxidans*
98.	*Arthrobacter* sp.			1565	211	ATCC	ATCC 21237	*Rhodococcus opacus*
99.	*Arthrobacter* sp.			1567	62	ATCC	ATCC 25581	*Rhodococcus opacus*
100.	*Arthrobacter* sp.			1568	63	ATCC	ATCC 27778	*Rhodococcus ruber*
101.	*Arthrobacter* sp.			1605	501	NCIB	NCIB 9666	*Corynebacterium glutamicum*
102.	*Arthrobacter* sp.			1649	311	DSM	DSM 20161	*Arthrobacter oxidans*
103.	*Arthrobacter* sp.			1745	315	ATCC	ATCC 14709	*Curtobacterium citreum*
104.	*Arthrobacter* sp.			1754	224	DSM	DSM 20142	*Arthrobacter psychrolactophilus (?)*
105.	*Arthrobacter* sp.			1938	516	ATCC	ATCC 15993	*Arthrobacter protophormiae*
106.	*Arthrobacter* sp.			1941	238, 517	K. Yamada	AJ 3124	*Arthrobacter protophormiae*
107.	*Arthrobacter* sp.			2015	317	DSM	DSM 20122	*Arthrobacter nicotianae*
108.	*Arthrobacter sulfureus*			1452	490	ATCC	ATCC 15170	
109.	*Arthrobacter sulfureus*		T	1705	538	DSM	DSM 20167	
110.	*Arthrobacter uratoxidans*		T	1130	484	ATCC	ATCC 21749	
111.	*Arthrobacter uratoxidans*			1697	507	ATCC	DSM 20648	
112.	*Arthrobacter ureafaciens*			1570	169	ATCC	ATCC 21124	*Arthrobacter histidinolovorans*
113.	*Arthrobacter ureafaciens*		T	1767	181	DSM	DSM 20126	
114.	*Arthrobacter ureafaciens*			1977	188	DSM	DSM 419	

No.	Species Name or Strain Denotation	Code	T†	WS No.	Glyc. No.‡	Source	Alternative Denotation	Reclassified as *
115.	*Arthrobacter ureafaciens*			2043	200	IAM	IAM 1390	*Arthrobacter histidinolovorans*
116.	*Arthrobacter woluwensis*		T	3337	769	DSM	DSM 10495	
117.	*Beutenbergia cavernae*		T	3374	819	HKI	0122	
118.	*Beutenbergia cavernae*			3378	821	HKI	0132	
119.	*Brachybacterium alimentarium*		T	3074	694	K. Schubert	CNRZ 925	
120.	*Brachybacterium conglomeratum*			1698	tot?	ATCC	ATCC 19101	
121.	*Brachybacterium conglomeratum*			2240	601	CCM	CCM 2135	
122.	*Brachybacterium conglomeratum*			2241	602	CCM	CCM 2136	
123.	*Brachybacterium conglomeratum*			2242	603	CCM	CCM 2432	*Rathayibacter tritici*
124.	*Brachybacterium conglomeratum*			2245	604	NCIB	NCIB 9859	
125.	*Brachybacterium conglomeratum*		T	3269	745	DSM	DSM 10241	
126.	*Brachybacterium faecium*		T	3241	734	DSM	DSM 4810	
127.	*Brachybacterium nesterenkovii*		T	3264	742	DSM	DSM 9573	
128.	*Brachybacterium paraconglomeratum*		T	3315	763	DSM	DSM 46341	
129.	*Brachybacterium rhamnosum*		T	3268	744	DSM	DSM 10240	
130.	*Brachybacterium tyrofermentans*		T	3075	695	K. Schubert	CNRZ 926	
131.	*Brevibacterium casei*			2124	523	NCDO	NCDO 2050	
132.	*Brevibacterium casei*			2125	524	NCDO	NCDO 2049	
133.	*Brevibacterium casei*			2127	525	NCDO	DSM 20658	
134.	*Brevibacterium casei*		T	2128	549	NCDO	DSM 20657	
135.	*Brevibacterium casei*	N3		2908	381	Company N		
136.	*Brevibacterium casei*	L3		3023	640	Company E		
137.	*Brevibacterium epidermidis*		T	3207	727	DSMZ	DSM 20660	
138.	*Brevibacterium iodinum*	V4_1		-	712	Dairy V		*Staphylococcus epidermidis*
139.	*Brevibacterium iodinum*	V4_2		-	717	Dairy V		*Brevibacterium linens*
140.	*Brevibacterium iodinum*		T	3203	726	DSMZ	DSM 20626	
141.	*Brevibacterium linens*			1500	302	F. Fiedler	AC B3	
142.	*Brevibacterium linens*			1501	303	F. Fiedler	AC B4	
143.	*Brevibacterium linens*			1503	304	F. Fiedler	AC 470	
144.	*Brevibacterium linens*			1504	305	F. Fiedler	AC 474	
145.	*Brevibacterium linens*			1692	117	UQM	UQM 26	
146.	*Brevibacterium linens*			1694	118	ATCC	ATCC 9174	
147.	*Brevibacterium linens*			1778	125	ATCC	DSM 20426	
148.	*Brevibacterium linens*		T	1782	128	ATCC	DSM 20425	
149.	*Brevibacterium linens*			1917	134	H. Seiler	Seiler 195	
150.	*Brevibacterium linens*			1939	135	AC	AC 825	
151.	*Brevibacterium linens*			1942	136	AC	AC 480	
152.	*Brevibacterium linens*			1945	157	K. Yamada	AJ 1540	
153.	*Brevibacterium linens*			1949	137	AC	AC 252	
154.	*Brevibacterium linens*			1950	138	AC	AC 478	
155.	*Brevibacterium linens*			1951	139	AC	AC 251	
156.	*Brevibacterium linens*			1952	140	AC	AC 578	
157.	*Brevibacterium linens*			1954	141	AC	AC 577	
158.	*Brevibacterium linens*			1968	142	NCDO	NCDO 1002	
159.	*Brevibacterium linens*			1975	143	AC	AC 831	
160.	*Brevibacterium linens*			1976	144	IFO	ATCC 8377	
161.	*Brevibacterium linens*			1978	145	AC	AC 575	
162.	*Brevibacterium linens*			2165	149	DSM	DSM 20158	
163.	*Brevibacterium linens*	M18		2890	818	I. Eppert		
164.	*Brevibacterium linens*	F1		2903	374	Company F		
165.	*Brevibacterium linens*	F2		2904	375	Company F		
166.	*Brevibacterium linens*	N1		2906	379	Company N		
167.	*Brevibacterium linens*	N2		2907	380	Company N		
168.	*Brevibacterium linens*	L1		2909	377	Company E		
169.	*Brevibacterium linens*	L2		2910	378	Company E		
170.	*Brevibacterium linens*	K1		2911	382	Dairy K		
171.	*Brevibacterium linens*	H2		2912	383	Company H		
172.	*Brevibacterium linens*	H3		2913	384	Company H		
173.	*Brevibacterium linens*	H4		2914	385	Company H		
174.	*Brevibacterium linens*	R5		2915	386	Dairy K		
175.	*Brevibacterium linens*	H1		2978	479	Company H		
176.	*Brevibacterium linens*	N4		3025	642	Company N		
177.	*Brevibacterium linens*	N5		3026	643	Company N		
178.	*Brevibacterium linens*	N6		3027	644	Company N		
179.	*Brevibacterium linens*	N7		3028	645	Company N		
180.	*Brevibacterium linens*	N8		3029	646	Company N		
181.	*Brevibacterium linens*	N9		3030	647	Company N		
182.	*Brevibacterium linens*	N10		3031	648	Company N		
183.	*Brevibacterium linens*	N11		3032	649	Company N		
184.	*Brevibacterium linens*	N12		3033	650	Company N		

No.	Species Name or Strain Denotation	Code	T†	WS No.	Glyc. No.‡	Source	Alternative Denotation	Reclassified as *
185.	*Brevibacterium linens*	N13		3034	651	Company N		
186.	*Brevibacterium linens*	N14		3035	652	Company N		*Brevibacterium casei*
187.	*Brevibacterium linens*	N15		3036	653	Company N		
188.	*Brevibacterium linens*	F4		3037	654	Company F		
189.	*Brevibacterium linens*	F5		3038	655	Company F		
190.	*Brevibacterium linens*	H7		3047	666	Company H		
191.	*Brevibacterium linens*	F9		3068	687	Company F		
192.	*Brevibacterium linens*	H8		3069	688	Company H		
193.	*Brevibacterium linens*	F13		3072	691	Company F		
194.	*Brevibacterium mcbrellneri*		T	3157	724	DSMZ	DSM 9583	
195.	*Brevibacterium otilidis*		T	3158	725	DSMZ	DSM 10718	
196.	*Brevibacterium* sp.			1098	66	ATCC	ATCC 14649	*Rhodococcus opacus*
197.	*Brevibacterium* sp.			1099	395	ATCC	ATCC 14902	*Corynebacterium glutamicum*
198.	*Brevibacterium* sp.			1100	396	ATCC	ATCC 19165	*Corynebacterium glutamicum*
199.	*Brevibacterium* sp.			1466	166	ATCC	ATCC 19239	*Arthrobacter aurescens*
200.	*Brevibacterium* sp.			1469	41,493	DSM	CSM 20165	*Rhodococcus ruber*
201.	*Brevibacterium* sp.			1472	167	F. Fiedler	ATCC 14604	*Arthrobacter oxidans*
202.	*Brevibacterium* sp.			1473	417	F. Fiedler	ATCC 21189	*Corynebacterium ammoniagenes*
203.	*Brevibacterium* sp.			1560	495	BKM	BKM 464	*Dietzia maris*
204.	*Brevibacterium* sp.			1577	90,212	ATCC	ATCC 21342	*Microbacterium oxidans*
205.	*Brevibacterium* sp.			1578	39	ATCC	ATCC 21089	*Rhodococcus ruber*
206.	*Brevibacterium* sp.			1602	310	ATCC	ATCC 15169	*Arthrobacter crystallopoietes*
207.	*Brevibacterium* sp.			1603	43	ATCC	ATCC 21222	*Rhodococcus erythropolis*
208.	*Brevibacterium* sp.			1655	214, 502	IAM	IAM 1391	*Microbacterium oxidans*
209.	*Brevibacterium* sp.			1656	215, 503	IAM	NCIB 10353	*Curtobacterium luteum*
210.	*Brevibacterium* sp.			1657	216, 504	IAM	NCIB 10352	*Microbacterium oxidans*
211.	*Brevibacterium* sp.			1672	44	ATCC	ATCC 15588	*Rhodococcus ruber*
212.	*Brevibacterium* sp.			1943	187	ATCC	ATCC 19390	*Arthrobacter aurescens*
213.	*Brevibacterium* sp.			1961	520	UQM	UQM 67	*Arthrobacter chlorophenolicus (?)*
214.	*Brevibacterium* sp. *_2*			1470_2	701	F. Fiedler	ATCC 21111	*Rhodococcus ruber*
215.	*Brevibacterium* sp. *–1*			1470_1	72	F. Fiedler	ATCC 21111	*Rhodococcus equi*
216.	*Brevibacterium stationis*		T	1962	464, 545	CCM	DSM 20302	*Corynebacterium ammoniagenes*
217.	*Cellulomonas biazotea*		T	1780	126	DSM	DSM 20112	*Arthrobacter crystallopoietes*
218.	*Cellulomonas biazotea*			2082	473, 522	UQM	UQM 49	
219.	*Cellulomonas cellasea*		T	1688	116	DSM	DSM 20118	
220.	*Cellulomonas cellulans*			1769	510	ATCC	DSM 20424	
221.	*Cellulomonas cellulans*			1781	127, 513	DSM	DSM 20106	
222.	*Cellulomonas cellulans*			1796	514	DSM	DSM 20155	
223.	*Cellulomonas cellulans*			1891	133	E. Stackebrandt	Prauser G62	
224.	*Cellulomonas cellulans*			1955	518	CIP	CIP 66.26	
225.	*Cellulomonas cellulans*			1956	519	CIP	CIP 66.27	
226.	*Cellulomonas cellulans*			1957	544	CIP	CIP 66.11	
227.	*Cellulomonas fermentans*		T	3226	790	DSM	DSM 3133	
228.	*Cellulomonas fimi*		T	1783	19	DSM	DSM 20113	
229.	*Cellulomonas flavigena*		T	1776	124	DSM	DSM 20109	
230.	*Cellulomonas flavigena*			2017	465	UQM	UQM 414	
231.	*Cellulomonas gelida*		T	1772	454	DSM	DSM 20111	
232.	*Cellulomonas* sp.			2131	476	DSM	exDSM 20034	*Cellulomonas gelida*
233.	*Cellulomonas turbata*			1890	132	E. Stackebrandt	Stackebrandt 891	
234.	*Cellulomonas turbata*		T	3285	759	DSM	DSM 20577	
235.	*Cellulomonas uda*			1770	511	DSM	DSM 20108	
236.	*Cellulomonas uda*		T	2084	474	DSM	DSM 20107	
237.	*Clavibacter michiganensis* subsp. *insidiosus*			1201	7	NCPPB	NCPPB 1110	
238.	*Clavibacter michiganensis* subsp. *insidiosus*			1209	488	NCPPB	NCPPB 1687	
239.	*Clavibacter michiganensis* subsp. *insidiosus*			1639	350	NCPPB	NCPPB 83	
240.	*Clavibacter michiganensis* subsp. *insidiosus*			1640	351	NCPPB	NCPPB 1686	
241.	*Clavibacter michiganensis* subsp. *insidiosus*			1641	352	NCPPB	NCPPB 1643	

No.	Species Name or Strain Denotation	Code	T†	WS No.	Glyc. No.‡	Source	Alternative Denotation	Reclassified as *
242.	*Clavibacter michiganensis* subsp. *insidiosus*			1664	354	NCPPB	NCPPB 1634	
243.	*Clavibacter michiganensis* subsp. *insidiosus*			1665	355	NCPPB	NCPPB 1635	
244.	*Clavibacter michiganensis* subsp. *insidiosus*			1666	356	NCPPB	NCPPB 1636	
245.	*Clavibacter michiganensis* subsp. *insidiosus*			1707	357	NCPPB	NCPPB 1660	
246.	*Clavibacter michiganensis* subsp. *insidiosus*			1827	362	DSM	DSM 20157	
247.	*Clavibacter michiganensis* subsp. *insidiosus*			2045	363	CCM	exCCM 1589	*Curtobacterium citreum*
248.	*Clavibacter michiganensis* subsp. *insidiosus*			2067	366	DSM	DSM 340	
249.	*Clavibacter michiganensis* subsp. *insidiosus*		T	2102	368	NCPPB	NCPPB 1109	
250.	*Clavibacter michiganensis* subsp. *insidiosus*			2103	369	NCPPB	NCPPB 1020	
251.	*Clavibacter michiganensis* subsp. *michiganensis*			1211	341	NCPPB	NCPPB 170	
252.	*Clavibacter michiganensis* subsp. *michiganensis*			1212	408	NCPPB	NCPPB 254	*Curtobacterium citreum*
253.	*Clavibacter michiganensis* subsp. *michiganensis*			1213	342	NCPPB	NCPPB 382	
254.	*Clavibacter michiganensis* subsp. *michiganensis*			1214	343	NCPPB	NCPPB 399	
255.	*Clavibacter michiganensis* subsp. *michiganensis*			1215	344	NCPPB	NCPPB 515	
256.	*Clavibacter michiganensis* subsp. *michiganensis*			1216	345	K. Yamada	ATCC 7430	*Arthrobacter ureafaciens*
257.	*Clavibacter michiganensis* subsp. *michiganensis*			1217	346	NCPPB	ATCC 7433	
258.	*Clavibacter michiganensis* subsp. *michiganensis*			1218	347	NCPPB	NCPPB 886	
259.	*Clavibacter michiganensis* subsp. *michiganensis*			1219	348	NCPPB	ATCC 14456	
260.	*Clavibacter michiganensis* subsp. *michiganensis*			1220	349	NCPPB	NCPPB 1379	
261.	*Clavibacter michiganensis* subsp. *michiganensis*			1221	409	NCPPB	NCPPB 1397	
262.	*Clavibacter michiganensis* subsp. *michiganensis*			1222	410	NCPPB	NCPPB 1468	
263.	*Clavibacter michiganensis* subsp. *michiganensis*			1223	411	NCPPB	NCPPB 1496	
264.	*Clavibacter michiganensis* subsp. *michiganensis*			1224	412	NCPPB	NCPPB 1572	
265.	*Clavibacter michiganensis* subsp. *michiganensis*			1225	5,413	NCPPB	NCPPB 1573	
266.	*Clavibacter michiganensis* subsp. *michiganensis*			1226	414	NCPPB	NCPPB 1574	
267.	*Clavibacter michiganensis* subsp. *michiganensis*			1227	415	NCPPB	NCPPB 2034	
268.	*Clavibacter michiganensis* subsp. *michiganensis*			1708	358	ATCC	ATCC 4450	
269.	*Clavibacter michiganensis* subsp. *michiganensis*			1709	704	K. Yamada	ATCC 492	
270.	*Clavibacter michiganensis* subsp. *michiganensis*			1788	360	DSM	DSM 20134	
271.	*Clavibacter michiganensis* subsp. *michiganensis*			2055	318	NCPPB	CCM 53	*Curtobacterium citreum*
272.	*Clavibacter michiganensis* subsp. *michiganensis*			2075	367	NCPPB	IFO 12471	
273.	*Clavibacter michiganensis* subsp. *michiganensis*			2146	370	ATCC	ATCC 7429	*Microbacterium lacticum*
274.	*Clavibacter michiganensis* subsp. *nebraskensis*		T	1585	420	ATCC	ATCC 27794	
275.	*Clavibacter michiganensis* subsp. *sepedonicus*			1178	400	NCPPB	NCPPB 299	
276.	*Clavibacter michiganensis* subsp. *sepedonicus*			1180	41	NCPPB	NCPPB 379	
277.	*Clavibacter michiganensis* subsp. *sepedonicus*			1181	486	NCPPB	NCPPB 1499	*Curtobacterium flaccumfaciens*

No.	Species Name or Strain Denotation	Code	T†	WS No.	Glyc. No.‡	Source	Alternative Denotation	Reclassified as *
278.	*Clavibacter michiganensis* subsp. *sepedonicus*		T	1182	402	NCPPB	NCPPB 2137	
279.	*Clavibacter michiganensis* subsp. *sepedonicus*			1183	487	NCPPB	NCPPB 2138	*Staphylococcus epidermidis*
280.	*Clavibacter michiganensis* subsp. *sepedonicus*			1185	152	NCPPB	NCPPB 2140	
281.	*Clavibacter michiganensis* subsp. *sepedonicus*			1187	404	NCPPB	NCPPB 2144	
282.	*Clavibacter michiganensis* subsp. *sepedonicus*			2087	431	ATCC	DSM 1757	
283.	*Corynebacterium accolens*		T	3043	660	DSMZ	DSM 44278	
284.	*Corynebacterium accolens*	B6		3053	672	K. P. Schaal	AI-1338	*Corynebacterium asperum*
285.	*Corynebacterium accolens*	B7		3054	673	K. P. Schaal	AL-1236	*Corynebacterium striatum*
286.	*Corynebacterium afermentans*	B5		3052	671	K. P. Schaal	I-3196	*Corynebacterium jeikeium*
287.	*Corynebacterium afermentans* subsp. *afermentans*		T	3044	661	DSMZ	DSM 44280	
288.	*Corynebacterium afermentans* subsp. *lipophilum*		T	3045	662	DSMZ	DSM 44282	
289.	*Corynebacterium ammoniagenes*	V8_1		-	714	Dairy V		*Corynebacterium variabilis*
290.	*Corynebacterium ammoniagenes*	V8_2		-	715	Dairy V		*Staphylococcus haemolyticus*
291.	*Corynebacterium ammoniagenes*		T	1571	532	ATCC	ATCC 6871	
292.	*Corynebacterium ammoniagenes*			1572	496	ATCC	ATCC 13746	*Corynebacterium glutamicum*
293.	*Corynebacterium ammoniagenes*			1604	500	IFO	DSM 20305	
294.	*Corynebacterium ammoniagenes*		T	1973	546	DSM	DSM 20306	
295.	*Corynebacterium amycolatum*		T	3041	658	DSMZ	DSM 6922	
296.	*Corynebacterium amycolatum*	B13		3060	679	K. P. Schaal	I-11722	*Corynebacterium asperum*
297.	*Corynebacterium amycolatum*	B14		3061	680	K. P. Schaal	B-408	
298.	*Corynebacterium amycolatum*	B15		3062	681	K. P. Schaal	AL-120	*Corynebacterium asperum*
299.	*Corynebacterium argentoratense*		T	3042	659	DSMZ	DSM 44202	
300.	*Corynebacterium auris*		T	3358	794	DSM	DSM 44122	
301.	*Corynebacterium bovis*		T	1695	535	ATCC	DSM 20582	*Brachybacterium conglomeratum*
302.	*Corynebacterium callunae*		T	1785	423	DSM	DSM 20147	
303.	*Corynebacterium coyleae*		T	3363	798	DSM	DSM 44184	
304.	*Corynebacterium diphtheriae*		T	3021	609	DSMZ	DSM 44123	
305.	*Corynebacterium flavescens*		T	1750	453	ATCC	DSM 20296	
306.	*Corynebacterium flavescens*			2031	577	DSM	exDSM 20172	*Microbacterium lacticum*
307.	*Corynebacterium glucuronolyticum*		T	3310	804	DSM	DSM 44120	
308.	*Corynebacterium glutamicum*	V1		-	710	Dairy V		*Corynebacterium variabilis*
309.	*Corynebacterium glutamicum*	V11		-	692	Dairy V		*Corynebacterium ammoniagenes*
310.	*Corynebacterium glutamicum*			1095	392	ATCC	DSM 20598	
311.	*Corynebacterium glutamicum*			1492	442	F. Fiedler	ATCC 13058	
312.	*Corynebacterium glutamicum*			1493	443	F. Fiedler	ATCC 13059	
313.	*Corynebacterium glutamicum*			1494	444	F. Fiedler	ATCC 13060	
314.	*Corynebacterium glutamicum*			1495	445	F. Fiedler	ATCC 13232	
315.	*Corynebacterium glutamicum*			1496	446	F. Fiedler	ATCC 13286	
316.	*Corynebacterium glutamicum*			1497	447	F. Fiedler	ATCC 13287	
317.	*Corynebacterium glutamicum*			1498	448	F. Fiedler	ATCC 13761	
318.	*Corynebacterium glutamicum*			1499	449	F. Fiedler	ATCC 15025	
319.	*Corynebacterium glutamicum*			1573	497	ATCC	ATCC 21642	
320.	*Corynebacterium glutamicum*			1575	498	ATCC	ATCC 21517	
321.	*Corynebacterium glutamicum*			1581	418	ATCC	ATCC 21065	
322.	*Corynebacterium glutamicum*			1582	419	ATCC	ATCC 21179	
323.	*Corynebacterium glutamicum*			1669	505	ATCC	ATCC 13826	
324.	*Corynebacterium glutamicum*			1670	506	ATCC	ATCC 15168	
325.	*Corynebacterium glutamicum*			1714	451	ATCC	ATCC 21644	
326.	*Corynebacterium glutamicum*			1756	509	DSM	DSM 20163	
327.	*Corynebacterium glutamicum*			1757	569	ATCC	ATCC 14067	
328.	*Corynebacterium glutamicum*			1790	571	DSM	DSM 20156	
329.	*Corynebacterium glutamicum*			1795	457	DSM	DSM 20137	
330.	*Corynebacterium glutamicum*			1940	574	ATCC	ATCC 13655	
331.	*Corynebacterium glutamicum*			2040	583	ATCC	DSM 20301	
332.	*Corynebacterium glutamicum*			2041	471	ATCC	ATCC 21492	
333.	*Corynebacterium glutamicum*			2046	428	ATCC	ATCC 19049	*Arthrobacter nicotianae*
334.	*Corynebacterium glutamicum*		T	2049	429	ATCC	DSM 20300	
335.	*Corynebacterium glutamicum* _1			1579_ 1	499	ATCC	ATCC 13869	
336.	*Corynebacterium glutamicum* _2			1579_ 2	552	ATCC	ATCC 13869	*Rhodococcus rhodochrous*
337.	*Corynebacterium hoagii*		T	3345	772	DSM	DSM 20295	
338.	*Corynebacterium jeikeium*		T	3016	610	DSMZ	DSM 7171	
339.	*Corynebacterium jeikeium*	B16		3063	682	K. P. Schaal	I-982	

No.	Species Name or Strain Denotation	Code	T†	WS No.	Glyc. No.‡	Source	Alternative Denotation	Reclassified as *
340.	*Corynebacterium jeikeium*	B17		3064	683	K. P. Schaal	B-694	
341.	*Corynebacterium kutscheri*		T	3020	611	DSMZ	DSM 20755	
342.	*Corynebacterium macginleyi*	B18		3065	684	K. P. Schaal	AL-1301	
343.	*Corynebacterium macginleyi*	B19		3066	685	K. P. Schaal	AL-1085	
344.	*Corynebacterium minutissimum*		T	3018	612	DSMZ	DSM 20651	
345.	*Corynebacterium mycetoides*			1789	424, 570	DSM	DSM 20148	
346.	*Corynebacterium mycetoides*		T	3349	773	DSM	DSM 20632	
347.	*Corynebacterium pilosum*		T	1700	536	ATCC	DSM 20521	
348.	*Corynebacterium propinquum*		T	3365	800	DSM	DSM 44285	
349.	*Corynebacterium pseudodiphteriticum*		T	1747	540	ATCC	ATCC 10700	
350.	*Corynebacterium pseudodiphtheriticum*	V2		-	711	Dairy V		*Corynebacterium variabilis*
351.	*Corynebacterium pseudodiphtheriticum*	B10		3057	676	K. P. Schaal	AL-186	
352.	*Corynebacterium pseudodiphtheriticum*	B11		3058	677	K. P. Schaal	AL-902	
353.	*Corynebacterium pseudodiphtheriticum*	B12		3059	678	K. P. Schaal	AL-1073	*Corynebacterium propinquum* or *pseudodiphtheriticum*
354.	*Corynebacterium pseudotuberculosis*		T	3019	613	DSMZ	DSM 20689	
355.	*Corynebacterium renale*			1826	573	ATCC	DSM 20298	
356.	*Corynebacterium renale*		T	2052	547	ATCC	DSM 20688	
357.	*Corynebacterium seminale*		T	3366	801	DSM	DSM 44288	
358.	*Corynebacterium* sp.			1089	391	ATCC	ATCC 14068	*Corynebacterium glutamicum*
359.	*Corynebacterium* sp.			1096	393	ATCC	ATCC 14066	*Corynebacterium glutamicum*
360.	*Corynebacterium* sp.			1109	23	ATCC	ATCC 21645	*Corynebacterium glutamicum*
361.	*Corynebacterium* sp.			1110	24	ATCC	ATCC 21654	*Corynebacterium glutamicum*
362.	*Corynebacterium* sp.			1131	30	ATCC	ATCC 14747	*Corynebacterium glutamicum*
363.	*Corynebacterium* sp.			1132	31	ATCC	ATCC 15529	*Rhodococcus erythropolis*
364.	*Corynebacterium* sp.			1133	73	ATCC	ATCC 21458	*Gordonia hydrophophica*
365.	*Corynebacterium* sp.			1188	405	NCPPB	NCPPB 2205	*Rathayibacter rathayi*
366.	*Corynebacterium* sp.			1189	406	NCPPB	NCPPB 2206	*Rathayibacter rathayi*
367.	*Corynebacterium* sp.			1481	168	ATCC	ATCC 21374	*Arthrobacter oxidans*
368.	*Corynebacterium* sp.			1484	440	F. Fiedler	NCIB 9647	*Corynebacterium glutamicum*
369.	*Corynebacterium* sp.			1485	441	F. Fiedler	ATCC 21084	*Corynebacterium ammoniagenes*
370.	*Corynebacterium* sp.			1486	300	F. Fiedler	ATCC 21188	*Arthrobacter* sp.
371.	*Corynebacterium* sp.			1488	301	F. Fiedler	ATCC 21341	*Dietzia maris*
372.	*Corynebacterium* sp.			1588	170	ATCC	ATCC 15167	*Arthrobacter ramosus/pascens*
373.	*Corynebacterium* sp.			1590	307	ATCC	ATCC 15927	*Microbacterium arborescens*
374.	*Corynebacterium* sp.			1592	308	ATCC	ATCC 21050	*Arthrobacter nicotianae*
375.	*Corynebacterium* sp.			1593	450	ATCC	ATCC 21251	*Corynebacterium callunae*
376.	*Corynebacterium* sp.			1594	309	ATCC	ATCC 21648	*Arthrobacter protophormiae*
377.	*Corynebacterium* sp.			1752	508	DSM	DSM 20128	*Corynebacterium glutamicum*
378.	*Corynebacterium* sp.			1792	54	ATCC	ATCC 21419	*Rhodococcus roseus*
379.	*Corynebacterium* sp.			1876	228	H. Seiler		*Arthrobacter* sp.
380.	*Corynebacterium* sp.			2038	580	AC	exAC250	*Arthrobacter nicotianae*
381.	*Corynebacterium* sp.			2042	628	D. F. Splitstoesser	Splitstoesser 64 (IV)	*Microbacterium dextranolyticum*
382.	*Corynebacterium* sp.			2050	472	D. F. Splitstoesser	Splitstoesser 88 (I)	*Corynebacterium callunae*
383.	*Corynebacterium* sp.			2053	250	D. F. Splitstoesser	Splitstoesser 28 (V)	*Microbacterium oxidans*
384.	*Corynebacterium* sp.			2056	588, 251,12	AC	exAC 264	*Arthrobacter nicotianae*
385.	*Corynebacterium* sp.			2059	252	D.F: Splitstoesser	Splitstoesser 25 (III)	*Microbacterium oxidans*
386.	*Corynebacterium* sp.			2063	592	AC	exAC 253	*Arthrobacter nicotianae*
387.	*Corynebacterium* sp.			2066	595	AC	exAC 262	*Corynebacterium variabilis*
388.	*Corynebacterium* sp.			2071	665	D. F. Splitstoesser	Splitstoesser 66 (VI)	*Rhodococcus erythropolis*
389.	*Corynebacterium* sp.			2072	57	D. F. Splitstoesser	Splitstoesser 60 (VI)	*Rhodococcus erythropolis*
390.	*Corynebacterium* sp.			2076	254	D. F. Splitstoesser	Splitstoesser 24 (V)	*Microbacterium* sp.
391.	*Corynebacterium* sp.			2079	58	F. Fiedler	NCIB 9646	*Rhodococcus erythropolis*
392.	*Corynebacterium* sp.			2083	629	D.F: Splitstoesser	Splitstoesser 61 (IV)	*Microbacterium dextranolyticum*
393.	*Corynebacterium striatum*		T	2101	548	NCTC	DSM 20668	
394.	*Corynebacterium striatum*			3017	614	DSMZ	DSM 7185	
395.	*Corynebacterium striatum*	B1		3048	667	K. P. Schaal	D-1825	

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396.	*Corynebacterium striatum*	B2		3049	668	K. P. Schaal	AL-1245	
397.	*Corynebacterium ulcerans*		T	3368	803	DSM	DSM 46325	
398.	*Corynebacterium urealyticum*		T	3013	615	DSMZ	DSM 7109	
399.	*Corynebacterium variabilis*	E4		-	722	Company E		
400.	*Corynebacterium variabilis*	V5		-	713	Dairy V		
401.	*Corynebacterium variabilis*		T	1646	534	DSM	DSM 20132	
402.	*Corynebacterium vitaeruminis*		T	1984	626	IFO	DSM 20294	
403.	*Corynebacterium xerosis*	V10		-	718	Dairy V		*Corynebacterium ammoniagenes*
404.	*Corynebacterium xerosis*		T	1600	617	K. Yamada	DSM 20743	
405.	*Corynebacterium xerosis*			1825	618	DSM	DSM 20170	
406.	*Corynebacterium xerosis*	B9		3056	675	K. P. Schaal	Va-167	*Corynebacterium asperum/amycolatum*
407.	*Curtobacterium albidum*		T	1720	222	CCM	DSM 20512	
408.	*Curtobacterium citreum*		T	1667	11	IFO	DSM 20528	
409.	*Curtobacterium flaccumfaciens*			1152	326	NCPPB	NCPPB 178	
410.	*Curtobacterium flaccumfaciens*			1153	327	NCPPB	NCPPB 390	
411.	*Curtobacterium flaccumfaciens*			1154	328	NCPPB	NCPPB 559	
412.	*Curtobacterium flaccumfaciens*			1155	329	NCPPB	NCPPB 567	
413.	*Curtobacterium flaccumfaciens*			1156	330	NCPPB	NCPPB 1412	
414.	*Curtobacterium flaccumfaciens*			1157	287	NCPPB	NCPPB 1435	
415.	*Curtobacterium flaccumfaciens*			1158	371	NCPPB	NCPPB 1441	
416.	*Curtobacterium flaccumfaciens*			1159	332	NCPPB	NCPPB 1442	
417.	*Curtobacterium flaccumfaciens*		T	1160	2	NCPPB	NCPPB 1446	
418.	*Curtobacterium flaccumfaciens*			1161	333	NCPPB	NCPPB 1597	
419.	*Curtobacterium flaccumfaciens*			1162	334	NCPPB	NCPPB 1751	
420.	*Curtobacterium flaccumfaciens*			1164	335	NCPPB	NCPPB 1844	
421.	*Curtobacterium flaccumfaciens*			1165	3	NCPPB	NCPPB 558	
422.	*Curtobacterium flaccumfaciens*			1166	336	DSM	DSM 20135	
423.	*Curtobacterium flaccumfaciens*			1229	293	NCPPB	NCPPB 2114	
424.	*Curtobacterium flaccumfaciens*			1230	294	NCPPB	NCPPB 2240	
425.	*Curtobacterium flaccumfaciens*			1231	295	NCPPB	NCPPB 2241	
426.	*Curtobacterium flaccumfaciens*			1232	4	NCPPB	NCPPB 2305	
427.	*Curtobacterium flaccumfaciens*			1794	361	DSM	DSM 20129	
428.	*Curtobacterium flaccumfaciens*			2047	584, 364	CCM	CCM 1588	
429.	*Curtobacterium flaccumfaciens*			2054	365	CCM	CCM 2403	
430.	*Curtobacterium flaccumfaciens* pvar. *betae*			1134	282	NCPPB	NCPPB 363	
431.	*Curtobacterium flaccumfaciens* pvar. *betae*			1135	283	NCPPB	NCPPB 364	
432.	*Curtobacterium flaccumfaciens* pvar. *betae*			1136	284	NCPPB	NCPPB 372	
433.	*Curtobacterium flaccumfaciens* pvar. *betae*			1137	285	NCPPB	NCPPB 373	
434.	*Curtobacterium flaccumfaciens* pvar. *betae*			1138	151	NCPPB	NCPPB 374	
435.	*Curtobacterium flaccumfaciens* pvar. *betae*			1139	286	NCPPB	NCPPB 375	
436.	*Curtobacterium flaccumfaciens* pvar. *betae*			2091	320	DSM	DSM 20141	
437.	*Curtobacterium flaccumfaciens* pvar. *oortii*		T	1430	296	NCPPB	ATCC 25283	
438.	*Curtobacterium flaccumfaciens* pvar. *poinsettiae*			1167	288	NCPPB	NCPPB 177	
439.	*Curtobacterium flaccumfaciens* pvar. *poinsettiae*			1168	289	NCPPB	NCPPB 844	
440.	*Curtobacterium flaccumfaciens* pvar. *poinsettiae*			1169	290	NCPPB	NCPPB 845	
441.	*Curtobacterium flaccumfaciens* pvar. *poinsettiae*			1170	291	NCPPB	NCPPB 846	
442.	*Curtobacterium flaccumfaciens* pvar. *poinsettiae*			1171	292	NCPPB	NCPPB 847	
443.	*Curtobacterium flaccumfaciens* pvar. *poinsettiae*			1172	337	NCPPB	NCPPB 848	
444.	*Curtobacterium flaccumfaciens* pvar. *poinsettiae*			1173	1	NCPPB	NCPPB 849	
445.	*Curtobacterium flaccumfaciens* pvar. *poinsettiae*			1174	338	NCPPB	ATCC 9070	
446.	*Curtobacterium flaccumfaciens* pvar. *poinsettiae*			1175	339	NCPPB	ATCC 9069	
447.	*Curtobacterium flaccumfaciens* pvar. *poinsettiae*			1648	563	DSM	DSM 20149	

No.	Species Name or Strain Denotation	Code	T†	WS No.	Glyc. No.‡	Source	Alternative Denotation	Reclassified as *
448.	*Curtobacterium flaccumfaciens* pvar. *violaceum*		T	1644	353	F. Fiedler	ATCC 23827	
449.	*Curtobacterium luteum*		T	1980	9,246	CCM	DSM 20542	
450.	*Curtobacterium pusillum*			1673	218	IAM	DSM 20529	
451.	*Curtobacterium pusillum*		T	1706	221	NCIB	DSM 20527	
452.	*Curtobacterium* sp.			1862	227	H. Seiler	cluster PM1	*Microbacterium liquefaciens*
453.	*Curtobacterium* sp.			2112	255	H. Seiler	Seiler K388/5	*Microbacterium liquefaciens*
454.	*Deinococcus radiodurans*		T	1731	R 1220	ATCC	DSM 20539	
455.	*Demetria terragena*		T	3380	823	HKI	0089	
456.	*Dermabacter hominis*		T	3251	735	DSM	DSM 7083	
457.	*Dermacoccus nishinomiyaensis*		T	3281	756	DSM	DSM 20448	
458.	*Dermatophilus congolensis*		T	3362	797	DSM	DSM 44180	
459.	*Dietzia maris*		T	3302	766	DSM	DSM 43672	
460.	*Gordonia aichiensis*		T	3356	777	DSM	DSM 43978	
461.	*Gordonia amarae*		T	3300	765	DSM	DSM 43392	
462.	*Gordonia bronchialis*		T	1802	17	CCM	DSM 43247	
463.	*Gordonia hirsuta*		T	3312	807	DSM	DSM 44140	
464.	*Gordonia hydrophobica*		T	3307	805	DSM	DSM 44015	
465.	*Gordonia rubropertincta*		T	1808	15	DSM	DSM 43197	
466.	*Gordonia rubropertincta*		T	1812	697	CCM	DSM 43248	
467.	*Gordonia sputi*		T	3355	776	DSM	DSM 43896	
468.	*Gordonia terrae*		T	1813	130	CCM	DSM 43249	
469.	*Intrasporangium calvum*		T	3295	764	DSM	DSM 43043	
470.	ISOLATE HS			-	R 509	H. Seiler	sputum	*Rothia dentocariosa*
471.	*Jonesia denitrificans*		T	2284	728	ATCC	DSM 20603	
472.	*Kocuria kristinae*		T	2086	630	K. H. Schleifer	DSM 20032	
473.	*Kocuria rosea*			1699	155	ATCC	ATCC 178 & 418	
474.	*Kocuria rosea*			1733	314	ATCC	ATCC 397	
475.	*Kocuria rosea*			2070	319	K. H. Schleifer	CCM 1744	
476.	*Kocuria rosea*			3271	746	DSM	DSM 11630	
477.	*Kocuria rosea*		T	3280	755	DSM	DSM 20447	
478.	*Kocuria varians*	E3		-	721	Company E		
479.	*Kocuria varians*		T	1049	91	ATCC	DSM 20033	
480.	*Kocuria varians*			1512	153	DSM	DSM 348	*Kocuria rhizophila*
481.	*Kocuria varians*			1730	636	ATCC	ATCC 15936	*Kocuria rhizophila*
482.	*Kocuria varians*			1735	637	ATCC	ATCC 399	
483.	*Kocuria varians*			2246	605	NCTC	NCTC 7281	*Brachybacterium conglomeratum*
484.	*Kurthia zopfii*			2064	593	NCTC	DSM 20487	
485.	*Kurthia zopfii*			2085	597	NCTC	ATCC 10538	*Corynebacterium ammoniagenes*
486.	*Kytococcus sedentarius*			2137	323	K. H. Schleifer	Schleifer TW 93	*Janibacter limosus (?)*
487.	*Kytococcus sedentarius*		T	3284	758	DSM	DSM 20547	
488.	*Lactobacillus coryniformis* subsp. *coryniformis*		T	2524		R. Vogel	DSM 20001	
489.	*Leifsonia aquatica*			1793	572	DSM	DSM 20146	
490.	*Leifsonia poae*		T	3414	824	VKM	VKM Ac-1401	
491.	*Leucobacter komagatae*		T	3423	833	DSMZ	DSMZ 8803	
492.	*Macrococcus caseolyticus*		T	1718	R 1212	ATCC	ATCC 13548	
493.	*Microbacterium arabinogalactanolyticum*		T	2730	259	DSM	DSM 8611	
494.	*Microbacterium arborescens*		T	2236	150, 550	DSM	DSM 20754	
495.	*Microbacterium aurum*		T	3262	752	DSM	DSM 8600	
496.	*Microbacterium barkeri*		T	1791	225	DSM	DSM 20145	
497.	*Microbacterium dextranolyticum*		T	2731	267	DSMZ	DSM 8607	
498.	*Microbacterium esteraromaticum*			2720	276	IFO	IFO 3752	
499.	*Microbacterium esteraromaticum*		T	2728	260	DSM	DSM 8609	
500.	*Microbacterium flavescens*		T	1703	220	NCIB	ATCC 13348	
501.	*Microbacterium imperiale*		T	1771	123	DSM	DSM 20530	
502.	*Microbacterium imperiale*			1959	624	UQM	Davis` collection NO:94	
503.	*Microbacterium keratanolyticum*		T	2726	261	DSMZ	DSM 8606	
504.	*Microbacterium lacticum*			1108	434	NCDO	ATCC 8181	
505.	*Microbacterium lacticum*			1913	620	H. Seiler	Seiler 91	
506.	*Microbacterium lacticum*			1914	458	H. Seiler	Seiler 630	
507.	*Microbacterium lacticum*			2018	466	UQM	Davis` collection NO.277	
508.	*Microbacterium lacticum*			2019	425	UQM	Davis` collection NO. 275	*Staphylococcus vitulinus/pulvereri*
509.	*Microbacterium lacticum*			2020	467	UQM	UQM 275	
510.	*Microbacterium lacticum*			2022	468	NCDO	NCDO 1103	
511.	*Microbacterium lacticum*			2024	469	NCDO	NCDO 960	

No.	Species Name or Strain Denotation	Code	T†	WS No.	Glyc. No.‡	Source	Alternative Denotation	Reclassified as *
512.	*Microbacterium lacticum*			2026	427	UQM	Davis` collection NO. 276	*Staphylococcus vitulinus/pulvereri*
513.	*Microbacterium lacticum*			2027	627	UQM	Davis` collection NO. 278	
514.	*Microbacterium lacticum*			2030	470	UQM	UQM 101	
515.	*Microbacterium lacticum*		T	2129	147	ATCC	DSM 20427	
516.	*Microbacterium laevaniformans*		T	2089	13,266	DSM	DSM 20140	
517.	*Microbacterium liquefaciens*			1489	209	F. Fiedler	K. Robinson 15	
518.	*Microbacterium liquefaciens*			1490	210	F. Fiedler	K. Robinson 20	
519.	*Microbacterium liquefaciens*		T	3288	760	DSM	DSM 20638	
520.	*Microbacterium luteolum*		T	1797	226, 515	DSM	DSM 20143	*Microbacterium oxidans*
521.	*Microbacterium oxidans*			1958	240	CIP	CIP 66.13	
522.	*Microbacterium oxidans*			1966	242	CIP	CIP 60.9	*Microbacterium liquefaciens*
523.	*Microbacterium oxidans*			1967	243	CIP	CIP 56.40	*Microbacterium liquefaciens*
524.	*Microbacterium oxidans*		T	1969	244	CIP	DSM 20578	
525.	*Microbacterium oxidans*			1971	245	CIP	ATCC 7762	
526.	*Microbacterium oxidans*			2130	256	CIP	NCIB 8535	*Microbacterium lacticum*
527.	*Microbacterium saperdae*		T	1650	213	DSM	DSM 20169	*Microbacterium oxidans*
528.	*Microbacterium schleiferi*			1894	229	H. Seiler	Seiler 171	
529.	*Microbacterium schleiferi*			1901	230	H. Seiler	Seiler 109	
530.	*Microbacterium schleiferi*			1902	231	H. Seiler	Seiler 105	
531.	*Microbacterium schleiferi*			1903	232	H. Seiler	Seiler 174	
532.	*Microbacterium schleiferi*			1904	233	H. Seiler	Seiler 111	
533.	*Microbacterium schleiferi*		T	1905	234	H. Seiler	Seiler 110	
534.	*Microbacterium schleiferi*			1906	235	H. Seiler	Seiler 107	
535.	*Microbacterium schleiferi*			1907	236	H. Seiler	Seiler 112	
536.	*Microbacterium schleiferi*			1908	237	H. Seiler	Seiler 115	
537.	*Microbacterium schleiferi*			2163	258	H. Seiler	Seiler 114	
538.	*Microbacterium* sp.			1105	433	ATCC	ATCC 15283	*Corynebacterium glutamicum*
539.	*Microbacterium* sp.			1107	557,97	ATCC	ATCC 21976	*Arthrobacter oxidans*
540.	*Microbacterium* sp.			1909	619	H. Seiler	Seiler 161	*Microbacterium schleiferi*
541.	*Microbacterium* sp.			1916	459	H. Seiler	Seiler 142	*Microbacterium schleiferi*
542.	*Microbacterium* sp.			1919	460	H. Seiler	Seiler 127	*Microbacterium lacticum*
543.	*Microbacterium* sp.			1921	461	H. Seiler	Seiler 139	*Microbacterium lacticum*
544.	*Microbacterium* sp.			1922	621	H. Seiler	Seiler 228	*Microbacterium lacticum*
545.	*Microbacterium* sp.			1923	622	H. Seiler	Seiler 166	*Microbacterium lacticum*
546.	*Microbacterium* sp.			1924	623	H. Seiler	Seiler 160	*Microbacterium lacticum*
547.	*Microbacterium* sp.			1925	462	H. Seiler	Seiler 143	*Microbacterium lacticum*
548.	*Microbacterium* sp.			1928	463	H. Seiler	Seiler 120	*Microbacterium lacticum*
549.	*Microbacterium* sp.			2023	426	NCDO	NCDO 627	*Brachybacterium nesterenkovii*
550.	*Microbacterium* sp.			2032	578	NCDO	NCDO 628	*Microbacterium lacticum*
551.	*Microbacterium* sp.			2162	477	H. Seiler	Seiler 145	*Microbacterium lacticum*
552.	*Microbacterium* sp.			2724	262	ATCC	ATCC 8315	*Microbacterium esteraromaticum*
553.	*Microbacterium* sp.			2725	263	ATCC	ATCC 8375	*Microbacterium esteraromaticum*
554.	*Microbacterium* sp. (white) (in the FT-IR identical to 1106_1)			1106_2	556	ATCC	ATCC 21376	*Arthrobacter oxidans*
555.	*Microbacterium* sp. (yellow) (in the FT-IR identical to 1106_2)			1106_1	555	ATCC	ATCC 21376	*Arthrobacter oxidans*
556.	*Microbacterium terrae*			2721	278	IFO	IFO 15301	
557.	*Microbacterium terrae*		T	2729	264	DSMZ	DSM 8610	
558.	*Microbacterium terregens*		T	2719	277	IFO	IFO 12961	*Microbacterium esteraromaticum*
559.	*Microbacterium testaceum*		T	1662	217, 247	DSM	DSM 20166	
560.	*Microbacterium testaceum*			1948	239	K. Yamada	AJ 1464	
561.	*Microbacterium testaceum*			2722	279	IFO	IFO 15302	
562.	*Microbacterium testaceum*			2723	280	IFO	IFO 15303	*Microbacterium liquefaciens*
563.	*Microbacterium trichothecenolyticum*		T	2727	265	DSMZ	DSM 8608	
564.	*Micrococcus luteus*		T	1513	306	K. H. Schleifer	DSM 20030	
565.	*Micrococcus luteus*			1722	421	ATCC	ATCC 400	
566.	*Micrococcus luteus*			1726	313	ATCC	ATCC 11880	
567.	*Micrococcus lylae*		T	2100	322	K. H. Schleifer	ATCC 27566	*Janibacter limosus (?)*
568.	*Micrococcus lylae*		T	3152	R 754	DSM	DSM 20315	
569.	*Micrococcus* sp.			2048	586	ATCC	ATCC 14396	*Kocuria rosea*
570.	*Micrococcus* sp.	B4		3051	670	K. P: Schaal	V-1447	*Staphylococcus saprophyticus*
571.	*Micrococcus* sp.	H10		3071	690	Company H		*Staphylococcus pulvereri*
572.	*Micrococcus* sp.	H11		3073	693	Company H		*Staphylococcus vitulinus/pulvereri*
573.	*Mycobacterium phlei*			1514	113	DSM	DSM 43214	*Rhodococcus rhodochrous*
574.	*Mycobacterium phlei*			1915	55	H. Seiler	Seiler 269	*Rhodococcus ruber*
575.	*Nesterenkonia halobia*		T	3283	757	DSM	DSM 20541	
576.	*Nocardia asteroides*			1609	75	DSM	DSM 43208	

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577.	*Nocardia asteroides –1*			1799_1	18	CCM	DSM 43063	*Rhodococcus* sp.
578.	*Nocardia asteroides –2*			1799_2	706	CCM	DSM 43063	*Nocardia otidiscaviarum*
579.	*Nocardia brasiliensis*		T	3354	788	DSM	DSM 43758	
580.	*Nocardia farcinica*		T	3352	787	DSM	DSM 43665	
581.	*Nocardia otidiscaviarum*		T	1805	16	DSM	DSM 43242	*Rhodococcus* sp.
582.	*Nocardia seriolae*		T	3359	789	DSM	DSM 44129	
583.	*Nocardioides simplex*			1989	190	AC	AC 157	*Arthrobacter oxidans*
584.	*Nocardioides simplex*			1990	316	AC	AC 16	*Arthrobacter oxidans*
585.	*Nocardioides simplex*			1997	195	AC	AC 159	*Arthrobacter oxidans*
586.	*Nocardioides simplex*			2034	521	AC	AC 4	*Microbacterium lacticum*
587.	*Nocardioides simplex*			2058	590	AC	AC 266	*Corynebacterium variabilis*
588.	*Nocardioides simplex*			2068	596	AC	AC 268	*Corynebacterium variabilis*
589.	*Ornithinicoccus hortensis*		T	3375	820	HKI	0125	
590.	*Ornithinicoccus hortensis*			3379	822	HKI	0131	
591.	*Promicromonospora citrea*		T	3296	762	DSM	DSM 43110	
592.	*Promicromonospora enterophila*		T	3304	781	DSM	DSM 4385	
593.	*Promicromonospora sukumoe*		T	3311	806	DSM	DSM 44121	
594.	raclette cheese isolate by M. Carnio	F.03		3459	813	M. Loessner		*Brevibacterium linens*
595.	raclette cheese isolate by M. Carnio	F.01		3460	811	M. Loessner		*Brevibacterium linens*
596.	raclette cheese isolate by M. Carnio	F.02		3461	812	M. Loessner		*Brevibacterium linens*
597.	raclette cheese isolate by M. Carnio	F.04		3462	814	M. Loessner		*Brachybacterium alimentarium*
598.	raclette cheese isolate by M. Carnio	F.05		3463	815	M. Loessner		*Brevibacterium linens* or *casei*
599.	raclette cheese isolate by M. Carnio	F.07		3464	816	M. Loessner		*Corynebacterium ammoniagenes*
600.	raclette cheese isolate by M. Carnio	F.08		3465	817	M. Loessner		*Brachybacterium tyrofermentans*
601.	*Rathayibacter iranicus*		T	1210	340	NCPPB	NCPPB 2253	
602.	*Rathayibacter rathayi*		T	3255	750	DSM	DSM 7485	
603.	*Rathayibacter toxicus*		T	3257	737	DSM	DSM 7488	
604.	*Rathayibacter tritici*			1193	560, 407	NCPPB	NCPPB 471	
605.	*Rathayibacter tritici*			1195	6	NCPPB	NCPPB 1953	
606.	*Rathayibacter tritici*			2051	430	IFO	ATCC 11402	
607.	*Rathayibacter tritici*			2164	432, 599	NCPPB	NCPPB 225	
608.	*Rathayibacter tritici*		T	3256	751	DSM	DSM 7486	
609.	*Rhodococcus coprophilus*		T	1822	83	ATCC	DSM 43347	
610.	*Rhodococcus equi*			1580	561	ATCC	ATCC 21521	*Rhodococcus rhodochrous*
611.	*Rhodococcus equi*			1587	115	ATCC	DSM 43199	
612.	*Rhodococcus equi*			1696	565	ATCC	ATCC 7698	
613.	*Rhodococcus equi*			1711	566	ATCC	ATCC 21107	
614.	*Rhodococcus equi*			1712	567	ATCC	ATCC 21280	*Corynebacterium ammoniagenes*
615.	*Rhodococcus equi*			1713	568	ATCC	ATCC 21690	
616.	*Rhodococcus equi*			1809	78	DSM	DSM 43199	
617.	*Rhodococcus equi*			1818	79	ATCC	exATCC 25694	
618.	*Rhodococcus equi*			1819	80	ATCC	exATCC 25728	
619.	*Rhodococcus equi*			2039	581	K. Yamada	ATCC 10146	
620.	*Rhodococcus equi*		T	2044	146	K. Yamada	DSM 20307	
621.	*Rhodococcus equi (CIP test strain)*			2289	551	CIP	CIP 58.69	
622.	*Rhodococcus erythropolis*			1079	96	ATCC	DSM 20665	
623.	*Rhodococcus erythropolis*			1440	111, 489	DSM	DSM 312	
624.	*Rhodococcus erythropolis*			1510	32	F. Fiedler	exDSM 43065	
625.	*Rhodococcus erythropolis*		T	1518	33	J. Baumgarten	DSM 43066	
626.	*Rhodococcus erythropolis*			1800	46	CCM	ATCC 19369	
627.	*Rhodococcus erythropolis*			1804	48	CCM	DSM 43060	
628.	*Rhodococcus erythropolis*			1944	576	DSM	DSM 313	
629.	*Rhodococcus erythropolis*			2036	579	IAM	IAM 1474	
630.	*Rhodococcus erythropolis*			2096	639	DSM	DSM 311	
631.	*Rhodococcus erythropolis*			2126	59	IAM	IAM 1399	
632.	*Rhodococcus erythropolis*			3369	809	DSM	DSM 743	
633.	*Rhodococcus erythropolis*			3370	810	DSM	DSM 43296	
634.	*Rhodococcus fascians*			1142	101	NCPPB	NCPPB 188	

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635.	*Rhodococcus fascians*			1143	102	NCPPB	NCPPB 469	
636.	*Rhodococcus fascians*			1144	103	NCPPB	NCPPB 764	
637.	*Rhodococcus fascians*			1145	104	NCPPB	NCPPB 765	
638.	*Rhodococcus fascians*			1146	105	NCPPB	NCPPB 766	
639.	*Rhodococcus fascians*			1147	106	NCPPB	NCPPB 1488	
640.	*Rhodococcus fascians*			1148	107	NCPPB	NCPPB 1675	
641.	*Rhodococcus fascians*			1149	108	NCPPB	NCPPB 1733	
642.	*Rhodococcus fascians*			1150	109	NCPPB	NCPPB 2210	
643.	*Rhodococcus fascians*			1151	110	NCPPB	NCPPB 2211	
644.	*Rhodococcus fascians*			1601	562	DSM	DSM 20131	*Arthrobacter citreus*
645.	*Rhodococcus fascians*		T	1823	156	IFO	DSM 20669	
646.	*Rhodococcus fascians*			2132	159	IAM	IAM 1079	
647.	*Rhodococcus globerulus*			1815	51	CCM	ATCC 19370	
648.	*Rhodococcus globerulus*			1821	82	ATCC	exATCC 25711	
649.	*Rhodococcus globerulus*			2145	148	DSM	DSM 20151	
650.	*Rhodococcus globerulus*		T	3306	782	DSM	DSM 43954	
651.	*Rhodococcus opacus*		T	3297	791	DSM	DSM 43205	
652.	*Rhodococcus percolatus*		T	3313	808	DSM	DSM 44240	
653.	*Rhodococcus rhodnii*		T	1824	84	NCIB	NCIB 11279	
654.	*Rhodococcus rhodochrous*			1116	26	ATCC	ATCC 21766	*Rhodococcus ruber*
655.	*Rhodococcus rhodochrous*		T	1515	114	DSM	DSM 43241	*Kocuria rhizophila*
656.	*Rhodococcus rhodochrous*			1519	34	J. Baumgarten	DSM 43202 & 363	
657.	*Rhodococcus rhodochrous*			1522	35	J. Baumgarten	Bayer RT 13	*Rhodococcus erythropolis*
658.	*Rhodococcus rhodochrous*			1803	47	CCM	CCM 2594	
659.	*Rhodococcus rhodochrous*			1806	49	DSM	DSM 43198	
660.	*Rhodococcus rhodochrous*			1816	52	ATCC	ATCC 9356	*Rhodococcus globerulus*
661.	*Rhodococcus rhodochrous*			1817	53	ATCC	ATCC 15998	*Rhodococcus ruber*
662.	*Rhodococcus roseus*			1807	50	DSM	DSM 43234	
663.	*Rhodococcus ruber*		T	3299	793	DSM	DSM 43338	
664.	*Rhodococcus* sp.			1094	22	ATCC	ATCC21195	*Rhodococcus erythropolis*
665.	*Rhodococcus* sp.			1120	100	ATCC	ATCC 15961	*Rhodococcus erythropolis*
666.	*Rhodococcus* sp.			1121	558	ATCC	ATCC 15108	*Rhodococcus erythropolis*
667.	*Rhodococcus* sp.			1517	61	J. Baumgarten	Bayer RT9	*Rhodococcus rhodochrous*
668.	*Rhodococcus* sp.			1583	631	ATCC	ATCC 21035	*Rhodococcus erythropolis*
669.	*Rhodococcus* sp.			1584	632	ATCC	ATCC 21402	*Gordonia rubropertincta*
670.	*Rhodococcus* sp.			1719	120	ATCC	ATCC 11890	*Dietzia maris*
671.	*Rhodococcus* sp.			1820	81	ATCC	exATCC 25723	*Gordonia sputi*
672.	*Rhodococcus zopfii*		T	3309	784	DSM	DSM 44108	
673.	*Rothia dentocariosa*		T	3303	780	DSM	DSM 43761	
674.	*Sanguibacter keddieii*		T	3338	770	DSM	DSM 10542	
675.	*Sanguibacter suarezii*		T	3339	771	DSM	DSM 10543	
676.	*Staphlococcus aureus* subsp. *aureus*		T	2438	R 1211	DSM	DSM 20231	
677.	*Staphylococcus arlettae*		T	3289	R 1171	DSM	DSM 20672	
678.	*Staphylococcus auricularis*		T	3286	R 1169	DSM	DSM 20609	
679.	*Staphylococcus capitis* subsp. *capitis*		T	3277	R 1165	DSM	DSM 20326	
680.	*Staphylococcus capitis* subsp. *ureolyticus*		T	3248	R 1157	DSM	DSM 6717	
681.	*Staphylococcus caprae*		T	3348	R 1182	DSM	DSM 20608	
682.	*Staphylococcus carnosus*		T	3145	R 756	DSM	DSM 20501	
683.	*Staphylococcus chromogenes*		T	3282	R 1168	DSM	DSM 20454	
684.	*Staphylococcus cohnii* subsp. *cohnii*		T	3273	R 1162	DSM	DSM 20260	
685.	*Staphylococcus cohnii* subsp. *urealyticum*		T	3249	R 1158	DSM	DSM 6718	
686.	*Staphylococcus delphini*		T	3292	R 1173	DSM	DSM 20771	
687.	*Staphylococcus epidermidis*		T	3341	R 1177	DSM	DSM 20044	
688.	*Staphylococcus equorum*	F.06		2733	733	M. Carnio	M. Carnio F96 19.06 ID97-48	
689.	*Staphylococcus equorum*		T	3003	R 787	DSM	DSM 20674	
690.	*Staphylococcus gallinarum*		T	3287	R 1170	DSM	DSM 20610	
691.	*Staphylococcus haemolyticus*		T	3343	R 1179	DSM	DSM 20263	
692.	*Staphylococcus hominis* subsp. *hominis*		T	3278	R 1166	DSM	DSM 20328	
693.	*Staphylococcus hyicus*		T	3347	R 1181	DSM	DSM 20459	
694.	*Staphylococcus intermedius*		T	3346	R 1180	DSM	DSM 20373	
695.	*Staphylococcus kloosii*		T	3290	R 1172	DSM	DSM 20676	
696.	*Staphylococcus lentus*		T	3279	R 1167	DSM	DSM 20352	

No.	Species Name or Strain Denotation	Code	T†	WS No.	Glyc. No.‡	Source	Alternative Denotation	Reclassified as *
697.	*Staphylococcus lugdunensis*		T	3151	R 753	DSM	DSM 4804	
698.	*Staphylococcus lutrae*		T	3270	R 1161	DSM	DSM 10244	
699.	*Staphylococcus muscae*		T	3250	R 1159	DSM	DSM 7068	
700.	*Staphylococcus pasteurii*		T	3340	R 1176	DSM	DSM 10656	
701.	*Staphylococcus piscifermentans*		T	3254	R 1160	DSM	DSM 7373	
702.	*Staphylococcus pulvereri*		T	3266	R 1183	DSM	DSM 9930	
703.	*Staphylococcus saprophyticus* subsp. *saprophyticus*		T	3342	R 1178	DSM	DSM 20229	
704.	*Staphylococcus schleiferi* subsp. *coagulans*		T	3330	R 1175	DSM	DSM 6628	
705.	*Staphylococcus schleiferi* subsp. *schleiferi*		T	3327	R 1174	DSM	DSM 4807	
706.	*Staphylococcus sciuri*		T	3153	R 755	DSM	DSM 20345	
707.	*Staphylococcus simulans*		T	3276	R 1164	DSM	DSM 20322	
708.	*Staphylococcus* sp.	V9		-	716	Dairy V		*Staphylococcus vitulinus/pulvereri*
709.	*Staphylococcus warneri*		T	3275	R 1163	DSM	DSM 20316	
710.	*Staphylococcus xylosus*	E1		-	719	Company E		*Staphylococcus succinus*
711.	*Staphylococcus xylosus*	E2		-	720	Company E		*Staphylococcus kloosii*
712.	*Staphylococcus xylosus*	L4		3024	641	Company E		*Staphylococcus succinus*
713.	*Staphylococcus xylosus*	F7		3040	657	Company F		
714.	*Stomatococcus mucilaginosus*		T	3291	761	DSM	DSM 20746	
715.	*Terrabacter tumescens*			1459	729	AJ	AJ 1460	*Arthrobacter oxidans*
716.	*Terrabacter tumescens*			1569	730	ATCC	ATCC 21109	*Rhodococcus equi*
717.	*Terrabacter tumescens*			1660	731	IAM	IAM 1447	*Arthrobacter* sp.
718.	*Terrabacter tumescens*		T	1755	732	ATCC	DSM 20308	
719.	*Terrabacter tumescens*			2057	589	AC	AC 256	*Corynebacterium variabilis*
720.	*Terrabacter tumescens*			2065	594	AC	AC 263	*Corynebacterium variabilis*
721.	*Terrabacter tumescens*		T	3274	754	DSM	DSM 20308	
722.	*Terracoccus luteus*		T	3424	834	DSMZ	DSMZ 44267	
723.	*Tsukamurella inchonensis*		T	3357	778	DSM	DSM 44067	
724.	*Tsukamurella paurometabola*			1586	40	ATCC	ATCC 15530	*Rhodococcus erythropolis*
725.	*Tsukamurella paurometabola*		T	1691	45	DSM	DSM 20162	
726.	*Tsukamurella paurometabola*			1801	633	CCM	DSM 43246	
727.	*Tsukamurella pulmonis*		T	3360	795	DSM	DSM 44142	
728.	*Tsukamurella tyrosinosolvens*		T	3364	799	DSM	DSM 44234	
729.	*Tsukamurella wratislaviensis*		T	3308	783	DSM	DSM 44107	
730.	*Turicella otitidis*		T	3334	785	DSM	DSM 8821	
731.	*Unidentified Bacterium*			1431	436	NCIB	ATCC 483	*Cellulomonas flavigena*

Notes: *: Reclassifications were carried out according to comparative 16S rDNA sequence analysis and/or FT-IR spectroscopy. Reclassifications are valid for the particular organisms stored in the Weihenstephan (WS) culture collection and do not necessarily apply for the strain in general (possible strain confusion between different culture collections, compare e. g. Macián *et al.,* 2000), especially not in the case of reclassified type strains. †:T marks type strains. ‡: Glycerol stock number. Glycerol stock numbers preceded by an "R" indicate L. Rieder's collection. Single quotes denote invalid species names. _1 and _2 in strain numbers indicate an originally mixed culture out of which two different strains were isolated.

Sources: AC: Collection of J. Antheunisse and W. H. J. Crombach, Univ. Wageningen, The Netherlands; **AJ, K. Yamada:** Central Research Laboratories, Ajinomoto Company, Kawasaki, Japan; **ATCC:** American Type Culture Collection, Manassas, VA, USA; **J. Baumgarten:** Bayer, Wuppertal, Germany; **BKM, VKM:** All-Russian Collection of Microorganisms, Pushchino, Moscow Region, Russia; **M. Carnio, I. Eppert, M. Loessner, L. Rieder, H. Seiler, WS:** Institute of Microbiology, FML Weihenstephan, Technical University of Munich, Freising, Germany; **CCM:** Czech Collection of Microorganisms Masaryk University, Brno, Czech Republic; **CIP:** Collection Institut Pasteur, Paris, France; **DSM(Z):** Deutsche Sammlung für Mikroorganisms (und Zellkulturen), Braunschweig, Germany; **F. Fiedler, K. Schubert:** Institut für Genetik und Mikrobiologie, Ludwig-Maximilians-Universität München, Munich, Germany; **HKI:** Hans-Knöll-Institut, Jena, Germany; **IFO:** Institute for Fermentation Osaka, Osaka, Japan; **T. A. Krulwich:** Department of Biochemistry, Mount Sinai School of Medicine, New York, USA; **NCDO:** National Collection of Dairy Organisms, Aberdeen, UK; **NCIB:** National Collection of Industrial Bacteria, Aberdeen, UK; **NCPPB:** National Collection of Plant Pathogenic Bacteria, Sand Hutton, UK; **NCTC:** National Collection of Type Cultures, London, UK; **IAM:** Institute of Applied Microbiology, Tokyo, Japan; **Rubber Inst.:** Rubber Institute, Kualalumpur, Malaysia; **K. P. Schaal:** Institut für Medizinische Mikrobiologie und Immunologie, Rheinische

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Acknowledgments

The work presented here proceeded under the supervision of Prof. Dr. Siegfried Scherer at the Institute of Microbiology, FML Weihenstephan, Technical University of Munich. I feel blessed having been given the opportunity to set up a Ph.D. thesis at this institute which is filled with a spirit of both hard work and enjoyable companionship.

I am grateful to Prof. Scherer for his continuous guidance and support. His door is always open and he dedicates much time and effort to his team.

I am also indepted to Prof. Dr. Karl-Heinz Schleifer for showing interest in my work and taking over the co-chair of the advisory commitee.

Dr. Wolfgang Ludwig kindly provided and introduced me to the ARB software environment together with the ARB 16S rDNA sequence database. Dr. Frank Mertens spent time and energy on Partial Least Square regression models for analyses of mixed microbial populations by FT-IR and has been a faithful co-author. Dr. Michael Kümmerle and Dr. Felix von Stetten both readily introduced me to FT-IR spectroscopy and molecular microbiology, respectively. Thank you also to Dr. Herbert Seiler.

The technical assistance of Sven Illgner, Patrizia Hägele and Louise Arnold is greatly appreciated. Lisa Rieder is acknowledged for her freely given assistance whenever necessary. Special thanks are due to Cornelia Fischer for excellent technical assistance.

I thank Dr. Lyudmila Evtushenko, Dr. Ingrid Groth, Prof. Klaus P. Schaal, and Dr. Karin Schubert for supplying me with strains from their collections and several German companies for providing starter culture strains for my FT-IR identification database.

Thank you to all co-workers at the institute who by their continuous support helped to make this time in Weihenstephan a very pleasant time for me. I will always remember the nice working atmosphere, helpful advice freely given, and delightful celebrations. It has been a pleasure to work with you.

I am grateful to my husband Dr. Joachim Charzinski for his support and his constant interest in my topic and I thank my parents for their life-long love and encouragement.

My work was supported by the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn), the AiF (Arbeitskreis für industrielle Forschung) and the German Ministry of Economics and Technology.

CURRICULUM VITAE

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Education

Ph.D. in Microbiology (Dr. rer. nat.)	Institute of Microbiology, FML Weihenstephan, Technical University of Munich	May 1997 – March 2001
Diploma in Technical Biology	University of Stuttgart	Oct. 1991 – April 1997
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Work experience

Dairy product analysist: Provided expert opinions for microbial analyses of food and dairy products	Institute of Microbiology, FML Weihenstephan, Technical University of Munich	Oct. 1997 – Oct. 2000
Diploma thesis: Bacteriocin-producing lactic acid bacteria in fine emulsified sausage (Brühwurst)	Gewürzmüller GmbH, Stuttgart	July 1996 – Feb. 1997
Laboratory assistant: Cultivation of soil isolates	Institute for Sanitary Engineering, Water Quality and Solid Waste Management, University of Stuttgart	Jan. 1994 – July 1994

Foreign exchange experience

Rotary exchange student	Rochester, NY, USA	Aug. 1988 – Aug. 1989

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