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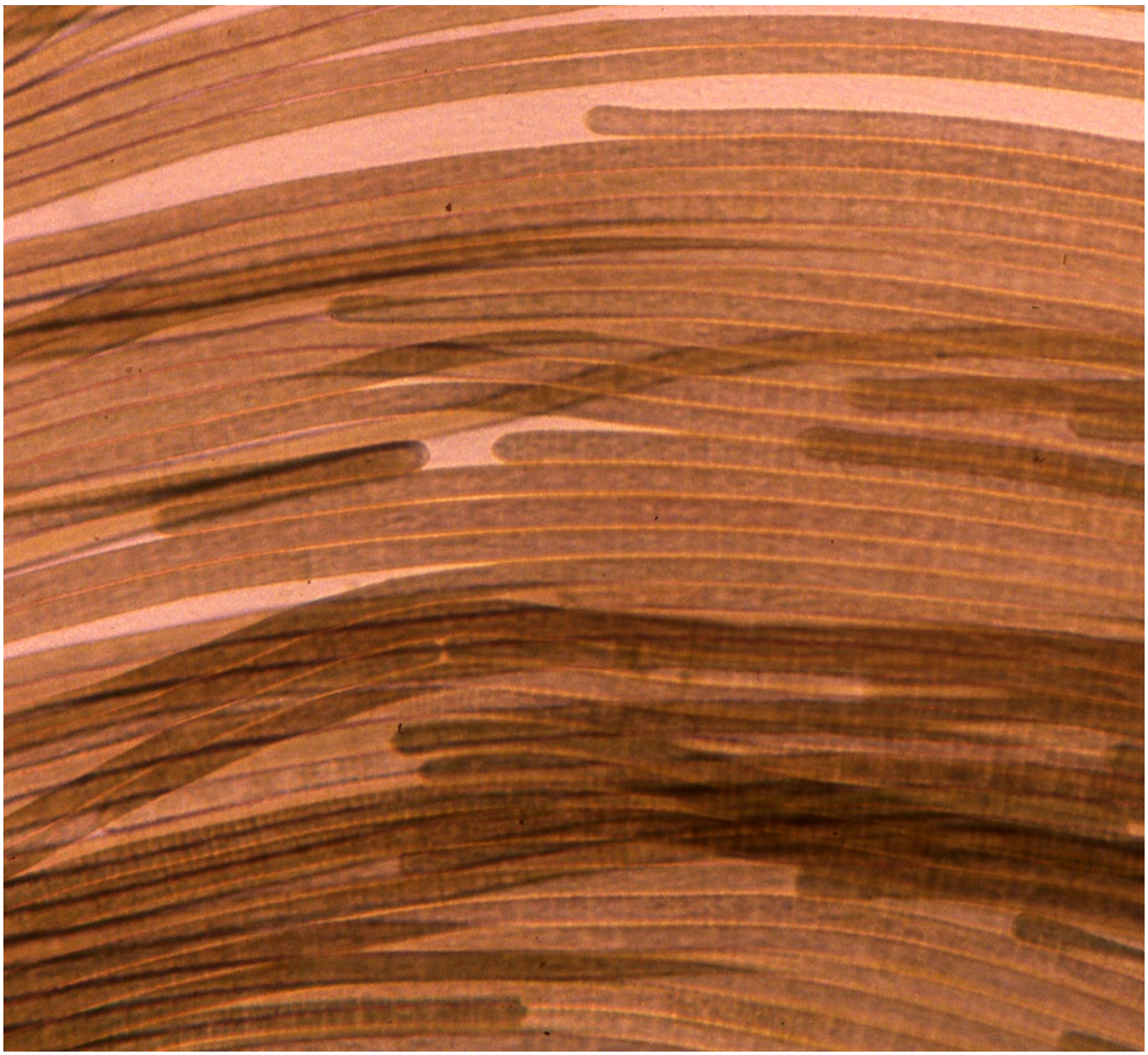

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On the cover

Oscillatoria cf. *boryana* from a tepid S-rich pool in New Zealand. Photograph supplied by Richard W. Castenholz – see his autobiography in this issue.

The Bulletin of BISMiS

Contents of Volume 2, part 2

The Bulletin of BISMiS (2011), Volume 2, part 2, pp. 87–106

A century of systematics of the genus *Bacteroides*: from a single genus up to the 1980s to an explosion of assemblages and the dawn of MALDI-TOF-Mass Spectrometry

Haroun N. Shah and Saheer E. Gharbia

The genus "*Bacteroides*" comprises the predominant group of micro-organism of the alimentary tract where they coexist to the mutual benefit of the host. Recent studies of the human gut microbiome is revealing the emergence of universal dominant clusters (or enterotypes) of which *Bacteroides*, *Prevotella* and *Ruminococus* are the dominant enterotypes (Arumugam et al., 2011). Perturbations in the local habitat are believed to trigger their translocation to new sites from where they cause numerous infections. The original "*Bacteroides*"* species were studied extensively in the rumen for nearly half a century and much of the fundamental science including anaerobic techniques were emulated to study infections in man (see e.g. Hungate, 1966). Today their physiological functions and significance are intensively studied in parallel with their phylogenetic relationships (see e.g. Karlsson et al., 2011) but this would not have been possible without painstaking work on their systematics from the mid-1970s. Once their heterogeneity had been ordered into coherent groups of species, more focused studies became possible and with the current interest in the human microbiome, their functional roles *in vivo* are becoming clearer. However, many taxa are non-fermentative and poorly circumscribed and new techniques are being explored to elucidate differential characters. This latter group has been one of the driving forces for the development of MALDI-TOF and LC-tandem mass spectrometry. This review charts the history of these taxonomic changes against the intransigence of established microbiologists and, the opposing views of newcomers to the field who systematically persevered to overturn the dogma of many decades into an accepted classification that today concurs with their phylogeny.

Introduction

Few microbiologists are taxonomists by training, most develop their interest and acquire their skills from sustained studies of a particular habitat. The overwhelming range of inter- and intra-species diversity together with the problems of assigning unknown isolates to a prior classification often provides the impetus for in-depth analysis of an ecosystem and fuels an interest in systematics. Developments in the field of taxonomy of the *Bacteroides* provide a poignant example in which huge debates were contested between those who were intransigent to taxonomic change and those who argued vehemently for modernization. Recognized very early on as a major component of the colon of man, workers in the field of veterinary (e.g. T. Mitsuoka, D. Love, E.M. Barnes, R.E. Hungate, M.P. Bryant, M. Sebald, M. Latham, C. Impey, et al.) and human sciences (e.g. L.V. Holdeman, W.E.C. Moore, S.M. Finegold, B. Drasar, J.G. Collee, D.W. Lambe, B.I. Duerden, W.P. Holbrook, H. Beerens, H. Werner, S. L. Gorback, F.P. Tally, V. R. Dowell, E. P. Cato, V.L. Sutter, T. Hofstad, D.L. Kasper, S.S. Socransky, K. Okuda, S. Tabaqchali, P. Borriello, M. Hill, H. Werner, R.J. Gibbons, J.B. MacDonald, D. Mayrand, T.J.M. van Steenbergen, G.K. Sundqvist, A.M. Yoshimura. I. Olsen, E. Nagy, et al.) produced voluminous literature in reference books, conference proceedings, original papers and were encapsulated in editions of *Bergey's Manual*, highlighting the ubiquitous presence of a continuum of species that were compatible with the general description of "*Bacteroides*".

From the onset, the broad description of the group, as Gram-stain-negative, non-spore-forming, nonmotile, obligatory anaerobic rods that may or may not ferment carbohydrates prevailed through all editions of *Bergey's Manual of Determinative Bacteriology*. This all-encompassing description of "*Bacteroides*", together with a paucity of reliable characters for species' descriptions resulted in the accumulation

*The name "*Bacteroides*" used here initially is all-encompassing to illustrate their early history.

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of a large and heterogeneous collection of species that could not be accommodated elsewhere (Bryant et al., 1958; Mitsuoka et al., 1974; Harrison and Hansen, 1963; Cato and Barnes, 1976).

The number of species varied periodically between editions of *Bergey's Manuals*, however, the type species, *Bacteroides fragilis*, remained unchanged at the center of variation as the intrageneric structure of this group transformed over the years. The inspired work of Veillon and Zuber (1898), which resulted in the isolation of Gram-stain-negative, nonsporing, obligately anaerobic bacteria from various infectious sites, stimulated other workers around the turn of the century. Notable examples included the work of Tissier (1908) who reported that the predominant micro-organisms of the human colon were anaerobes and described five "types" while Krumwiede and Pratt (1913) and Tunnicliff (1919) reported various anaerobic fusiform bacteria. Castellani and Chalmers 1919 assimilated descriptions from several of these studies, particularly that of Veillon and Zuber (1898) for the 1st edition of *Bergey's Manual* (Bergey et al., 1923). This was reinforced by Primbram (1929, 1933) who reported studies on the classification of these micro-organisms but was not cited as their source until the 8th edition of *Bergey's Manual* (1974).

The first radical change was witnessed in the 5th edition of the *Manual* (Bergey et al., 1939) when an attempt was made to construct a combined schema incorporating the extensive work of Prévot (1938) and Eggerth and Gannon (1933). The new definition now encompassed only Gram-stain-negative species and the black pigment of colonies grown on blood agar was introduced as a new determinative character to identify *Bacteroides melaninogenicus*. Largely because of Prévot's work, data on animal pathogenicity tests were included for the first time in *Bergey's Manual*. However, the schema produced in the following edition of the *Manual* (Breed et al., 1948) was confusing as it also included Prévot's work (1938) in the same chapter that provided a "key to the species of the genus "*Bacteroides*". *Bacteroides* species were described under the generic names "*Ristella*", "*Bacterium*", "*Bacillus*", "*Zuberella*" or "*Coccobacillus*" (Bergey et al., 1939). Thus, *Bacteroides fragilis* and *Bacteroides melaninogenicus* were synonyms of *Ristella fragilis* and *Ristella melaninogenica* respectively.

The arrival of compact, largely automated gas-liquid chromatographs facilitated the rapid analysis of volatile, short-chained fatty acid end products of metabolism such as formic, acetic, propionic, *n*-butyric, iso-butyric, and iso-valeric acids, etc., in microbiology laboratories. The diagnostic profiles of species were effectively used by Holdeman and Moore to again fundamentally restructure the genus *Bacteroides* (1974). The family *Bacteroidaceae* now comprised three genera based on the profile of their end products of metabolism. Genus 1, *Bacteroides*, produced a complex mixture of short-chained volatile fatty acids. Genus 2, *Fusobacterium*, produced butyric acid as a major end product and generally had a simpler profile, while Genus 3, *Leptotrichia*, yielded lactic acid as its only major end product. While *Fusobacterium* and *Leptotrichia* were fairly well defined, the *Bacteroides* were often deduced by failure to match profiles to the two former genera. Within the genus *Bacteroides*, species profiles of acids were diverse and matching an unknown to a given pattern for identification was often subjective. Thus, although *Bacteroides*, moved to a another stage of classification, it was evident that species still retained considerable inherent heterogeneity. Several subspecies were conveniently created to minimize the number of species but these only served to obscure their intra-species diversity. The newly described genus *Bacteroides* excluded 18 of the 30 species described in the 7th edition of the *Manual* (1957) while four species comprised subspecies. Thus, *Bacteroides melaninogenicus* was listed as three subspecies, *viz. Bacteroides melaninogenicus* subsp. *melaninogenicus* for the highy saccharolytic strains, *Bacteroides melaninogenicus* subsp. *intermedius* for moderately fermentative strains, and *Bacteroides melaninogenicus* subsp. *asaccharolyticus* for strains that were non-fermemtative. The species, *Bacteroides fragilis*, *Bacteroides ruminicola*, and *Bacteroides clostridiiformis* were also now redefined to include subspecies.

In the 1970s to 1980s, chemotaxonomic analyses were extensively reported on members of the genus of *Bacteroides*. The heterogeneity of this group was reflected in their DNA base compositions which ranged from 28–61 mol% G+C, while large differences in long-chained cellular fatty acids, menaquinone composition, polar lipids, peptidoglycan chemotypes, SDS-PAGE patterns, and metabolic enzymes were reported (for reviews, see Shah 1991a, 1991b). Consequently, the diversity among specific taxa was such that several were reclassified and in some cases new species were proposed (see Table 1). In the 1st edition of *Bergey's Manual of Systematic Bacteriology* (Holdeman et al., 1984), the number of species increased by 75% (Table 1) over the previous (8th) edition of *Bergey's Manual of Determinative Bacteriology* (1974) by virtue of many being elevated to species rank but mainly due to the large numbers reported from the oral cavity. However, the criteria and structure of the genus followed along the same lines as the 8th edition of the *Manual* (1974) and the tremendous diversity reported within the genus was merely highlighted as footnotes to the chapter. This was echoed by the International Committee on

Systematic Bacteriology Subcommittee on the Taxonomy of Gram-negative Anaerobic Rods, which persisted in making minimal changes to improve the taxonomy of the genus (see e.g. International Committee on Systematic Bacteriology, 1976). Table 1 provides a summary of the sequential changes reported from the 1st edition of *Bergey's Manual* (Bergey et al., 1923) to the 1st edition of *Bergey's Manual of Systematic Bacteriology* (1984).

Bacteroides melaninogenicus, focus of the first change

This broad acceptance of heterogeneity permeated to the species level. Thus, the species *Bacteroides melaninogenicus* encompassed strains that were metabolically very diverse but resided in juxtaposition to each other as "subspecies" to avoid taxonomic change. In any other branch of taxonomy it would be irreconcilable that a species could encompass saccharolytic (*Bacteroides melaninogenicus* subsp. *melaninogenicus*), moderately saccharolytic (*Bacteroides melaninogenicus* subsp. *intermedius*) and also non-fermentative *Bacteroides melaninogenicus* subsp. *asaccharolyticus*) strains into a single species. The overriding property of the black pigment, observed after growth on blood agar plates by *Bacteroides melaninogenicus* was the basis in which the heterogeneity of this species was accepted. The scientific community held steadfastly to the accepted dogma and was so refractory to change, it would seem prudent that detailed characterization of the pigment of *Bacteroides melaninogenicus*, its most weighted yet contentious character, might provide the momentum for change.

This species was first described in 1921 by Oliver and Wherry as *Bacteroides melaninogenicum* because it was thought that the distinctive black pigment produced by cells grown on blood agar plates was melanin (Oliver and Wherry, 1921). Although the pigment was incorrectly identified (Schwabacher et al., 1947), the accepted rules of nomenclature overruled the suggested change of the specific epithet to *Bacteroides nigrescens*, and "*melaninogenicus*" was retained. The nature of the pigment was contested by Tracy (1969) who reported that the pigment was not specific for *Bacteroides melaninogenicus* and could be induced in other non-pigmented species such as *Bacteroides fragilis*. Tracy (1969) stated that the pigment was extracellular and identified it as colloidal ferrous sulfide. However, most studies demonstrated that the pigment was only produced when cells were cultured on blood agar and was specific for *Bacteroides melaninogenicus* (Duerden, 1975). Our studies (Shah et al., 1979) showed that the depth and duration of pigmentation varied among strains and long-wave UV radiation was pronounced before the characteristic jet-black

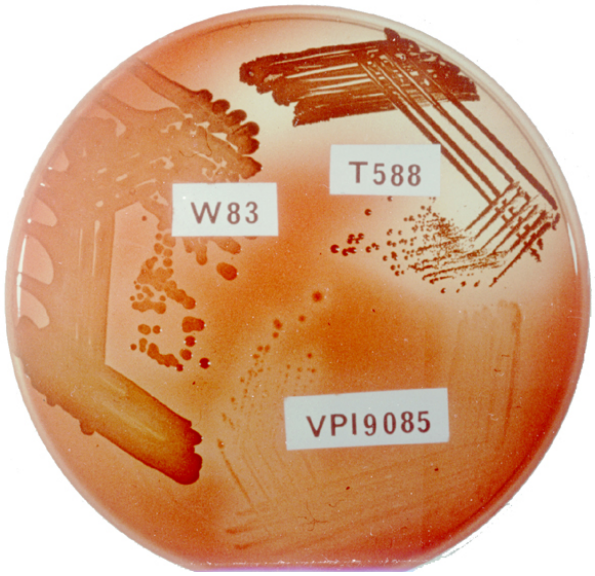


Figure 1. Variation in colonial morphology and depth of pigmentation among strains of "*Bacteroides melaninogenicus*" grown on blood agar plates. Strain VPI 9085 was a reference strain of "*Bacteroides oralis* that pigmented, albeit light brown and was eventually used to create the new species "*Bacteroides loescheii*" after Loesche et al. (1964) who proposed the species, *Bacteroides oralis*. Strain W83 showed mucoid dark brown colonies, characteristic of the asaccharolytic strains. T588, a representative of the moderately saccharolytic group formed very small, jet-black, pigmented colonies.

pigmentation arose. Large-scale extraction, crystallization and mass spectral determination of the pigments eventually confirmed them to be protohaemin (non-fluorescent) while the bright fluorescent compound was shown to be protoporphyrin (Shah et al. 1979). Of the three, subspecies "*melaninogenicus*" produced mainly protoporphyrin hence colonies on blood agar were often light brown to beige rather than black (Figure 1). Some laboratories reported that strains of *Bacteroides melaninogenicus* showed no pigmentation while conversely isolates of the non-pigmented species *Bacteroides oralis* were found to pigment so consistently that extensive re-examination of this equally contentious group began.

A key property of *Bacteroides oralis* was its inability to form black-pigmented colonies on blood agar or to ferment the pentose sugars, xylose and arabinose. (Loesche et al., 1964). The latter was described as a significant differential property of the rumen spcies, *Bacteroides ruminicola* (Bergey's 1984). However, strains designated *Bacteroides oralis* (including the Type strain 7CM) were very heterogeneous and found to be incompatible with its original description.

Table 1. Summary of the sequential changes reported from the 1st edition of *Bergey's Manual of Determinative Bacteriology* (1923) to the 1st edition *Bergey' Manual of Systematic Bacteriology* (1984)

Initially, most species descriptions were based on Veillon and Zuber (1898) and updated by Castellani and Chalmers (1919) for their inclusion in the 1st edition in 1923. Parallel identification schemes were devised elsewhere, the most significant of these being Prévot (1938) and Eggerth and Gannon 1933. Where there were obvious synonyms based on morphological and biochemical data, later editions of Bergey's (from 4th edition) began incorporating them in a single system with the genus name *Bacteroides* taking precedence.

Edition details, contributors, and notes	Family/tribe/genus/species	Salient phenotypic tests	Habitat
1st edition of *Bergey's Manual of Determinative Bacteriology* (1923), pp. 255–264. D.H. Bergey, F.C. Harrison, R.S. Breed, B.W. Hammer and F. M. Huntoon.	Family IV: *Bacteriaceae* Tribe IX: *Bacteroideae* Genus XVIII: *Bacteroides* Castellani and Chalmers. Species: 17 were described as follows: (1) *B. fragilis* (Type); (2) *B. cornutus*; (3) *B. variabilis*; (4) *B. dimorphus*; (5) *B. laevis*; (6) *B. pseudoramosus*; (7) *B. tortuosus*; (8) *B. angulosus*; (9) *B. acumicatus*; (10) *B. bifidus*; (11) *B. bullosus*; (12) *B. variegatus*; (13) *B. thetaiotaomicron*; (14) *B. liquefaciens*; (15) *B. rigidus*; (16) *B. tenuis*; (17) *B. multiformis*. Even though Oliver and Wherry had described the black-pigmented species *B. melaninogencus* in 1921, it was not included in *Bergey's Manual* until the 5th edition (1939).	Definition taken from Castellani and Chalmers 1919; motile and non-motile rods, without endospores. Shows good growth on ordinary culture media; without pigment formation. All obligate anaerobes. Key tests were: motility, coagulation of milk, indole and acid from glucose. About half of the species listed including the type species, *B. fragilis* were described as Gram-positive.	All isolates were from the intestinal track of man and animals; *B. fragilis* isolated from a gangrenous appendix. A few species were facultatively anaerobic.
2nd edn (1926), pp. 271–279. D.H. Bergey, F.C. Harrison, R.S. Breed, B.W. Hammer and F. M. Huntoon.	Family IV: *Bacteriaceae* Tribe XI: *Bacteroideae* Genus XXI: *Bacteroides* Castellani and Chalmers 1919.	Unchanged from the 1st edition. Several species continue to be listed as "Gram-positive".	Unchanged from the 1st edition.
3rd edn (1930), pp. 369–378. D.H. Bergey, F.C. Harrison, R.S. Breed, B.W. Hammer and F. M. Huntoon.	Family IV: *Bacteriaceae* Tribe XIII: *Bacteroideae* Genus XXIV: *Bacteroides* Castellani and Chalmers 1919. Species: 19: Two new species, *B. oviformis* and *B. fundibuliformis* were added.	Unchanged from the previous editions. Indole production and acid in milk were key characters. Morphology and limited range of carbohydrate fermentation reactions used for speciation.	All species were intestinal except *B. fundibuliformis* which was isolated from a vaginal abscess.
4th edn (1934), pp. 404–413. D.H. Bergey, R.S. Breed, B.W. Hammer, F. M. Huntoon, E,G.D.Murray and F.C. Harrison.	Family IV: *Bacteriaceae* Tribe XIII: *Bacteroideae* Genus XXVI: *Bacteroides* Castellani and Chalmers 1919. Species: all 19 species described before were retained.	*B. fragilis* was listed for the first time as "Gram-variable".	Unchanged from the 3rd edition.

Edition details, contributors, and notes	Family/tribe/genus/species	Salient phenotypic tests	Habitat
5th edn (1939), pp. 556–586. D.H. Bergey, R.S. Breed, E,G.D. Murray and A. P. Hitchens. The extensive work of Prévot (1938) and Eggerth and Gannon (1933) were now incorporated. Prévot's classification placed *Bacteroides* in two Orders comprising eight genera.	Family XI: *Bacteriaceae* Tribe: (Nil) Genus VIII: *Bacteroides* Castellani and Chalmers 1919. Species: 21 listed but only four, *B. fragilis*, *B. variabilis*, *B. fundibuliformis* and *B. thetaiotaomicron* were retained from the 4th edition. 19 new species were listed: *B. serpens*; *B. siccus*; *B. coagulans*; *B. varius*; *B. inaequalis*; *B. insolitus*; *B. vescus*; *B. exiguus*; *B. uncatus*; *B. vulgatus*; *B. incommunis*; *B. distasonis*; *B. tumidus*; *B. convexus*; *B. ovatus*; *B. uniformis*; *B. gulosus*; *B. caviae* and *B. melaninogenicus*.	A more extensive list of fermentation tests were now included. Some that were hitherto considered Gram-positive (e.g. *B. fragilis*), were reassessed and excluded if they Gram-positive.	A number of human sites were now included; e.g. urinary tract, genitalia, surgical wounds pulmonary infections, septicemias, abscesses. For the first time, the oral cavity was cited as a habitat for *B. melaninogenicus*.
6th edn (1948), pp. 564–581. I. Huddleson, F. Johnson, F.H. Kauffmann and C.D. Kelly. Both systems [Prévot (1938) and Eggerth and Gannon (1933)] were not tested in parallel hence the schema drawn up for their identification was considered "tentative" at that stage.	Family XI: *Parvobacteriaceae* Tribe III: *Bacteroideae* Genus I: *Bacteroides* Castellani and Chalmers. 1919. (revised by T.E. Roy and C.D. Kelly). Species: the 23 species listed in the 5th edition were retained unchanged.	Phenotypic properties were retained from the 5th edition but there were several nomenclatorial changes.	Unchanged from the 5th edition.
7th edn (1957), pp. 423–436. N.R. Smith. The uncertainty of the previous edition was replaced by a more definitive, concise scheme.	Family VI: *Bacteroidaceae* Tribe: Nil Genus I: *Bacteroides* Castellani and Chalmers 1919. Species: 30 species. 14 new species were listed: *B. furcosus*;*B. perfoetens*; *B. trichoides*; *B. terebans*; *B. halosmophilus*; *B. putidus*; *B. putredinis*; *B. capillosus*; *B. cylindroides*; *B. succinogenes*; *B. glutinosus*; *B. destillationis*: *B. viscosus*; *B. variegates*. 7 species listed in the 6th edition were excluded: *B. fundibuliformis*, *B. siccus*, *B. varius*, *B. gulosus* and *B. caviae*, *B. inaequalis* and *B. vescus*.	Key hierarchal tests included: motility, encapsulation, gelatin liquefaction, requirement for serum or ascitic, carbohydrate fermentation tests and cellular morphology. Cellulose was included to identify species such as *B. succinogenes* that can digest it while rumen fluid was added to improve the grown of micro-organisms from this site.	The habitat of the group was described as "found in the alimentary and urogentical tracts of man and other animals". Work on rumen species by Bryant et al. (1958) and Hungate (1966) were widely reported.

Edition details, contributors, and notes	Family/tribe/genus/species	Salient phenotypic tests	Habitat
8th edn (1974), pp. 384–404 L.V. Holdeman and W.E.C. Moore. (Based on "*The Virginia Polytechnic Institute* and *State University Anaerobe Laboratory*", Blacksburg, VA, 1st edition, 1972). It included extensive work from Prévot (1967) and Sebald (1962) where metabolic end products and DNA base compositions were already being used for classification.	Family 1: *Bacteroidaceae* Pribram 1933, 10. Genus 1: *Bacteroides* Castellani and Chalmers 1919. Species: 22 listed with a number of subspecies. *B. fragilis was* retained as the type species but 5 phenotypically closely related species were reduced to subspecies rank as follows: *B. fragilis* subsp. *fragilis*; *B. fragilis* subsp. *vulgatus*; *B. fragilis* subsp. *distasonis*; *B. fragilis* subsp. *ovatus*; *B. fragilis* subsp. *thetaioatomicron*. In addition to the above, the following 7 species in 7th edition were retained: *B. putredinis*; *B. furcosus*; *B. succinogenes*; *B. serpens* and *B. melaninogenicus*. 18 species listed in previous edition were excluded. 14 new species were listed: *B. ruminicola* (2 subspecies); *B. ochraceus*; *B. oralis*; *B. amylophilus*; *B. hypermegas*; *B. termitidis*; *B. biacutus*; *B. clostridiiformis* (2 subspecies); *B. constellatus*; *B. praeacutus*; *B. corrodens*; *B. nodosus*; *B. pneumosintes* and *B. niger*.	The key to delineation of species placed heavy reliance on pigment formation on blood agar plates. Cellular morphologies based on microscopy were sketched. An extensive list of carbohydrates were used to differentiate species along with their major acid end product/s. Where known the mol% G+C content for species were given and fell within the range 40–55%. Other tests were given by Dowell and Hawkins (1968) and Sutter et al. (1975)	Strain from animals extended and included one species from the intestinal contents of termites (*B. termitidis*) which had a low G+C content of 34 mol%.
1st edition (1984) of *Bergey's Manual of Systematic Bacteriology*, pp. 602–631 L.V. Holdeman, R. W. Kelley and W.E.C. Moore. (Based on '*The Virginia Polytechnic Institute* and *State University Anaerobe Laboratory*', Blacksburg, Virginia, 4th Ed. 1977).	Family 1: *Bacteroidaceae* Pribram 1933, 10. Genus 1: *Bacteroides* Castellani and Chalmers 1919. Species: 39 described. A number previously designated subspecies such as *B. fragilis* group were elevated to species rank again. Seven species in the 8th edition were excluded. Strains of *B. serpens*, *B. niger a*nd *B. constellatus* were no longer extant while spores were found in *B. clostridiiformis* and *B. biacutus* and both species reassigned to other genera. Similarly *B. ochraceus* was considered to be capnophilic rather than anaerobic and was reclassified in genus *Capnocytophaga*. 21 new species were described, some of which were previously assigned subspecies status such as *B. asaccharolyticus* and *B. intermedius*.	The same criteria (extensive carbohydrate fermentation tests and metabolic end product profiles) were used to circumscribe species in the 8th edition were retained but complete data were now added to most species. In particular their metabolic end products and DNA base compositions were listed for most species. Cellular morphologies based on microscopy were extended. A vast amount of chemotaxonomic data such as long-chained cellular fatty acids, respiratory quinones were available but not utilized.	Man, animal and a large number of species from environmental habitats.

Figure 3 shows the IEF-protein patters (in a narrow pH gradient of 4.5–6.0) of 13 strains that were widely circulated as authentic cultures of *Bacteroides oralis* and highlights this incongruity. Four groups were resolved and shown by DNA–DNA reassociation to comprise the nucleus of five distinct species that were eventually proposed as *Bacteroides buccalis*, *Bacteroides denticola* and *Bacteroides pentosaceus* (the latter was subsequently changed to *Bacteroides buccae* (Bergey's 1984; Holdeman and Johnson, 1982; Shah and Collins, 1981). Related species *Bacteroides veroralis* sp. nov. was proposed by Watabe et al. (1983) and *Bacteroides oulorum* sp. nov. by Shah et al. (1985).

The unambiguous identification of the pigments of *Bacteroides melaninogenicus* led to studies of their function. Porphyrins are often present as the prosthetic group of cytochromes and cell extracts of both pigmented and non-pigmented species were found to have cytochrome *c* and cytochrome *b* (Shah, 1991b). At that time, anaerobic bacteria were thought to utilize ferrodoxin-type mechanisms for energy conservation. The presence of porphyrins supported the view of an anaerobic electron transport chain in Gram-stain-negative anaerobes and represented a major divergence from other anaerobic species. The electron-transport complex was shown to consist of another electron transport carrier, menaquinone, derived from its growth requirement for vitamin K1 (Shah and Collins, 1980). The presence of enzymes such as malate dehydrogenase and fumarate reductase with succinate as an end product, together with radiolabeling studies, confirmed the presence of only a partial TCA cycle (often referred to as the succinate pathway). Furthermore, the abolition of fumarate reductase activity by inhibitors such as 2-n-heptyl-4-hydroxyquinoline *N*-oxide, and the concomitant reduction in cytochrome, suggested that fumarate acted as an electron sink in accepting reducing equivalents from NADH generated during catabolism by *Bacteroides melaninogenicus* (Shah and Williams, 1987a, 1987b; Al-Jalili and Shah, 1988). These results confirmed beyond doubt that more than one mechanism of energy conservation existed in anaerobic bacteria and helped to explain its dependence on heme (for the prosthetic porphyrins) and menadione (vitamin K_1) for menaquinones biosynthesis which are naturally acquired in the intestinal tract. Differences in pH optima and enzyme stabilities of the *Bacteroides melaninogenicus* subspecies further emphasized the heterogeneity of this group (Shah and Williams, 1982; Shah, 1993).

Studies of the mol% G+C content and multilocus enzyme electrophoresis of these groups revealed considerable heterogeneity with this taxon. The saccharolytic (*Bacteroides melaninogenicus* subsp. *melaninogenicus*) resolved into three groups, the moderately saccharolytic (*Bacteroides melaninogenicus* subsp. *intermedius*) into two clusters while the nonfermentative strains were delineated into three additional groups in accord with their menaquinone profiles (Figure 2) and DNA–DNA reassociation studies eventually led to establishment of these clusters as eight distinct species (Bergey's, 2011).

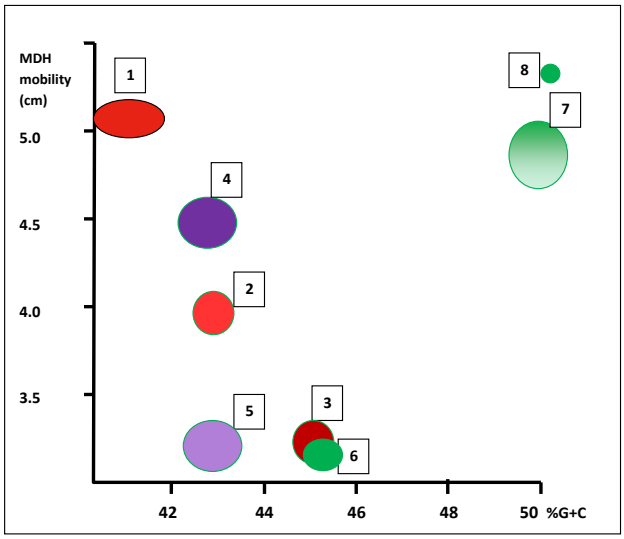


Figure 2. DNA base composition against the electrophorectic mobility of malate dehydrogenase of the *Bacteroides melaninogenicus*" complex. (Adapted from Shah et al., 1976.) Red – saccharolytic strains that were eventually assigned to (1) *Bacteroides melaninogenicus*, (2) *Bacteroides denticola*, and (3) *Bacteroides loescheii*. Purple – moderately saccharolytic strains that were later proposed as two distinct species, (4) *Bacteroides intermedius* and (5) *Bacteroides nigrescens*. Green – non-fermentative strains that subsequently became (6) *Bacteroides gingivalis*, (7) *Bacteroides asaccharolyticus*, and (8) *Bacteroides endodontalis*.

Clarification of the taxonomy of this group represents an excellent example of the immense value of systematics in revealing the site-specificity and hence the potential contribution of a particular species to disease. Earlier, MacDonald et al. (1956) and Socransky and Gibbons (1965) demonstrated that "*Bacteroides melaninogenicus*" was an essential component of the flora of mixed infections using a guinea pig model. Similar experiments were not reproducible subsequently and were attributed to differences in strains used. Over the years, it became apparent that the saccharolytic strains appear to be less virulent and more associated with the normal flora. By contrast, the asaccharolytic species such as *Porphyromonas gingivalis*, *Porphyromonas asaccharolytica*, and *Porphyromonas endodontalis* possess a large number of virulence determinants and were reported

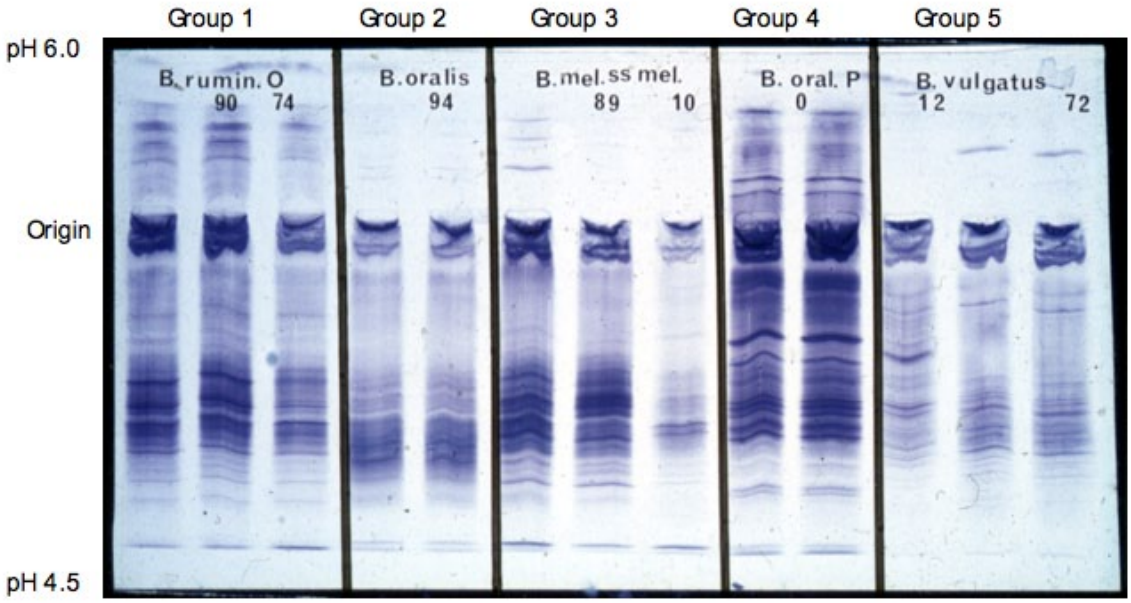


Figure 3. Narrow range (pH 4.5–6.0) isoelectric-focusing of proteins of strains designated "*Bacteroides oralis*" and their relatedness by DNA–DNA reassociation (given above each lane). (From Shah et al., 1982.) The lane without a number is ^{3}H-labeled DNA reference probe. These resolved into five groups a shown above; from left to right; (1) Oral isolates that were phenotypically similar to *Bacteroides ruminicola* – subsequently proposed as *Bacteroides pentosaceus* (=*Bacteroides buccae*). (2). Two strains designated *Bacteroides oralis* that shared 94% reassociation; eventually proposed as *Bacteroides buccalis*. (3). Saccharolytic strains of *Bacteroides melaninogenicus*, one of which had only 10% DNA–DNA reassociation and became *Bacteroides loescheii*. 4. Strains that were also phenotypically similar to *Bacteroides oralis* (in group 2) but did not hybridize with these strains; a new species *Bacteroides oulorum* was created for these. (5). The ^{3}H-labeled reference DNA of the type strain of *Bacteroides oralis*, strain 7CM showed high affinity with *Bacteroides vulgatus* and was transferred to this species.

more widely from infections. Today as direct molecular analysis of mixed infections are undertaken, combinations involving nonfermentative strains appear to substantiate the work of MacDonald et al. (1956) and Socransky and Gibbons (1965) and suggest that their strain may have been an asaccharolytic isolate. More directly *Porphyromonas gingivalis*, delineated from this complex (Figure 3), is specific to the oral cavity and considered a key component of periodontal disease and this facilitated detailed studies into its pathogenic mechanisms. For example, the vesicle-bound cysteine proteinase of *Porphyromonas gingivalis* "gingivain" (Shah et al., 1993) was shown to be a potent tissue and cellular destructive enzyme and considerable work has been devoted to elucidating its biological role. *Porphyromonas gingivalis* is a nonfermentative species whose growth requirements are markedly influenced by the presence of peptides (Shah, 1993). It seems likely that *in vivo* these micro-vesicles, which are drawn into the interstitial spaces of the tissues, hydrolyze native proteins to release peptides/amino acids to maintain the growth of cells. Consequently, current therapeutic strategies are being focused on inhibiting proteolysis.

Bacteroides fragilis, the type species and other non-pigmented "bacteroides"

Around the period from 1970 to 1980, while oral microbiologists worked almost exclusively on pigmented species, intestinal and rumen scientists focused largely on *Bacteroides fragils* and *Bacteroides ruminicola*, respectively. Comparative studies of the *Bacteroides melaninogenicus*, *Bacteroides fragilis*, and *Bacteroides ruminicola* groups revealed vast differences in biochemical and chemical properties (see Shah, 1976, 1991a). The most striking differences were in their DNA base compositions (approx. 28–61 mol% G+C) which is almost as diverse as the bacterial kingdom. However, the majority, including the type species *Bacteroides fragilis*, had DNA base compositions within the approximate limiting range of 40–50 mol% and appeared to comprise the nucleus of the genus. Others such as *Bacteroides praeacutus*, *Bacteroides hypermegas*, *Bacteroides furcosus*, and *Bacteroides ureolyticus*, etc., were similar to fusobacteria in possessing DNA base compositions of ~30 mol% G+C. At the extreme limits were other species such as *Bacteroides capillosus*, *Bacteroides multiacidus*, and *Bacteroides microfusus* that possessed base compositions of ~60 mol% G+C.

To further study their diversity, systematic analysis of these groups were undertaken with particular emphasis on the analysis of their long-chained cellular fatty acids and respiratory quinones (see e.g. Shah and Collins, 1983). The non-hydroxylated fatty acids of species with mol% G+C contents of 40–50 mol% consisted predominantly of straight-chain, anteiso- and iso-methyl branched-chain acids; monounsaturated fatty acids were found in only small amounts. The major fatty acids of species such as *Bacteroides ochraceus*, *Bacteroides distasonis*, and *Bacteroides splanchnicus* and the asaccharolytic subspecies of *Bacteroides melaninogenicus* was 13-methyltetradecanoic acid (C_{15} iso) whereas the saccharolytic subspecies of *Bacteroides melaninogenicus* and oral isolates that were phenotypically similar to the *Bacteroides ruminicola* and *Bacteroides fragilis* groups possessed mainly 12-methyl-tetradecanoic acid (C_{15} anteiso). Interestingly, the rumen strains of "*Bacteroides*" (*viz. Bacteroides ruminicola* subsp. *ruminicola* and *Bacteroides ruminicola* subsp. *brevis*) were characterized by major levels of C_{15} anteiso and pentadecanoic ($C_{15:0}$) fatty acids.

Menaquinones were the only isoprenoid quinones present among all taxa with most species possessing menaquinones with between 10 and 13 isoprene units (Shah and Collins, 1980, 1983). The most unusual was the capnophilic species, *Bacteroides ochraceus* which contained menaquinones with six isoprene units and was subsequently reclassified in the genus *Capnocytophaga* (Leadbetter et al., 1979; Collins et al., 1982). The *Bacteroides fragilis* group of species was characterized by comparable amounts of menaquinones with 10 and 11 isoprene units whereas the rumen species, *Bacteroides ruminicola* subsp. *ruminicola* and *Bacteroides ruminicola* subsp. *brevis* had mainly 11 and 12 isoprene units. Oral isolates that were phenotypically similar to *Bacteroides ruminicola* possessed high amounts of menaquinones with 12 and 13 isoprene units (Shah and Collins 1980). DNA base compositions subdivided the genus into a low G+C group (~30 mol%), a high G+C group of 60 mol%, and, a third intermediate group of approximately 40–50 mol% G+C, many of which appear to possess considerable biochemical and chemical heterogeneity. The latter contained the type species, *Bacteroides fragilis* hence taxonomic studies focussed largely on this group.

Redefinition and its impact on the biology of the group with particular reference to mid-range mol% G+C group

Physiologically, "*Bacteroides*" species are metabolically versatile, being able to utilize nitrogenous substrates such as aspartate and glutamate but vary in their capacity to metabolize carbohydrates. They appear to lack functional enzymes for a complete Tricarboxylic Acid Cycle but have a partial system that leads to succinate as an end product of metabolism. Malate is an intermediate of this pathway hence malate dehydrogenase is universally detected as is glutamate dehydrogenase from glutamate metabolism (Shah and Williams, 1982). Such diverse physiological differences, together with supporting genomic and phenotypic data made it increasingly difficult to retain this diverse group as a single genus. Thus, taxonomic changes, resisted so long and retained up to the 1st edition of the new *Bergey's Manual of Systematic Bacteriology* (1984), were inevitable. In 1983, a review of the genus was undertaken and a new definition was proposed that recognized only species that were biochemically closely related to *Bacteroides fragilis*, the type species (Shah and Collins, 1983). A compilation of the known properties of the group led to the following redefinition of the genus *Bacteroides* based upon the following criteria:

- Produce major amounts of succinic and or, butyric acids (together with substantial levels of acetic and occasionally other short-chain acids;
- Contain malate dehydrogenase and glutamate dehydrogenase;
- Possess DNA base composition within the approximate limiting range of 40–52 mol% G+C;
- Possess sphingolipids;
- Produce major amounts of straight-chain saturated, iso- and anteiso-methyl branched long-chain fatty acids;
- Contain menaquinones as the sole respiratory quinones.

This new definition allowed the retention of the *Bacteroides fragilis* group of species and the pigmented/non-pigmented species that are biochemically related to *Bacteroides melaninogenicus*. However, within this more restricted definition, three groups were clearly apparent:

(1) Highly saccharolytic species: e.g. *Bacteroides fragilis* and closely related taxa metabolize a range of sugars, have a functional glycolytic pathway and possess enzymes of the hexose monophosphate pathway such as glucose-6-phosphate dehydrogenase and 6-phoshogluconate dehydrogenase. Malate dehydrogenase and glutamate dehydrogenase are present in all species of the "*Bacteroides fragilis* group". All grow in the presence of bile. They were reclassified as the genus *Bacteroides sensu stricto* (Shah and Collins, 1989).

(2) Moderately saccharolytic species: *Bacteroides melaninogenicus*, *Bacteroides intermedius*, *Bacteroides oralis*, *Bacteroides buccalis*, and *Bacteroides denticola*, etc. These

Table 2. Redefinition of the genus *Bacteroides* (Shah and Collins, 1983) excluded a vast number of species that remain *incertae sedis* even up to the current edition of *Bergey's Manual* (2011) – furthermore, many of these taxa were found to be so unique that several have retained their status as monospecific genera

"*Bacteroides* species"	Taxonomic designation following redefinition	Current status (phylum/family/genus)
Bacteroides amylophilus	*Ruminobacter amylophilus*	*Gammaproteobacteria/Succinivibronaceae/Ruminobacter*
Bacteroides capillosus	*Bacteroides capillosus* (*Pseudoflavonifractor capillosus*); closest relatives: *Clostridium orbiscindens* and *Eubacterium plautii*	*Firmicutes/Clostridiaceae/Pseudoflavonifractor*
Bacteroides coagulans	*Bacteroides coagulans* (misclassified)	*Bacteroidetes/Bacteroidaceae/Bacteroides*
Bacteroides furcosus	*Anaerorhabdus furcosa*	*Bacteroidetes/Bacteroidaceae/Anaerorhabdus*
Bacteroides hypermegas	*Megamonas hypermegas* (not officillay assigned a family)	*Firmicutes/Veillonellaceae/Megamonas*
Bacteroides microfusus	*Rikenella microfusus*	*Bacteroidetes/Rikenellaceae/Rikenella*
Bacteroides multiacidus	*Mitsuokella multiacida*	*Firmicutes/Veillonellaceae/Mitsuokella*
Bacteroides nodosus	*Dichelobacter nodosus*	*Gammaproteobacteria/Cardiobacteriaceae/Dichelobacter*
Bacteroides pneumosintes	*Dialister pneumosintes*	*Firmicutes/Veillonellaceae/Dialister*
Bacteroides praeacutus	*Tissierella praeacuta*	*Firmicutes*/Family XI *incertae sedis/Tissierella*
Bacteroides putredinis	*Alistipes putredinis*	*Bacteroidetes/Rikenellaceae/Alistipes*
Bacteroides ruminicola	*Prevotella ruminicola*	*Firmicutes/Prevotellaceae/Prevotella*
Bacteroides succinogenes	*Fibrobacter succinogenes*	*Fibrobacteres/Fibrobacteraceae/Fibrobacter*
Bacteroides termitidis	*Sebaldella termitidis*	*Fusobacteria/Leptotrichiaceae/Sebaldella*
Bacteroides ureolyticus	*Bacteroides ureolyticus* (misclassified)	*Proteobacteria* (delta/epsilon)/*Campylobacteraceae/Campylobacter*

metabolize a limited range of carbohydrates that includes mucins. Interestingly, they lack glucose-6-phosphate dehydrogenase and 6-phoshogluconate dehydrogenase but possess both malate dehydrogenase and glutamate dehydrogenase. Growth is inhibited by bile. They were placed in the genus *Prevotella* (Shah and Collins, 1990).

(3) Asaccharolytic species: *Bacteroides asaccharolyticus* and *Bacteroides gingivalis*, etc. Radiolabeling studies with ^{14}C-glucose revealed that although glucose was taken up, it remained in cell wall polymers and not present in their metabolic end products (Shah and Williams, 1987b). Some species metabolize a limited range of carbohydrates that includes mucins. They lack glucose-6-phosphate dehydrogenase and 6-phoshogluconate dehydrogenase but possess both malate dehydrogenase and glutamate dehydrogenase. These were reclassified in a new genus, *Porphyromonas* (Shah and Collins, 1988).

These proposals left the following species *incertae sedis*: *Bacteroides amylophilus*, *Bacteroides capillosus*, *Bacteroides coagulans*, *Bacteroides furcosus*, *Bacteroides hypermegas*, *Bacteroides microfusus*, *Bacteroides multiacidus*, *Bacteroides nodosus*, *Bacteroides pneumosintes*, *Bacteroides praeacutus*, *Bacteroides putredinis*, *Bacteroides succinogenes*, *Bacteroides termitidis*, and *Bacteroides ureolyticus*. These species were so incompatible with other members that subsequent work over the following two decades failed to retain a single species within the three new genera. Many were displaced into very distant lineages in one of the most radical taxonomic changes witnessed within a genus that hitherto comprised an established group that dates back to

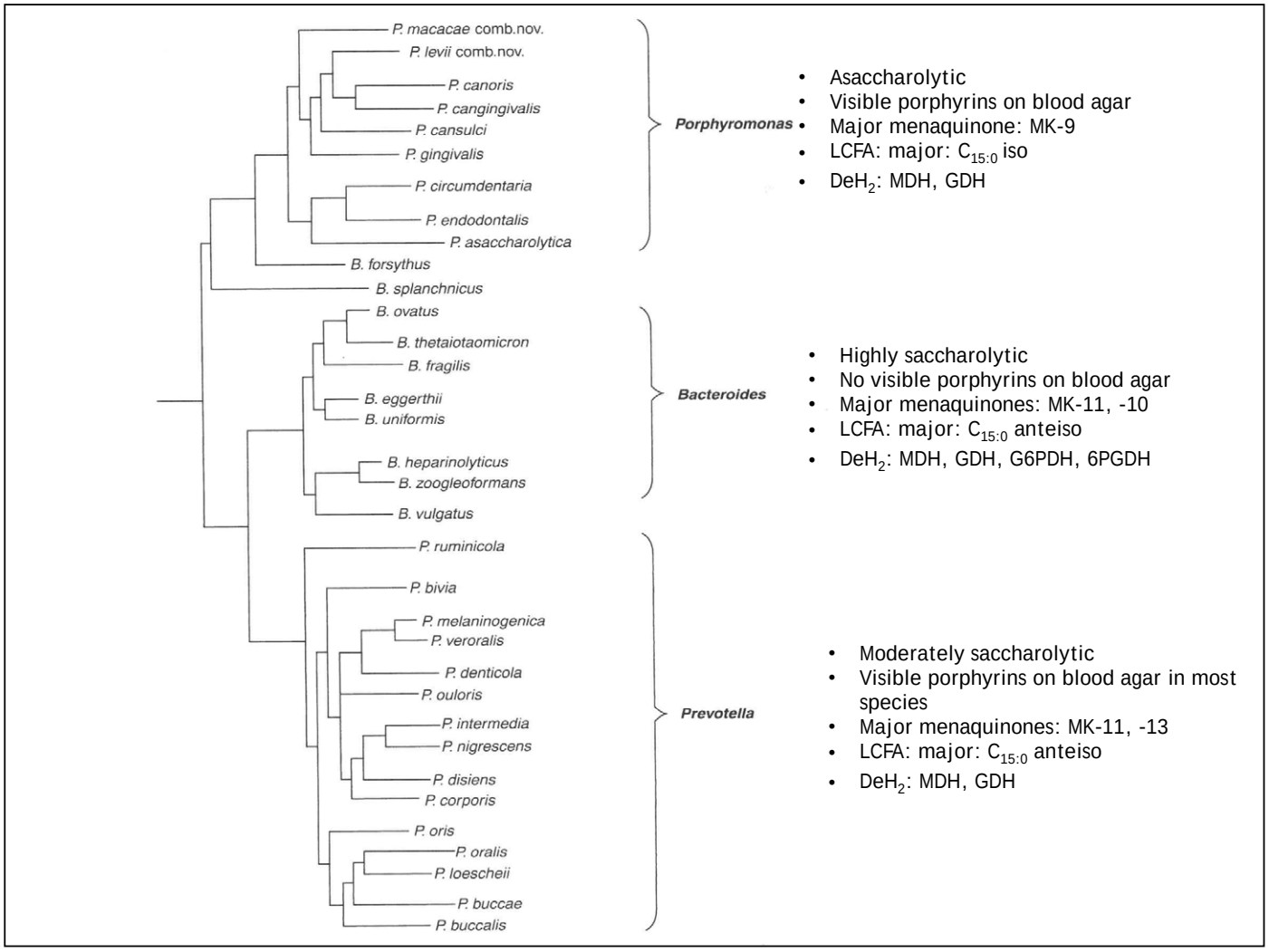


Figure 4. Early 16S rRNA tree and its correlation with key biochemical and chemotaxonomic data. The three clusters, *Bacteroides sensu stricto*, *Prevotella* and *Porphyromonas* (Shah and Collins, 1988, 1989 and 1990) were proposed ahead of 16S rRNA gene sequence analysis (adapted from Shah et al., 1998). Abbreviations: LCFA; long-chained fatty acid; DeH2: dehydrogenase; MDH, malate dehydrogenase; GDH, glutamate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phoshogluconate dehydrogenase.

the 1st edition of *Bergey's Manual* (Bergey et al., 1923). Some species were even recovered among Gram-positive taxa (e.g. *Clostridiaceae*), various lineages within the *Proteobacteria* and *Firmicutes*, while new orders and families were created to accommodate some such as *Bacteroides succinogenes* (*Fibrobacteres*), see summary in Table 2.

The impact of molecular systematics on restructuring of *Bacteroides* and related taxa

Comparative DNA sequencing analysis of the small-subunit rRNA (16S rRNA) became the driving force of bacterial systematics in the early 1990s and nearly all taxonomic changes necessitated its application. Proposals to restructure the "*Bacteroides*" was done prior to comparative 16S rRNA gene sequence analysis, consequently the strength of the proposed changes would be scrupulously challenged in the ensuing years. Figure 4 shows good concordance between the key biochemical/chemical features used to circumscribe the three groups of "*Bacteroides*" into the new genera and the initial data obtained by 16S rRNA (Shah et al., 1998). However, this was just the initial phase of an extensive literature that followed (see e.g. Paster et al., 1994; Sakamoto and Ohkuma, 2010, 2011; Sakamoto et al., 2007; Gupta, 2004; Gupta and Lorenzini, 2007), culminating in their acceptance in *Bergey's Manual of Systematic Bacteriology* (2011) and witnessed the remarkable elevation of some of these taxa from species/subspecies to family level. Most striking was the displacement of many taxa that were

hitherto excluded from the redefined genus (Shah and Collins, 1983) to very distant lineages (see Table 2). The arrival of PCR and the ease and simplicity with which comparative 16S rRNA sequencing could be achieved led to large numbers of species periodically being added to each of the newly proposed genera. Thus, whereas the 1st edition of *Bergey's Manual of Systematic Bacteriology* (1984) listed 39 species, the current edition (2011) lists 83 species in three genera as follows: 25 species as *Bacteroides* (plus eight labeled *incertae sedis*), 34 as *Prevotella* species, and 16 as *Porphyromas* species. The remainder are dispersed into immensely diverse lineages and are summarized in Table 2.

The topology of the phylogenetic tree using comparative sequencing alignment of several gene targets such as *hsp60* (Sakamoto and Ohkuma, 2010), *gyrB* (Lee and Lee, 2010), *rpoB* (Ko et al., 2007), etc., mirrors those derived by 16S rRNA. More recently multilocus sequence analysis of internal fragments of the gene targets *dnaJ*, *gyrB*, *hsp60*, *recA*, *rpoB*, and 16S rRNA were concatenated and various algorithms used to test the robustness of the phylogenetic relationships among *Bacteroides* species (Sakamoto and Ohkuma, 2011). The level of discrimination between species was greater compared with single-gene trees and a level of 97.5% gene sequence similarity, based on the fragments of these six genes, was used to demarcate members of the genus *Bacteroides sensu stricto*. In future, such work may be simplified since concatenation of three gene targets alone, *dnaJ*, *gyrB*, and *hsp60*, paralleled the above results (Sakamoto and Ohkuma, 2011). Interestingly, the *gyrB* gene was found to be taxonomically the most informative. At higher taxonomic rank, the branching of these lineages were substantiated using indels that correlate with the DNA Gyrase B protein. A 4-amino-acid insert in GyrB and a 45-amino -acid insert in the SecA proteins were uniquely shared by this group of species and clearly differentiated them from other phyla (Gupta et al., 2004, 2007).

The wealth of molecular systematic data over the last decade has establish beyond doubt high confidence in the taxonomic structure of this group. However, most of the biochemical/chemical and, molecular techniques such as DNA–DNA reassociation that initially revealed the immense heterogeneity of this group are now outside the realms of most laboratories. Phenotypic tests reveal expression and as such are still pragmatic characters for the identification of wild-type strains and variants and help elucidate the inherent diversity of a taxon. This is exacerbated where taxa are non-fermentative since traditional biochemical tests, based on carbohydrate fermentation reactions are futile. New approaches based on the proteome represent a cornucopia of biomolecules that have not been systematically explored. Methods such as multilocus enzyme electrophoresis, SDS-PAGE and flat-bed-IEF (see, e.g. Figure 2, Figure 3 and Swindlehurst et al., 1977), have provided compelling proof of concept of their value for microbial systematics but the technology for high-throughput identification of discriminatory marker proteins had to await the arrival of new forms of mass spectrometry (MS) such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). MALDI-TOF-MS is today being established as a reliable adjunct to molecular-based methods and some of its pioneering developments were entrenched in applications to seek new characters for the delineation of non-fermentative/poorly defined taxa such as *Porphyromonas*, *Prevotella*, and *Bacteroides*.

Mass spectral analysis of "*Bacteroides*" and the dawn of MALDI-TOF-MS

During the flourishing era of chemotaxonomy around the 1980s, MS analysis of cellular components such as porphyrins, respiratory quinones, long-chain cellular fatty acids (gas chromatography-MS; MS, e.g. Shah and Collins, 1983), polar lipids (Fast Atomic Bombardment-MS; e.g. Druker et al., 1997) or whole-cell profiles by Pyrolysis mass spectrometry (e.g. Gutteridge et al., 1985) were extensively reported for "*Bacteroides*". However, a limitation of these methods was their inability to analyze large biomolecules such as proteins.

Thus, whereas earlier MS methods were limited to ionization of small molecules (<1500 Da) such as menaquinones, new MS methods such as MALDI-TOF-MS and LC-MS/MS theoretically had no upper limit.

In the early applications of MALDI-TOF-MS, numerous parameters needed optimization to obtain reproducible spectral profiles. Thus, sample preparation, the design of the MS target plate for spotting the analyte, the type, concentration and method of application of the matrix solution used for ionization, cultural conditions such as the medium composition, temperature, pH, age of the culture, strain variation etc together with MS parameters such as laser energy, reproducibility between instruments and the application of various algorithms for data analysis all required considerable research to eventually establish a standardized protocol. In our preamble to assemble a microbial database of MS profiles, we hypothesized that since many virulence determinants of pathogenic microbes are surface-associated, a database of MS profiles based on these molecules would serve the dual purpose of providing unique species-specific signatures while also highlighting pathogenic variants. Proof of

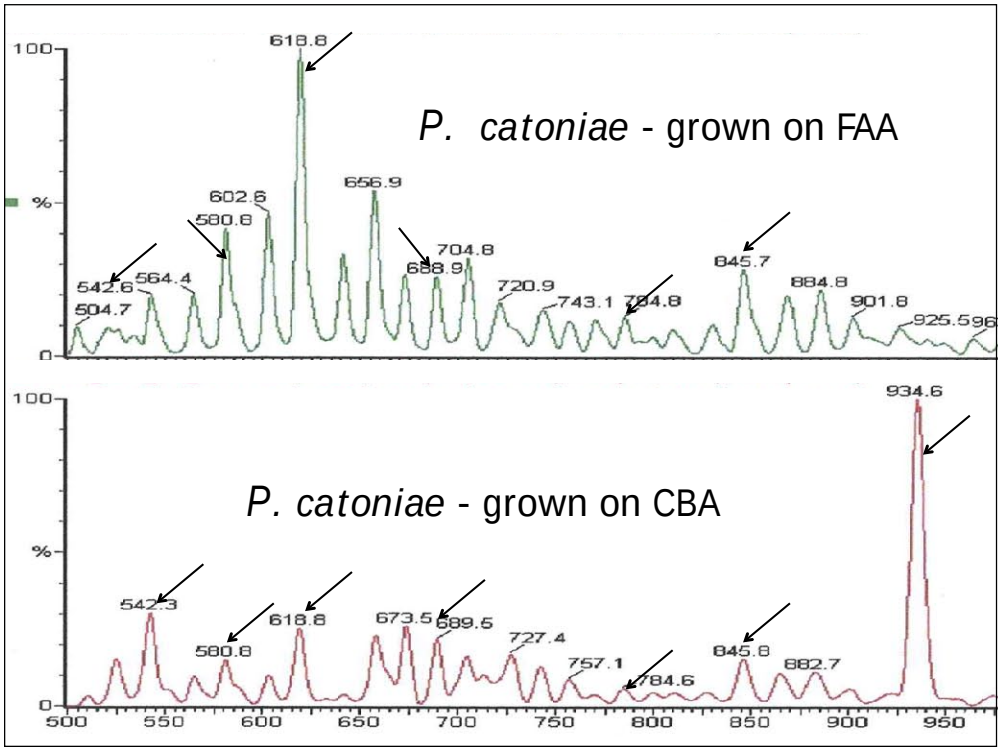


Figure 5. Changes in the MALDI-TOF-MS profile of the same strain of *Porphyromoas catoniae* (NCTC 12856) grown of Fastidious Anaerobic Agar (FAA) and Columbia Blood Agar (CBA). Many of the significant mass ions, e.g. 542, 580, 618, 689, 784, and 845 Da, are retained. However, significant mass ions such as 935 Da are present only in cells grown on CBA.

principle was established by being able to delineate smooth (virulent) and rough (beguine) variants of *Peptostreptococcus micros* (Rajendram, 2003).

However, culture conditions markedly affects the surface composition of cells and, for the same strain different spectral profiles were obtained (see, e.g. Figure 5; Shah et al., 2000). In order to develop a standard protocol that could be used for all bacteria, considerable in-depth analysis was undertaken using a broad range of bacterial species to establish a database of mainly type and reference strains of some 500 species from the National Collection of Type Cultures (see, e.g. Keys et al., 2004).

A watershed in the development of this work was the delineation of species of the genus *Porphyromonas* which provided unequivocal support for pursuing this approach (Shah et al., 2002). Since members of this genus are virtually indistinguishable except by DNA–DNA reassociation, their unambiguous separation by MALDI-TOF-MS heralded a new simple approach to study the systematics of such poorly characterized taxa (see, e.g. Figure 6; Shah et al., 2002). This work was extended to include several other genera where they was a paucity of reliable characters for delineating species (Keys et al., 2004).

The method was trialled in a clinical laboratory in 2007–2008 and while it was successful for *Staphylococcus aureus* (Rajakurana et al. 2009), *Clostridium difficile* isolates were poorly differentiated. The latter was resolved by changing the matrix solution from 5-chloro-2-mercaptobenzothiazole (CMBT) to 2,5-dihydroxy benzoic acid in acetonitrile:ethanol:water (1:1:1) with 0.3 % TFA which had the beneficial effect of shifting the mass spectral profile away from surface-associated to intracellular molecules (mainly ribosomal proteins) and resulted in a dramatic improvement in the stability of the spectral ions. Thus, whereas before it was necessary to culture cells on a single medium under standardized conditions to obtain a reproducible mass spectral profile, cells could now be cultured on any medium and the stability of the ribosomal proteins permitted their identification to the species level (see review Kallow et al., 2010). Significant developments in the hardware and software by mass companies has propelled MALDI-TOF-MS to the forefront of clinical laboratories in Europe and now replacing traditional biochemical tests that have been used for decades. Among the *Bacteroides* and related taxa, not only are species being resolved but the potential to identify antibiotic-resistant strains is evident (Nagy et al., 2009).

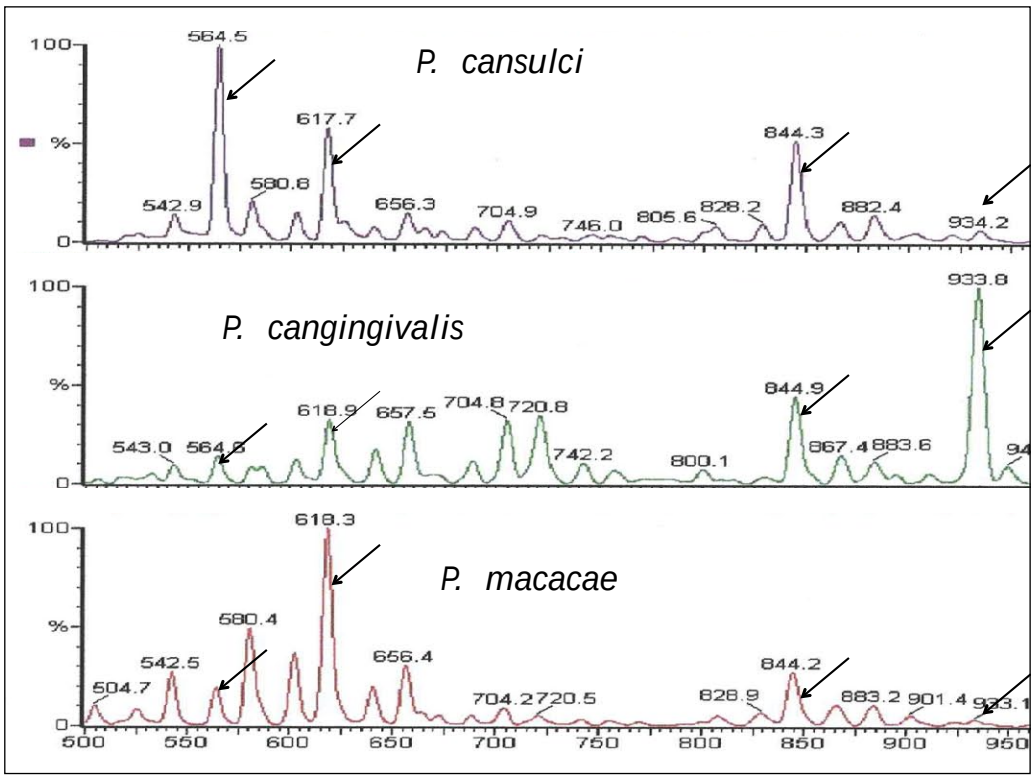
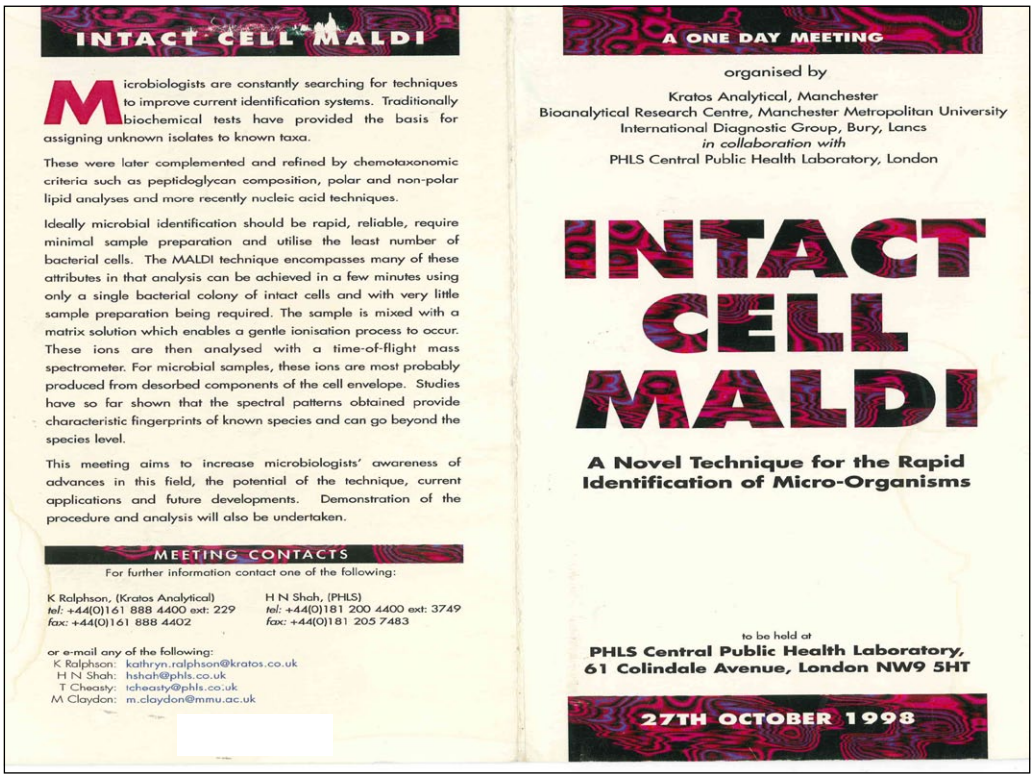


Figure 6. Examples of the distinctive MALDI-TOF-MS profiles of intact cells of *Porphyromoas* sp. containing both genus-specific (e.g. 618 and 844 Da mass ions) but also a significant number of species-specific mass ions (examples indicated by arrows). Members of the genus *Porphyromoas* now comprise 16 species, in addition to several others whose names have not yet been validly published (*Bergey's Manual*, 2011). However, with the exception of DNA–DNA reassociation, they cannot be reliably delineated. The MALDI-TOF-MS spectra shown above and those reported earlier (Shah et al., 2002) shows that each species can be unambiguously distinguished. It was this poorly characterized group that became one of the compelling forces for the development of this technique for microbial identification.

In 1998, the first conference to explore the potential clinical application for micro-organisms took place at the Health Protection Agency (formerly Public Health Laboratory Service, London; see Figure 7) and using a simple basic bench-top MALDI-TOF mass spectrometer, the separation of *Porphyromonas* species was demonstrated live to a scientific audience. The potential of the technique was evident, however, it took a decade of continuous development to propel this technology to the forefront of microbial diagnostics. In the future, this approach is likely to be superseded by LC-MS-MS techniques as this will markedly extend its applications by permitting identification of the protein/peptide biomarkers, pathogenic determinants, antibiotic resistance, etc., in a single step (Al-Shahib et al., 2010). However, like MALDI-TOF-MS, the former will require development of both hardware and software to make it more amenable for the clinical laboratory.

Coda

From 1898 when Veillon and Zuber first described the type species of *Bacteroides*, *Bacteroides fragilis* and paved the way for over a century of work on these micro-organisms, their taxonomic structure today is considered robust and able to provide a sound phylogenetic framework from which to elucidate functional aspects of these species in their natural habiat. Their dominance in the intestinal track is so pronounced that several such as *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Bacteroides uniformis*, *Bacteroides distasonis*, *Bacteroides uniformis*, etc., were reported before the 1940s (*Bergey's Manuals* – Bergey et al., 1923, 1939) before anaerobic techniques were well established. In one of the most extensive studies of the gut microbiome todate, Qin et al. (2010) reported 57 of the most frequent microbial genomes among 124 individuals and listed 24 "*Bacteroides*" species of which the most frequent was *Bacteroides uniformis*. It's recent taxonomic structure further enabled new species such as *Bacteroides xylanisolvens*, *Bacteroides finegoldii*, *Bacteroides pectinophilus*, *Bacteroides intestinalis*, *Parabacteroides johnsonni*, and *Bacteroides dorei* to be discerned while six groups were described only as "*Bacteroides* sp.", indicating further diversity within this taxon. Similarly, in the oral cavity, several of the former "*Bacteroides*" (now *Prevotella*


INTACT CELL MALDI

Microbiologists are constantly searching for techniques to improve current identification systems. Traditionally biochemical tests have provided the basis for assigning unknown isolates to known taxa.

These were later complemented and refined by chemotaxonomic criteria such as peptidoglycan composition, polar and non-polar lipid analyses and more recently nucleic acid techniques.

Ideally microbial identification should be rapid, reliable, require minimal sample preparation and utilise the least number of bacterial cells. The MALDI technique encompasses many of these attributes in that analysis can be achieved in a few minutes using only a single bacterial colony of intact cells and with very little sample preparation being required. The sample is mixed with a matrix solution which enables a gentle ionisation process to occur. These ions are then analysed with a time-of-flight mass spectrometer. For microbial samples, these ions are most probably produced from desorbed components of the cell envelope. Studies have so far shown that the spectral patterns obtained provide characteristic fingerprints of known species and can go beyond the species level.

This meeting aims to increase microbiologists' awareness of advances in this field, the potential of the technique, current applications and future developments. Demonstration of the procedure and analysis will also be undertaken.

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A ONE DAY MEETING

organised by

Kratos Analytical, Manchester
Bioanalytical Research Centre, Manchester Metropolitan University
International Diagnostic Group, Bury, Lancs
in collaboration with
PHLS Central Public Health Laboratory, London

INTACT CELL MALDI

A Novel Technique for the Rapid Identification of Micro-Organisms

to be held at
PHLS Central Public Health Laboratory,
61 Colindale Avenue, London NW9 5HT

27TH OCTOBER 1998

Figure 7. Conference flyer of the first conference (1998) held to explore the potential application of MALDI-TOF mass spectrometry for microbial identification. It took a decade of research and development for this technology to gain widespread acceptance in the clinical microbiology laboratory.

and *Porphyromonas*) that were previously reported to be specific to this site have been confirmed by studies of the oral microbiome using 16S rRNA (Dewhirst et al., 2010), while proteome analysis revealed the site-specificity of the oral species, *Porphyromonas gingivalis* (Nandakumar et al., 2009). The latter emanated from one of the MLEE groups of the *Bacteroides melaninogenicus* complex reported some 35 years ago (Figure 2; Shah et al., 1976), reinforcing the interrelationship between in-depth taxonomic studies and microbial ecology.

The biochemical properties of many species provides clues to their potential function *in vivo* and emphasizes the need to retain phenotypic tests as part of the description of a species. The division of the "*Bacteroides*" into saccharolytic, moderately saccharolytic and non-fermentative appears to be directly related to their functions *in vivo*. Degradation of polysaccharies is a key functional role of saccharolytic "*Bacteroides*" and genome analysis of species such as *Bacteroides thetaiotaomicron* (6.2 Mb), *Bacteroides vulgatus* (5.2 Mb), *Bacteroides fragilis* (5.2 Mb) and *Bacteroides distasonis* (4.8 Mb) have been extensively studied against a background of these properties (see, e.g. Magnus et al., 2006; Karlsson et al., 2011). All species possess very high levels of glycosidases and transglycosidases but *Bacteroides thetaiotaomicron* has the most extensive array of carbohydrate enzymes (e.g. enzymes involved in glycan biosynthesis and metabolism, *N*-glycan and glycosaminoglycan degradation and metabolism of galactose, glycerolipid, glycosphingolipid, starch, pentose and glucuronate interconversions) including eight loci that are involved in capsular polysaccharide synthesis. "*Bacteroides distasonis*" can degrade polysaccharides but the species has the lowest capacity to do so and phylogenetically branches off the main cluster of species with lower sequence similarity and was eventually assigned to a closely related branch as *Parabacteroides distasonis* (Sakamoto and Benno, 2006). In the gut, about one-third of dietary fiber are present as plant cell-wall polysaccharides of which xylan and arabioxylan are among the most abundant. Species such as *Bacteroides xylanisolvens* was discovered by enrichment (Chassard et al., 2008) and shows the highest DNA sequence similarity to *Bacteroides ovatus*, irrespective of the gene targets used (unpublished). Interestingly they both have the highest capacities to degrade xylan.

By contrast *Porphyromonas* (formerly part of the "*Bacteroides*") lacks the capacity to degrade carbohydrates and in the case of *Porphyromonas gingivalis*, the most intensively studied species, its genome (2.34 Mb) is only half the size of that of the *Bacteroides*. It was shown previously that this

species utilizes nitrogenous substrates such as peptides and amino acids as sources of energy in accordance with the ecological site in which it thrives (Shah and Williams, 1987b; Gharbia et al., 1989). Analysis of its genome function revealed that this species is rich in pyruvate/oxoglutarate oxidoreductases, β-alanine, glycine, serine and threonine and tryptophan metabolism. As studies on the human and animal microbiome continues, relating such functions to precisely defined species is now the focus of considerable attention.

Today there is general consensus among microbiologists that there is a constant and representative core of genes that defines a species. Below this level of discrimination, functional properties vary somewhat among strains suggesting that distinct niches are occupied. One of the challenges of systematics today (in the absence of DNA–DNA reassociation) is to find ways to quantatively describe the limits of a species and relate this to their ecological role. The control circuits within an organism are dynamic hence a cell cannot be considered as a simple projection of its genome. Cellular activity is discharged through a myriad of transport, signaling, regulatory and metabolic pathways each embodied in the functional and structural relationships of many specific molecules that provide a holistic function for the survival of the cell. It's likely that physiological function and analysis of their proteome and transcriptome will feature more prominently in the future to help derive a definition and, in the case of the human microflora, provide insight into their function in the microbiome. One reason why DNA–DNA reassociation was so successful was that it provided clear demarcation of a species based on the whole genome, its drawback was that it was limited to a defined set of test strains and gave no clues about the function of the species. Future taxonomists should profile the global expression of cells to search for new parameters to quantitatively define a unified set of functions for a given species.

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Functional gene sequence studies of pure cultures are the basis of systematic studies of environmental communities of phototrophic bacteria and their species-specific analyses

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From the establishment of proper cultivation conditions of phototrophic sulfur bacteria 50 years ago up to today significant improvements have been made to systematically treat the phototrophic green and purple sulfur bacteria and identify them in environmental communities. Important steps for these improvements were first of all the description of a large number of pure cultures representing a proper fraction of environmental diversity, their correct taxonomic treatment and the clear definition of the taxa. Further important steps were the establishment of a phylogenetics-based taxonomy supported by 16S rRNA gene sequences and the demonstration of congruence between phylogenies based on 16S rRNA genes and functional genes. The formation of a large database of *fmoA* genes of green sulfur bacteria and of *pufLM* genes of purple sulfur bacteria and their obvious phylogenetic congruence with the 16S rRNA gene enabled detailed studies of environmental communities of these bacteria and the recognition of species and genera in natural habitats. The comprehensive studies of selected habitats yielded promising results and demonstrated the potential of this approach for the systematic characterization of environmental communities.

Introduction

Because only a small fraction of environmental bacteria is brought into pure culture and grown on culture media in the lab, culture-based approaches have significant disadvantages for analyses of environmental communities. Alternatively, molecular genetic approaches have been developed with a large database of 16S rRNA gene sequences as a reference. These approaches have the advantage of covering a much larger fraction of the natural communities, but they often cannot be well correlated with pure culture approaches, in particular, because both approaches address different fractions of a bacterial community. Molecular tools such as 16S rRNA gene sequences, which cover a wide phylogenetic range of bacteria, fail in many cases to depict the diversity of specific functional groups of bacteria in environmental communities. The most important reason is the fact that they are specific to physiological groups of ecological relevance to a limited extent. Here, more specific approaches have to be applied. Such tools have been elaborated for the green sulfur bacteria (*Chlorobi*) on the basis of the *fmoA* gene (Alexander et al., 2002) and for the phototrophic purple bacteria on the basis of the *pufLM* genes (Tank et al., 2009) and were applied to selected habitats.

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From classical taxonomy to molecular systematics

Treatment of phototrophic green and purple sulfur bacteria advanced significantly when Norbert Pfennig (Pfennig, 1961) established proper media and cultivation conditions for these bacteria and isolated a large number of strains from aquatic, mainly freshwater habitats. Together with Hans-Georg Trüper the classical taxonomy of these bacteria has been established, and a large number of species were properly described and the higher taxa were defined (Pfennig and Trüper, 1971a, 1971b, 1974). Primarily phenotypic properties were used to define and characterize the species, because molecular genetic data and DNA sequence information were not available at that time. Properties used for identification included cell shape and size, other morphological criteria, the presence of gas vesicles, internal membrane structures, chlorosomes, flagellation, carotenoid and photosynthetic pigment composition, absorption spectra, vitamin requirement, G+C content of the DNA, as well as physiological properties including substrate utilization and the deposition of elemental sulfur, a storage product and intermediate during sulfide oxidation, inside or outside the cells (Pfennig and Trüper, 1974, 1989).

Despite the fact that morphological properties through microscopic observations are among the first information available for phototrophic bacteria in environmental samples, they are of limited value for the recognition of species.

Consequently, most of our knowledge on occurrence of phototrophic bacteria in nature is based on culture studies and identification of the isolates (Pfennig, 1977; Imhoff, 2001a), though in recent years an increasing number of investigations use genetic approaches to characterize environmental communities, often in combination with the isolation and culture of these bacteria (e.g. Martínez-Alonso et al., 2005; Ranchou-Peyruse et al., 2006). Although culture approaches yielded a rough idea on the bacteria present and eventually even on their relative proportion, the question always remained whether cultivation conditions were appropriate to cover the bacteria of relevance in the analyzed sample even if variation of culture media was used to increase the number of bacteria cultured from a particular source. It is generally assumed that the selective properties of media and cultivation conditions give rise only to a limited portion of the phototrophic bacteria present in the sample.

A phylogenetic system for phototrophic sulfur bacteria

The use of sequence information of the 16S rRNA molecule and its gene into bacterial phylogeny by Woese (1987) and the application of this sequence information for systematic considerations not only revealed the great phylogenetic diversity of the phototrophic bacteria but also promoted changes in their taxonomy. The first taxonomic consequences from the recognition of the phylogenetic relationships among the phototrophic bacteria included the separation of the genus *Ectothiorhodospira* from the *Chromatiaceae* and its recognition as a distinct family, the *Ectothiorhodospiraceae* with *Ectothiorhodospira* and the new genus *Halorhodospira* as genera (Imhoff, 1984). In addition, the purple nonsulfur bacteria were reorganized. Most significantly members of *Alpha-* and *Betaproteobacteria* were separated. Some of these, in the classical system, had been combined as a single genus. For example, the former *Rhodospirillum tenue* and *Rhodopseudomonas gelatinosus*, previously thought to be close relatives to the alphaproteobacterial purple nonsulfur bacteria, were reclassified as *Betaproteobacteria* and were transferred to the genus *Rhodocyclus* and renamed *Rhodocyclus tenuis* and *Rhodocyclus gelatinosus* (Imhoff et al., 1984; later *Rubrivivax gelatinosus* Willems, Gillis and De Ley 1991). *Rhodocyclus purpureus* as the only and type species of this genus was recognized itself as a member of the *Betaproteobacteria* among the majority of the alpha-proteobacterial purple nonsulfur bacteria. With increasing precision and accuracy of the sequence data, further rearrangements among the purple sulfur bacteria (Guyoneaud et al., 1998, Imhoff et al., 1998b) and the purple nonsulfur bacteria (Imhoff et al., 1998a) were proposed. All of these taxonomic changes carefully considered phenotypic properties as well as available molecular data (e.g. fatty acids and quinone composition, LPS structure, cytochrome *c* amino acid sequences) and genetic sequence data of the 16S rRNA gene. Altogether the new molecular taxonomy of phototrophic bacteria by and large reflected the phylogeny of the 16S rRNA gene (Imhoff, 2001b).

Phylogeny of functional genes of phototrophic sulfur bacteria

Several aspects promoted the introduction of other genes into systematic considerations and the phylogenetic comparison of these genes with that of the 16S rRNA gene. First, the phylogeny of the 16S rRNA gene, although a well conserved and ideal molecule, does not necessarily exactly reflect the phylogeny of the bacteria as a whole. Second, the 16S rRNA gene is not suitable for studies on communities of phototrophic bacteria: in particular, purple bacteria do not form a coherent phylogenetic group and are closely related to non-phototrophic chemotrophic *Alpha-*, *Beta-* and *Gammaproteobacteria*. Therefore, it was necessary to look for more specific molecules and genes characteristic for these bacteria. Third, the largest collection of pure cultures and type strains of phototrophic purple and green sulfur bacteria was available in our laboratory, including all strains initially isolated and maintained by Norbert Pfennig.

Phylogeny of the fmoA gene in green sulfur bacteria

The green sulfur bacteria were the first group that was carefully analyzed this way. The *fmoA* gene encoding a bacteriochlorophyll-*a* protein appeared suitable to establish a phylogeny distinct from the 16S rRNA gene phylogeny. The FMO protein mediates energy transfer between the chlorosomes and the reaction center in the cytoplasmic membrane of green sulfur bacteria (Fenna et al., 1974). The *fmoA* gene is present in green sulfur bacteria and the recently described "*Candidatus* Chloracidobacterium thermophilum" (Bryant et al., 2007). It is absent in another major phylogenetic line of phototrophic green bacteria containing chlorosomes, the *Chloroflexi* (Blankenship et al., 1995). Therefore it is an appropriate target to specifically analyze environmental communities of the green sulfur bacteria (Alexander and Imhoff, 2006). Because a comprehensive phylogeny of the green sulfur bacteria, based on 16S rRNA gene sequences, was not available, phylogenies of both 16S rRNA and *fmoA* genes were established including all available types (Alexander et al., 2002). The results were quite remarkable in that the phylogenies of the two independent genes were largely congruent and species and strains could be identified by either of the two genes. A comparative view of the phylogenetic trees of green sulfur bacteria according to 16S rRNA and *fmoA* genes is presented by Imhoff (2003).

First of all, the available information on sequences from both genes was used to rearrange the strains of green sulfur bacteria among the species, redefine the species and in a few cases establish new species and one new genus (Imhoff, 2003). Misclassification of a large number of strains was recognized and considerable taxonomic changes were required, because the classification by morphological and simple physiological properties (such as formation of gas vesicles, thiosulfate utilization, carotenoid and bacteriochlorophyll molecules present) in many instances was not in congruence with the genetic relatedness supported by these two independent genes. A careful comparison of the properties of all available strains and of their phylogenetic relations according to both *fmoA* and 16S rRNA genes was presented together with the proposed taxonomic changes (Imhoff, 2003). The clear correlation of phylogenetic relations and taxonomy were preconditions for the possible recognition of species in natural samples by molecular approaches based on genetic sequence information.

Phylogeny of the pufLM genes in purple sulfur bacteria

Phototrophic purple bacteria are distributed among the alpha-, beta- and gamma-groups of the *Proteobacteria* and many are closely related to non-phototrophic chemotrophic bacteria. As a consequence, higher taxa of family and order rank contain phototrophic and non-phototrophic genera, though the genus definitions of the phototrophic purple bacteria maintain the presence of a photosynthetic apparatus and the ability to perform photosynthesis as defining properties (Imhoff and Caumette, 2004). [Unfortunately, numerous violations published to this recommendation and to the given genus descriptions, in particular among the aerobic phototrophic bacteria, have caused considerable confusion and made necessary the renaming of the bacteria of concern in a number of cases.]

In order to selectively approach the phylogeny of the phototrophic purple bacteria and to develop tools for the analysis of natural communities of these bacteria, the *pufLM* genes were selected, which encode the light (L) and medium (M) subunits of the photosynthetic reaction center type II structural proteins of all phototrophic proteobacteria (purple sulfur bacteria, purple nonsulfur bacteria as well as the aerobic phototrophic purple bacteria producing bacteriochlorophyll and forming a photosynthetic apparatus) and the phototrophic members of *Chloroflexi*. The establishment of a comprehensive database of *pufLM* gene sequences of most of the recognized type strains of the purple sulfur bacteria and the determination of their phylogenetic relationships again was possible due to the large culture collection maintained. A good correlation was found between phylogenies of 16S rRNA and *pufLM* genes of the purple sulfur bacteria and is demonstrated by comparison of the phylogenetic trees of both genes (Tank et al., 2009). This supported the 16S rRNA-based taxonomy (Imhoff, 1998b; Guyoneaud et al., 1998; Imhoff, 2001b) and at the same time qualified the *pufLM* genes as a valuable tool for studies of environmental communities of phototrophic purple bacteria. Species recognition of these bacteria even in complex mixtures of environmental communities is now possible.

Molecular ecology and species recognition of phototrophic sulfur bacteria in the natural environment

After the introduction of PCR in the 1980s and in combination with cloning techniques, it became possible to use DNA extracted from environmental samples to identify individual sequences in a habitat and to compare these with sequences known from characterized species. In principle, this opened up the possibility of analyzing the species composition of environmental communities, if 16S rRNA gene sequences are accepted not only as a phylogenetic marker but also as a taxonomic characteristic. In fact, an extensive comparison of DNA–DNA reassociation data and 16S rRNA gene similarities of pure cultures demonstrated that sequence similarities >98.7% (not the previously considered 97%) could be recommended as a point at which DNA–DNA hybridization studies would be expected to exceed 70% and thus would be an acceptable borderline to recognize species (Stackebrandt and Ebers, 2006). Taking these numbers as a rough rule of thumb, species could be recognized in environmental samples on the basis of 16S rRNA gene sequence similarities. Pitfalls of applying 16S-rRNA-based approaches to the analysis of communities of phototrophic bacteria, in particular phototrophic purple bacteria, were due to the lack of specific primers, respectively the lack of specific sequence stretches that would allow a specific analysis of this phylogenetically diverse group of bacteria in complex mixtures and environmental samples. Their position in the *Proteobacteria* and in part close relationship to non-phototrophic bacteria made it impossible to identify phototrophic purple bacteria from 16S rRNA gene sequences obtained from the environment. In consequence, this necessitated the use of functional genes, e.g. based on genes related to photosynthesis of these bacteria for such studies. In addition, due to the great phylogenetic distance between major groups of phototrophic prokaryotes it is likely that separate approaches (primers, target genes) are required to cover for these major groups.

Sequence information is pre-destined to link bacterial systematics and ecological studies because (i) sequence infor-

mation is now well established as a property in bacterial systematics and forms a backbone for bacterial phylogeny and systematic treatment, and (ii) sequence information becomes available from individual clones of environmental communities. While bacterial systematics primarily is dealing with strains and cultures of bacteria, molecular genetic studies of natural communities are relying on sequence information. Environmental sequences can be arranged in phylotypes, which unify sequences (respectively the bacteria associated with the sequences) above a defined similarity and are used to measure the genetic diversity within environmental samples. If distinction of phylotypes is made at a sequence level that compares to the level of distinction between species with pure cultures, phylotypes can be used to describe the species diversity of environmental communities. Anyhow, if environmental clone sequences are sufficiently similar to known species, represented by their type strains, it is quite likely that they are representatives of this species or close relatives thereof. If considerations concerning sequence similarities as a rough guide for species differentiation of pure cultures are transferred to sequences from the environment, they could help to obtain a rough estimate of the species diversity in environmental samples and help in species recognition. Based on the accepted rules established for the 16S rRNA gene, similar considerations can be made for functional genes, if their evolutionary rate in comparison to the 16S rRNA gene is known or can be delineated from a database of sequences. Accordingly thresholds of 86% and 95% sequence similarity of the *pufLM* genes have been proposed for the distinction of genera and species of purple sulfur bacteria (Tank et al., 2009, 2011; Zeng et al., 2007).

Environmental communities of green sulfur bacteria

With the comprehensive study of *fmoA* and 16S rRNA gene phylogenies of pure cultures of green sulfur bacteria as a solid basis, a first detailed molecular genetic study on the species composition of green sulfur bacteria communities was made with samples from saline habitats of different geographical location using the established primers (Table 1; Alexander and Imhoff, 2006). Quite interestingly, all of the clone sequences (more than 370 16S rDNA sequences and more than 130 *fmoA* sequences) from marine and saline habitats all over the world, of the Baltic Sea, the Mediterranean Sea, Sippewissett salt marsh (Massachusetts, USA) and Bad Water (Death Valley, California) were associated with salt-dependent phylogenetic lines of green sulfur bacteria which had been previously established with pure cultures (Alexander et al., 2002). The clear dominance of representatives of the true marine green sulfur bacteria, in particular of the genus *Prosthecochloris* (Alexander et al., 2002, Imhoff, 2003) was in line with the experience from culture approaches, which regularly yielded *Prosthecochloris aestuarii* in enrichments and as isolated cultures from many marine habitats (see Imhoff, 2001a). Though the culture studies always had left doubt whether the specific cultivation approaches had selected just strains of *Prosthecochloris*, the genetic analyses supported these results, conclusively demonstrating that members of *Prosthecochloris* are the dominant green sulfur bacteria in many marine and saline habitats.

However, the phylogenetic diversity of marine green sulfur bacteria belonging to *Prosthecochloris* apparently is significantly higher than known from pure cultures so far. Available sequence information allowed the recognition of at least four different groups within this genus, probably representing different species (Alexander and Imhoff, 2006). Among these were the established species *Prosthecochloris aestuarii* and *Prosthecochloris vibrioforme*. In addition, *Prosthecochloris indica* was described later with new isolates from India (Kumar et al., 2009).

Environmental communities of phototrophic purple sulfur bacteria

Though the *pufM* gene has been recognized as a potential tool to study anoxygenic phototrophic bacteria in natural environments, environmental studies attempting species recognition were hampered by the lack of a comprehensive database of *pufM* gene sequences (Achenbach et al., 2001; Karr et al., 2003; Oz et al., 2005; Hu et al., 2006; Asao et al., 2011). Though these studies revealed important information on the diversity of phototrophic bacteria in the investigated habitats, systematic studies and the species-specific analysis of complex environmental communities of phototrophic purple bacteria were enabled only by the establishment of a comprehensive database using the almost complete sequences of the combined *pufL* and *pufM* genes of most purple sulfur bacteria and the verification of their phylogenetic relationship (Tank et al., 2009).

Salt lakes of the Salar de Atacama

The first detailed study of environmental communities of these bacteria which referred to this large *pufLM* database and used the established primers (Table 1) was the analysis of salt lakes of the Chilean highlands (Thiel et al., 2010). Like other hypersaline environments, lakes of the Salar de Atacama (Laguna Chaxa and Laguna Tebenquiche) exhibited the presence of extended purple-red colored microbial mats in and on the surface of the lake sediments. The composition of the clone libraries demonstrated a highly diverse and variable community of anoxygenic phototrophic bacteria.

Table 1. Primer systems recommended for the analysis of environmental communities of phototrophic bacteria

Gene	Primer sequence	Primer name	Reference
Green sulfur bacteria			
fmoA	ATGGTCCTTTTYGG	F-start-fmo	Alexander et al. (2002)
	CCGACCATNCCGTGRTG	R-889-fmo	
Purple bacteria			
pufLM	TTCGACTTYTGGRTNGGNCC	pufL67F	Tank et al. (2009)
	CCAKSGTCCAGCGCCAGAANA	pufM781R	
Anoxygenic phototrophic bacteria			
bchY	CCNCARACNATGTGYCCNGCNTTYGG	bchY_fwd	Yutin et al. (2009)
	GGRTCNRCNGGRAANATYTCNCC	bchY_rev	

The communities of phototrophic bacteria from both lakes were characterized by the presence of representatives related to the type strains of the moderately and extremely halophilic *Chromatiaceae Halochromatium salexigens*, *Halochromatium glycolicum*, *Thiohalocapsa halophila*, *Ectothiorhodospira mobilis*, *Ectothiorhodospira variabilis* and *Halorhodospira halophila* as "closest relatives" (Caumette et al., 1988, 1991, 1997; Gorlenko et al., 2009). Evidence was also obtained for the presence of several phylotypes of BChl *b*-containing anoxygenic phototrophic bacteria distant to (<80% sequence similarity) the genera *Thiococcus*, *Thioflavicoccus*, and *Thioalkalicoccus*, which form a distinct phylogenetic branch among the purple sulfur bacteria (Imhoff et al., 1998b; Bryantseva et al., 2000; Tank et al., 2009). These bacteria are known as inhabitants of marine sediments (Imhoff and Pfennig, 2001; Nicholson et al., 1987; Pfennig et al., 1997) and therefore their presence in these lake sediments is not surprising, though they have been rarely isolated from coastal marine sediments and soda lakes (Bryantseva et al., 2000).

The analysis of purple bacteria communities from Chilean salt lakes of the Salar de Atacama most significantly revealed that all but one (a betaproteobacterium very distantly to *Rubrivivax*) out of 25 phylotypes identified were affiliated with the *Gammaproteobacteria* (Thiel et al., 2010). Only two of these phylotypes qualified according to the sequence similarity to be identified as one of the recognized species (*Ectothiorhodospira mobilis* and *Thiohalocapsa halophila*), while the majority of the phylotypes (17) were at such a low similarity (<80%) to known purple sulfur bacteria that quite likely they might represent new genera. Most remarkable was the dominance and diversity (11 phylotypes) of a novel, so far unknown lineage of *pufLM* containing *Gammaproteobacteria*, which was highly diverse and prevalent in different lakes of the Salar de Atacama (Thiel et al., 2010). In conclusion, most of the bacteria recognized by *pufLM* sequences in the two salt lakes represent new bacteria, more than two-thirds even at the genus level or higher taxonomic rank. This depicts the extraordinary situation of the habitats in the Chilean highlands with extreme climatic and environmental conditions and great geographic distance to all so-far investigated habitats of phototrophic bacteria as well as the uniqueness of their bacterial communities.

Baltic Sea coastal lagoon

In another study based on the *pufLM* gene approach, the phototrophic bacterial community of a brackish water Baltic Sea coastal lagoon was characterized (Tank et al., 2011). Interestingly, major components of the purple sulfur bacterial community of this brackish water lagoon affiliated to genera and species of phototrophic purple sulfur bacteria typically isolated from such habitats including *Marichromatium*, *Thiocystis*, *Thiorhodococcus*, *Allochromatium*, *Thiocapsa*, and *Thiorhodovibrio*; some sequences were related to moderately halophilic *Halochromatium* and *Thiohalocapsa* species, which were not commonly seen thus far in brackish waters (Caumette et al., 1988, 1991, 1997; Imhoff, 2001a). The overall diversity in the lagoon was comparable to that found in other studies on communities of anoxygenic phototrophic purple bacteria using molecular techniques (Martínez-Alonso et al., 2005; Ranchou-Peyruse et al., 2006). Altogether 26 *pufLM* phylotypes were identified in the habitat sample, of which 14 were purple sulfur bacteria.

Quite importantly, most of the phylotypes in this study could be clearly assigned to known genera and only 5 out of 20 phylotypes of purple sulfur bacteria had sequence similarities slightly below the proposed limit of 86% (83.4–85.6%) *pufLM* sequence similarity to the closest known type strain and therefore might represent novel genera. In conclusion, the purple sulfur bacteria in the lagoon more or less are known at the genus level but novelty of these bacteria is high at the species level with only 5 out of 20 phylotypes clearly assigned to known species.

Interesting results were obtained by performance of enrichment experiments along gradients of temperature (13–44°C) and salinity (0–7.5% NaCl) with samples from this habitat and the molecular analysis of changes within the community. These experiments demonstrated that the media and culture conditions were quite appropriate for almost all purple sulfur bacteria recognized by the genetic approach. With the exception of three phylotypes found as single clones in the environmental sample, all were retrieved at least from one of the enrichments. In addition, six phylotypes of purple sulfur bacteria were retrieved only after various enrichments. Among these were three phylotypes most similar to the known type strains of *Thiorhodococcus mannitoliphagus* (99.8%), *Thiorhodococcus kakinadensis* (98.2%), and *Marichromatium gracile* (100%). An interesting property of *Marichromatium gracile* was demonstrated by these experiments. This bacterium became most prominent in enrichments at elevated temperatures above 41°C but was not detected at lower temperatures. The clear preference of *Marichromatium gracile* for elevated temperatures (see also Serrano et al., 2009) points to its obvious competitive advantage in habitats heated during the daytime by the sun.

Conclusions

A solid taxonomic system based on well characterized pure cultures is of inestimable value to attempt species recognition in the environment. Taxonomy of anoxygenic phototrophic bacteria has over many decades established a well organized and clearly structured system of the bacteria depending on pure cultures. In recent years phylogenetic aspects were systematically included into the taxonomy of these bacteria and this work with pure cultures forms the basis for studies of environmental communities. Their consequent application to the characterization of natural bacterial communities certainly will promote our knowledge on species distribution of these bacteria and their environmental importance.

The genetic approaches using *fmoA* and *pufLM* gene sequences and in particular a database of these genes from taxonomically characterized and well defined species (most important from the type strains) made possible the specific analysis of environmental communities of green and purple sulfur bacteria. With the background of sequences from most of the type strains of known species, it is possible to recognize these species in the natural communities and to relate other sequences to these known types. Thereby the novelty of the clone sequences as well as the diversity of the communities can be estimated. First comprehensive investigations, as outlined above, have demonstrated the power of these methods.

Both novelty and diversity were different in the two groups of phototrophic bacteria and in the different habitats studied. The sequences of green sulfur bacteria from different habitats were restricted to known genera and fitted nicely into the established marine and saltwater lineages of the green sulfur bacteria. In addition, the diversity of these bacteria was rather low with only a few phylotypes present in a single habitat. The purple sulfur bacterial communities were more diverse in both habitats. The Baltic Sea coastal lagoon represents a repeatedly investigated type of habitat from which quite a number of phototrophic bacteria have been isolated and characterized. As far as can be concluded from the genetic sequence analyses, the majority of the components of this community was new at the species level but known at the genus level. The Chilean salares represent an extraordinary and extreme habitat with special conditions regarding salt concentration and composition, irradiation and drastic diurnal changes. The great majority of purple sulfur bacterial phylotypes of this habitat was new at the genus level or even at higher taxonomic ranks.

Though we are far from being able to systematically describe natural communities even of the well-characterized anoxygenic phototrophic bacteria, results of the work discussed here are promising and demonstrate that this might be a feasible goal for the future. An important point of support for this optimistic view is the fact that coordination of cultivation-independent and cultivation-dependent approaches demonstrated that most of the phylotypes seen *in situ* also were found after enrichment with culture conditions and media established by Norbert Pfennig (Pfennig and Trüper, 1981; Tank et al., 2011). In addition, most of the phylotypes present in the different habitats studied represent close relatives to bacteria in culture with a clear taxonomic identity. Extended cultivation studies may give rise to cultures representing novel phototrophic bacterial species detected with molecular ecology approaches in the natural environment. From such extreme habitats as the salt lakes of the Chilean Altiplano, even representatives of new phylogenetic lineages, once genetically detected in environmental samples, may be grown in culture by applying just slight modifications of media and conditions currently used.

Another promising approach used the sequence of the *bchY* gene, which is more widely distributed in phototrophic bacteria containing either a type 1 or a type 2 photosystem and was demonstrated to provide amplification products from various purple bacteria, green sulfur bacteria and also green nonsulfur bacteria (Yutin et al., 2009), though the total length of the amplification products was only 480–510 nucleotides. These primers (Table 1) were also successfully applied to environmental samples (Yutin et al., 2009), but a

comprehensive database of sequences from pure cultures is still lacking. When the phylogeny of this gene is established with the available type strains of phototrophic bacteria, the *bchY* gene may prove to be an additional valuable tool to systematically analyze natural communities of phototrophic bacteria.

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The importance of cultured representatives of the human intestinal microbiota in the metagenomic era

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There has been remarkable progress in the application of molecular approaches, mainly based on analysis of the 16S rRNA gene, to characterize complex microbial ecosystems including that of the human colon. This has been augmented recently by high throughput metagenomic approaches. Although there is an obvious need to link sequence information to functionality, much less emphasis has been given to the isolation and culturing of the gut anaerobes from the colon. Lack of research effort in this area may be due to the fact that the majority of these bacteria are anaerobes that have rather exacting growth requirements. Nevertheless, it is becoming evident that the most dominant bacterial species that are likely to play a significant role in the formation of short-chain fatty acids, including butyrate and propionate, and in the degradation of recalcitrant substrates such a resistant starch and dietary fiber can be isolated and cultured. Many of the bacterial strains representing the dominant species have also recently been genome-sequenced. Moreover, these genome sequences are publicly available and mining this information will provide researchers with the most tremendous insight into the genetic make-up of the inhabitants of our gut. It is important however to continue to culture anaerobic micro-organisms as well as applying molecular methods, as it is only then that we can truly assess the role of these fascinating micro-organisms in the colon. Some novel bacterial isolates may prove to have potential for biotechnological applications or as probiotics.

Introduction

The developments over recent years in rapid, culture-independent molecular methods for describing microbial communities have been truly remarkable. Initial progress relied largely on the analysis of 16S rRNA gene sequences (Zoetendal et al., 2006) directly amplified from environmental samples by PCR. More recently the use of barcoded primers in conjunction with pyrosequencing represents a further technical advance that circumvents the need for cloning. More targeted approaches include qPCR, fluorescence *in situ* hybridization (FISH), and the development of microarrays which may use many thousands of oligonucleotide probes to target different taxonomic tiers. Rapid progress is now being made with non-targeted metagenomic sequencing of DNA recovered from environmental samples, again driven by remarkable developments in sequencing technology and bioinformatic analysis.

In contrast, few research laboratories now engage in isolating and culturing bacteria, and this area of endeavour has largely fallen out of fashion. Indeed there is even a strand of opinion that cultural microbiology should be regarded as defunct (Ritz, 2007). We believe however that it is important to critically examine the assumptions on which this view is based particularly in relation to gut communities. At the start of this revolution in molecular ecology it was frequently stated that less than 1% of micro-organisms present in most environmental samples could be cultivated in the laboratory (Eilers et al., 2000). This may indeed be true for ecosystems such as oceans and soils that have low nutrient availability and often very low growth rates. In these cases metagenomics may represent the only approach available to characterize these communities. Our purpose here, however, is to consider the contribution of cultural approaches, allied to molecular methodologies, to studying the microbial communities of the human intestine.

The human colon contains a high microbial cell density of around 10^{11} cells per ml of contents and in adults the colon carries around 250 ml of contents. The system is in a state of constant turnover, requiring resident organisms to have a certain minimum growth rate to avoid being washed out. In contrast to many other ecosystems, the colon is nutritionally rich, and develops a highly anoxic environment with an oxidation-reduction potential of around −200 mV. The pH along the colon will also fluctuate from mildly acidic to neutral values. It is possible that many colonic bacterial species have rather exacting nutritional requirements; nevertheless, culturing these metabolically diverse anaerobes, if

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feasible, must have a major role to play in understanding their role in human health. A representative cultured isolate enables study of pathogenicity potential, substrate preferences, growth requirements, fermentation pathways, interactions with the immune system, environmental tolerance, resistance traits and a host of other characteristics. The genome can also be sequenced rapidly and inexpensively, thus contributing to interpretation of physiological as well as metagenomic data.

What is the culturability of bacteria that inhabit the human intestine?

Analysis of 16S rRNA gene sequences has indicated that around 30% or less of phylotypes recovered from human fecal samples correspond to cultured species (Suau et al., 1999; Hayashi et al., 2002; Eckburg et al., 2005; Tap et al., 2009; Walker et al., 2011). Such estimates have been based on "old-fashioned" Sanger sequencing of at least 500 bases up to full-length sequences, using 97–99% identity cut-offs (depending on the precise region sequenced) that distinguish known species. Pyrosequencing data from more recent surveys generally comprise shorter reliable read lengths that do not allow phylotype discrimination at this level.

Frequency distributions from such analyses have identified a group of phylotypes that are typically more abundant than others in fecal samples from adult humans. These phylotypes are not found to be dominant in 100% of individuals, and so cannot be said to constitute a strict "core". Nevertheless they have now been reported in multiple studies and can reasonably be described as "dominant" phylotypes within the human colonic microbiota. Tap et al. (2009) reported more than 60 phylotypes that occurred among the dominant fecal microbiota in nine or more of their group of 17 healthy adults. Walker et al. (2011) found 50 phylotypes that accounted for >0.5% of amplified 16S rRNA gene sequences from 6 overweight males. Two thirds of these corresponded either to previously described species, or to cultured isolates that will form the basis for new species descriptions in the future. This analysis showed therefore that the low cultural coverage of the human intestinal microbiota applies mainly to the less abundant phylotypes (i.e. those that individually comprised <0.5% of total sequences). Importantly, this suggests that low cultural representation may simply be the result of the small number of cultured isolates for which 16S rRNA gene sequences are available, i.e. human gut bacteria should be considered as "undercultivated" rather than "unculturable".

Another very striking observation is that four of the top five most abundant fecal bacterial species reported by Moore and Moore (1996) and by Walker et al. (2011) in Caucasian populations were the same (Table 1). What makes this remarkable, apart from the fact that the populations were from different continents and involved different recruitment criteria, is that the Moore and Moore (1996) study was entirely culture-dependent, whereas Walker et al. (2011) were reporting analysis of amplified 16S rRNA gene sequences. While much has been made of the problem of cultural bias, it appears that this has not been such a major factor with regard to the recovery of the dominant species of human fecal bacteria, provided that strict anaerobic culture techniques are used. The very large culture collections obtained in earlier studies that pre-dated routine 16S rRNA sequencing may therefore have contained a much wider range of cultured variation than is currently available.

Geographic variation

A recent 16S rRNA-based study by de Fillipo et al. (2009) demonstrated major differences in fecal microbiota composition between groups of African and Italian children. While many species were common to both groups, the ratio of *Bacteroidetes* to *Firmicutes* was higher in the African children. Furthermore the dominant *Bacteroidetes* were *Prevotella* spp. in the African children and *Bacteroides* spp. in the Italian children. Since little intensive cultivation work has been done recently with subjects outside North America, Europe and Japan, this suggests that there may be considerable undescribed diversity that might be recovered readily by cultivation in other human populations. The authors proposed that differences in staple diet were the main factors underlying these differences, although the evidence for this was circumstantial. The earlier culture-based study of Moore and Moore (1996) examined adult rural native Africans, rural native Japanese and Japanese Hawaians in addition to North American Caucasians. Their data also suggested some geographic or ethnic differences, e.g. the lack of *Eubacterium rectale* among the dominant bacteria detected in the rural Japanese group, and a lack of *Bacteroides vulgatus* in the rural Africans.

Metagenomics

Metagenomics is the analysis of the total DNA recovered from environmental samples (Frank and Pace, 2008). Metagenomic libraries may either be analyzed by functional screening with a view to novel gene discovery, or analyzed by random sequencing, with the latter approach proving far more rapid and increasingly popular. Sequences can be analyzed to determine the prevalence of different types of gene present (Gill et al., 2006). Taxonomic assignment of sequences to bacterial species is possible but this is depen-

Table 1. Dominant bacterial phylotypes and species detected in human fecal samples either by sequencing of directly amplified 16S rRNA or by anaerobic culturing

16S rRNA data from Walker et al. (2011) are for six overweight males (Scottish, Caucasian) and cultural data from Moore and Moore (1996) are for 17 North American Caucasians. All species are listed that individually accounted for more than 2% of total bacteria in each study.

Species	Total bacterial sequences (16S rRNA) (%)	Total bacterial isolates (cultured) (%)
Faecalibacterium prausnitzii	7.98*	3.0†
Eubacterium rectale	4.43	5.9‡
Clostridium clostridioforme	3.83	
Collinsella aerofaciens	3.67	10.0§
Bacteroides vulgatus	3.21	8.2
Anaerostipes sp. (SS2/1 related)	2.25	
Ruminococcus bromii	2.11	5.2
Eubacterium hallii	2.0	
Eubacterium rectale 1		2.9||
Bifidobacterium longum		2.6
Peptostreptococcus DZ		2.2
Bifidobacterium adolescentis		2.2

*Sum of three phylotypes corresponding to this species.
†Described as *Fusobacterium prausnitzii*.
‡"*Eubacterium rectale* 2" taken as corresponding to the current definition of *Eubacterium rectale*.
§Sum of "*Eubacterium aerofaciens* 1 and 2".
||Thought likely to correspond to one or more of the redefined *Roseburia* spp.

dent either on comparison of the 16S rRNA gene sequences present, or on the availability of reference genomes from cultured isolates (Qin et al., 2010). Such high-throughput sequencing allows large numbers of samples to be analyzed without the problem of PCR bias. Arumugam et al. (2011) analyzed fecal samples of volunteers from four countries and found that the microbiota composition could be grouped into three distinct clusters or enterotypes. One enterotype was enriched in *Bacteroides* spp., a second in *Prevotella* spp. whilst the third was enriched in *Lachnospiraceae* related to *Ruminococcus obeum.* It was suggested that factors such as body mass index, age, or gender had little impact in defining these enterotypes.

Functional screening of metagenomic libraries is dependent on appropriate high-throughput screening assays, as have been applied in the other ecosystems such as soil (Riesenfeld et al., 2004). An alternative type of metagenomic approach is to use a PCR-based approach to target specific functional genes as this gives a phylogenetic perspective to specific functional groups. This approach has been used to analyze hydrogen-metabolizing bacteria including sulfate-reducing bacteria and methanogens (Denman et al., 2007; Deplancke et al., 2000) and will also be discussed further below in relation to butyrate-producing bacteria.

Composition of the human colonic microbiota

The most abundant Gram-negative phylum of bacteria in the human colon are the *Bacteroidetes*, represented mainly by four genera, *Bacteroides*, *Parabacteroides*, *Prevotella*, and *Alistipes*. These genera include more than 20 cultured species among which *Bacteroides dorei*, *Bacteroides cellulolyticus*, and *Bacteroides faecis* have been described in recent years. The genome size among the *Bacteroidetes* is comparatively large and indicates activities against a wide range of plant- and host-derived polysaccharides. *Proteobacteria* are generally less numerous, but inhabitants of the large intestine include *Enterobacteriaceae* and sulfate-reducing bacteria (SRB) such as *Desulfovibrio piger*. The Gram-negative phyla *Verrucomicrobia* and *Lentisphaerae* have two species so far described from the human gut, the mucin-degrader *Akkermansia muciniphila* (Derrien et al., 2004) and *Victivallis vadensis* (Zoetendal et al., 2003), respectively. Among the high mol% G+C content, Gram-stain-positive *Actinomycetes* are two abundant genera, *Collinsella* and *Bifidobacterium,* which can represent 5% or more of the colonic microbiota (Flint et al., 2007). The genus *Bifidobacterium* includes many cultured species of which *Bifidobacterium adolescentis* and *Bifidobacterium longum* are generally abundant in the adult large intestine.

Eckburg et al. (2005) reported finding the greatest 16S rRNA gene sequence diversity within the low mol% G+C content *Firmicutes* which accounted for 76% of 395 phylotypes defined in their study of colon and fecal samples from three individuals. The corresponding figure from Walker et al. (2011) for six individuals was 76% out of 320 phylotypes. In such studies the largest number of *Firmicutes* phylotypes fall into two families, the *Ruminococcaceae* and *Lachnospiraceae*. The *Ruminococcaceae* show a rather low representation of cultured species (Lay et al., 2007), but include *Faecalibacterium prausnitzii*, a dominant colonic species that has a requirement for acetate for optimal growth (Duncan et al., 2002) (Table 1). Human colonic *Ruminococcus* species include *Ruminococcus callidus* and the starch-degrading *Ruminococcus bromii* strains. Related ruminococci from the rumen (*Ruminococcus albus* and *Ruminococcus flavefaciens*) however possess the ability to hydrolyze crystalline cellulose (Flint et al., 2008). Interestingly, Chassard et al. (2012) recently isolated a new cellulolytic ruminococcus species, *Ruminococcus champanellensis*, from a human fecal sample, and ruminococcal 16S rRNA gene sequences appear to be far more abundant on particulate material compared to the liquid phase of human fecal samples (Walker et al., 2008).

The *Lachnospiraceae* comprise a disparate collection of bacterial genera and species and has been estimated to also make up 25% or more of bacteria found in the colon (Flint et al., 2007). A number of different genera belong to this cluster including *Anaerostipes*, *Clostridium*, *Coprococcus*, *Eubacterium*, *Roseburia*, *Blautia*, and *Dorea*. *Roseburia* spp. and *Eubacterium rectale* are a major component of this cluster that produce butyrate and make up around 7–10% of the fecal microbiota (Aminov et al., 2006). *Blautia hydrogenotrophica* is of particular interest as it is an acetogen. *Ruminococcus* spp. belonging to the *Lachnospiraceae* (but clearly in need of generic reclassification) include *Ruminococcus obeum*, *Ruminococcus torques*, and *Ruminococcus gnavus*. Other human gut *Firmicutes* fall into families that are generally less abundant. *Veillonellaceae* (clostridial cluster IX) are poorly characterized from the human colon but based on other ecosystems such as the rumen that harbors *Selenomonas*, *Veillonella*, and *Megasphaera* species, it would be expected that this group might make an important contribution to propionate formation in the colon. Species that belong to clostridial cluster XV include *Clostridium bartlettii* (Song et al., 2004) and *Anaerofustis stercorihominis*, which is a bile-tolerant, butyrate producer (Finegold et al., 2004). Other *Firmicutes* regularly identified in human stool include *Eubacterium cylindroides* belonging to cluster XVI and *Clostridium ramosum* belonging to clostridial cluster XVIII (Wilderboer-Veloo et al., 2003; Hayashi et al., 2002).

The diversity of archaea in the human colon has so far received less intensive study. The dominant methanogen is reported to be *Methanobrevibacter smithii* with *Methanosphaera stadtmanae* also detected. *Methanobrevibacter smithii* uses H_2 and CO_2 or formate to form methane.

Butyrate-producers from the human large intestine

Most bacteria that inhabit the colon are anaerobes that ferment substrates to form mainly short-chain fatty acids and gases. One of these SCFA products, butyrate, is the major energy source for colonocytes and has a role in regulating host gene expression and apoptosis (Scheppach, 1994; Hamer et al., 2008). Considerable efforts have been made to isolate butyrate producers from the human colon, all of which were found to be *Firmicutes* (Barcenilla et al., 2000; Pryde et al., 2002; Louis and Flint, 2009). This work led to the identification of butyryl CoA:acetate CoA transferase as the final step in butyrate synthesis in most human colonic butyrate-producing bacteria (Louis et al., 2004; Charrier et al., 2006). Recently, a set of degenerate PCR primers was developed that could recognize this gene from phylogenetically diverse *Firmicutes* (Louis et al., 2010). Remarkably, the majority (88 %) of the butyryl CoA:acetate CoA transferase sequences obtained by PCR amplification from fecal samples of 10 healthy individuals corresponded to 12 species that were already cultured, with the most abundant being *Eubacterium rectale*, *Roseburia faecis*, *Eubacterium halli* and a new species *Anaerostipes* to be proposed shortly (Duncan et al., 2006; Allen-Vercoe et al., in preparation).

In terms of function we can assign these butyrate-producers to three broad groupings. The flagellated *Roseburia*/*Eubacterium rectale* group (Aminov et al., 2006) belongs to the *Lachnospiraceae* and their population has been shown to decrease in response to reductions in dietary starch intake in human intervention studies (Duncan et al., 2007). A second group of *Lachnospiraceae* includes *Eubacterium halli* and two *Anaerostipes* species that appear to share the ability to utilize lactic acid together with acetate to form butyrate (Duncan et al., 2004). The third group are *Ruminococcaceae* related to *Faecalibacterium prausnitzii*. This is one of the most abundant species in the healthy colon (Table 1) and is proposed to have anti-inflammatory activity (Sokol et al., 2008). Its abundance is diminished in individuals with active IBD (Sokol et al., 2007; Willing et al., 2009). Other less dominant butyrate producers include those belonging to the genus *Coprococcus*. Unlike the species discussed above, *Coprococcus eutactus* forms butyrate using the butyrate kinase enzyme (Louis et al., 2004).

Conclusions

The availability of cultured isolates from the human colon has contributed enormously to our understanding of the taxonomy, physiology, metabolism, and ecology of the gut microbiota. Cultured isolates also facilitate the design of molecular tools such as FISH probes and primers for qRT-PCR that can be used to target functional groups. Cultured isolates may also be used to define interactions between bacterial species and between bacteria and host cells that are difficult or impossible to predict unless tested biologically (Belenguer et al., 2006; Falony et al., 2006; Munoz-Tamayo et al., 2011). Large genome programs including the NIH-funded Human Microbiome Project (https://commonfund.nih.gov/Hmp/) and the EU funded MetaHIT project (http://www.metahit.eu/) are collectively sequencing several hundred human intestinal isolates. These genome sequences are publicly available from GenBank and are generating valuable information that reveals that these cultured intestinal species are in possession of genes encoding a wide array of glycosyl hydrolases with predicted activities against a broad range of polysaccharides, and also with pathways for vitamin and cofactor synthesis.

Whilst bacterial species from the large intestine and other ecosystems remain uncultured there will always be a need to strive to culture these elusive cells. Understanding of the microbial ecosystem of the human colon will continue to benefit from a range of molecular and cultivation approaches which should be developed in parallel (Goodman et al., 2011). In the future certain gut bacterial isolates are highly likely to be exploited for biotechnological applications or perhaps as new probiotics.

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Working towards a Molecular Systematics Library for Prokaryotes: a perspective

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With the breakthrough of high-throughput DNA sequencing technologies and development of bioinformatics tools, the dramatically increasing availability of genomic data has provided an opportunity and challenge for systematists to renovate the taxonomic system of prokaryotes especially for the classification of species and their related genera and subspecies. With a better understanding of the genetic and ecological diversity of prokaryotes based on meta- and pan-genomic analyses, comprehensive insights into prokaryotic speciation will revitalize the taxonomic system for prokaryotes in the near future. By integrating more comprehensive genotypic information with corresponding phenotypes of the subject taxa at the genomics level, and relying on related physiological and ecological knowledge, we may upgrade our classical taxonomic treatment in encyclopedia-style books to a "Molecular Systematics Library for Prokaryotes" indexed by phylogenomic information and integrated with ever increasing knowledge of biological diversity, which may eventually lead us to a comprehensive understanding of prokaryotic speciation.

Introduction

The main task of microbial taxonomy is to establish a logical and hierarchical framework to accommodate micro-organisms with huge diversity to facilitate their biological study (Cowan, 1970). Practically, taxonomy refers to three areas or goals: the sorting of individuals into likes and unlikes (*classification*); the labeling or naming of the groups sorted (*nomenclature*); and the comparison of the unknown with the known followed by their corresponding *identification* (Cowan, 1970). The nomenclature of prokaryotes has had a solid foundation through the unremitting efforts of several generations of dedicated microbiologists (e.g. Euzéby, 1997; Tindall et al., 2006). And now, the achievement of naming micro-organisms has provided a platform for scientific communication, insofar as the correct use of any name may open access to a catalog of information about that organism. Meanwhile, the continuous development of novel technologies used in taxonomy, including the investigation of prokaryotic communities by using improved culture-dependent approaches for extended environment conditions, has significantly affected the foundation of taxonomy. For instance, the classification and identification of *Actinobacteria* has been continuously updated to accommodate the dramatic increase of novel isolates during the past several decades (Stackebrandt et al., 1997; Zhi et al., 2009).

The development of taxonomic research is closely linked to the advancement of new techniques, and the methods used to study micro-organisms taxonomically have reflected current developments in microbiology. In the late 19th century, initial emphasis was placed on morphology both micro-morphology, relying heavily upon microscopic observation such as cell shapes and the structures of cellular components (e.g.

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flagella or pili), and cultural characteristics, relying upon the introduction of solid media for colony isolation. Phenotypic studies developed later with the publication of new physiological and biochemical techniques, such as the growth properties and chemical composition of cell walls and membranes, etc. Along with the development of genetic knowledge and sequencing techniques, it is well established that the phenotypes of prokaryotes are primarily determined by their genotypes (and to a certain extent, the epigenetic properties). Given that, more and more attention is being paid to the genotypic characteristics and biomarkers for phylogeny by taxonomists. In that direction, with the aid of the strong correlation between sequence variations in the conserved 16S rRNA genes and the phylogenetic/evolutionary relationships among the prokaryotes, there is a current trend to differentiate prokaryotic taxa more and more on the basis of the genotype, in particular, by sequence comparisons of the 16S rRNA genes (Kämpfer, 2010). On the other hand, it is widely recognized that not all species can be differentiated merely based on their 16S rRNA gene sequences, and thus for the purpose of proposing a novel species, a phenotypic description and possibly DNA–DNA hybridization remain essential. Recently, taking the advantage of the availability of large amount of genomic sequences from divergent species of prokaryotes, many researchers have extended their focus to other housekeeping genes (usually, single copied among the taxa being studied) for addressing taxonomic and evolutionary issues (Naser et al., 2007; Martens et al., 2008; Rong and Huang, 2010; Labeda, 2011). For the sake of durability, many orthologous genes functioning in chromosomal replication (e.g. *dnaK* and *gyrB*), recombination and repair (e.g. *recA*) and transcription (e.g. *rpoB*) are employed to perform multilocus sequence typing (MLST) in epidemiological identification of strains within defined populations (Maiden et al., 1998). When the natural and evolutionary relationships among micro-organisms are not well resolved by the 16S rRNA gene-based phylogeny, additional housekeeping genes have been employed (Foster et al., 2009; Alam et al., 2010; Zhang et al., 2011).

With respect to the integration of genomic information, there are at least two advantages obvious for prokaryotic taxonomy: the absolutely large body of information for phylogenetic analysis, and genome-scale metabolic pathway and/or network as the backbone for answering questions about evolution and ecological relationships (Barona-Gómez et al., 2012). Because methods of statistics can only lead to improved precision but not necessarily improved accuracy, the use of appropriate statistical methods, including concatenation, separate gene-by-gene analyses, and modelling, for data analysis (especially for large datasets) is important (Rannala and Yang 2008). However, phylogenetic tree construction with analytical methods may not be consistent with that based on 16S rRNA gene sequences, and perhaps even incongruent with each other (Rannala and Yang 2008).

Although the current polyphasic taxonomy has combined the biological characters of a taxon via multiple approaches ranging from phenotypic properties to genomic information, it is still generally difficult to consolidate the genotype and phenotype into one uniform system. In other words, the taxonomic system is yet to be comprehensive, covering both the phylogeny backbone and the physiological and metabolic properties strongly related to the ecological influences. Generally, there are two key issues yet to be solved: (a) the specific correlation of taxonomic characteristics (phenotypes) to the genomic sequences (genotypes) based on accurate annotations, and (b) the proper stratification of different sets of genomic markers (in many cases, simply genes) for prokaryotic phylogeny at different taxonomic levels. In addition, the advent of genomics has revolutionized biological studies of micro-organisms and will also revitalize prokaryotic taxonomy to tackle the new scientific problems raised by the genomic research. The genomes (or genomic markers) of isolates, including those yet to be cultured, not only provide comprehensive even complete and accurate genomic information in the form of DNA sequences and annotated genes, but also an opportunity for investigating and understanding the biological process encoded by the genome. One obvious example is the reconstruction of metabolic networks from genome sequences, which can provide novel *in silico* chemotaxa useful as potential evolutionary markers (Barona-Gómez et al., 2012). Furthermore, as phenotypes represent the expression of genotypes under certain environmental conditions, phylogenomic studies based upon central and/or peripheral metabolism should be in agreement with the phenotypic characteristics of polyphasic taxonomy. Under these circumstances, the modern prokaryotic taxonomic system should neither be based merely on 16S rRNA or selected housekeeping genes nor be portrayed as a phenotypic character-based traditional reference book. By introducing the theories and techniques of genomics to bridge the gap between genotypes and phenotypes, modern taxonomy will be enriched and vitalized in the true sense of biology, i.e. to reflect the evolutionary history and natural ecological relationships among the taxa.

Understanding the genetic diversity of prokaryotes

The breakthrough of sequencing technology has made more and more genome sequences of prokaryotes available which directly extends the genetic information for the study of phylogeny from merely the 16S rRNA gene and/or a few

housekeeping genes to the complete genomes encoding all the biological processes. It allows us to differentiate, at all levels of resolution, the genetic make-up between two strains and thus substantially contributes towards the definition of a microbial species (Konstantinidis et al., 2006).

A comprehensive understanding of biodiversity based on our knowledge of genomics and the practice of taxonomy is the key for future development of taxonomy in the genomics era. *Genetic diversity*, as the molecular foundation of *ecological diversity*, is a basic representation of biodiversity based on genetic variations of *species* interacting under variable environmental conditions.

Estimates of diversity for natural bacterial communities have traditionally depended on cultivable species. However, molecular ecological studies mainly probed by 16S rRNA genes suggests that the culture-based estimation actually led to a longstanding underestimation of bacterial diversity (Torsvik et al., 2002). Therefore, the genetic diversity of a population (e.g. at or below the species level) can be explored thoroughly in the era of genomics. This is particularly true if the functional aspects of physiology, metabolism and growth in a complex environment are considered along with the genomic sequences for comprehending biological diversity (Mocali and Benedetti, 2010). Meta-genomic analysis of a given community may offer an alternative approach towards that direction by describing the genetic diversity of a microbiota and characterizing some of the major contributing species without physical isolation of the strains (Petrosino, 2009).

Within a bacterial species (or even genus), the *core genome* that is responsible for the basic functions (Gil et al., 2004) may not change dramatically except for some obligate bacterial symbionts (Moran, 2003). On the other hand, the gene reservoir available for inclusion in its *pan-genome* (Tettelin et al., 2005) is vast and dramatically variable. More and more *individual genome*-specific genes, including mobile genetic elements, plasmids and bacteriophages, may continue to be identified after sequencing hundreds of genomes (Tettelin et al., 2008). Hence, the *peripheral genome* is considered to play an important role (e.g. conferring adaption to different niches) in the maintenance of genetic diversity. The genomic variability of *Streptococcus pneumoniae* has been studied with a large group of isolates; its pan-genome can greatly vary in size, and that guarantees the species a quick and efficient response to diverse environments (Donati et al., 2010). The longer an individual has evolved independently, the more new genes it will potentially contribute to the species' pan-genome. The size of the pan-genome, therefore, reflects the degree to which the sequenced strains are phylogenetically related, and it yields information on the evolution of the species (Muzzi and Donati, 2011). Recently, Luo et al. (2011) demonstrated that the genomes of the intestinal commensal *Escherichia coli* encode some functional genes benefitting the fitness in the human (and animal) gut, while their environmental counterparts possess another set of specific genes that is instrumental in resource acquisition and survival in the environment. Thus, although all of these *Escherichia coli* isolates belong to the same species based on our current phylogenomic standards for species demarcation (Goris et al., 2007), it is clear that the diversity of their peripheral genomes may provide more insight into the biological diversity within a population, such as of a species, and may facilitate our understanding about the evolutionary dynamics among individuals, either representing cryptic new species or a process of speciation under different environmental conditions. These two examples imply that it is essential to know the gene content of a bacterium in order to understand how it speciates. To the higher ranks in the taxonomic system, two taxa sharing closer evolutionary relationships should possess, in theory, more genes in common. The core genome will likely be valuable in revealing the divergence amongst different taxa.

For the most clinically important species, some prevalent sequence-based approaches, e.g. MLST, have been instrumental in studying the population structure and evolution of pathogens and even used for identification (Chan et al., 2001). However, the genetic variations typically used entail small-sized samples and limited fractions of the genomes and cannot distinguish among closely related isolates. The use of next-generation sequencing technologies to generate whole-genome DNA sequence data for large population samples of bacteria has enabled subspecies typing reveal the global geographic structure and transmission process of pathogenic bacteria (Harris et al., 2010).

Understanding the ecological diversity of prokaryotes

The various environmental conditions in which microbes reside are supposed to be one of the factors contributing to the ambiguity of genetic diversity of prokaryotes and to their mechanisms of microbial speciation. James Staley (2010) recently pointed out that a comprehensive understanding of microbial diversity should be the fourth goal of microbial taxonomy. In our opinion, there are two levels of implications, i.e. the discovery and description of the diversity, and the rationale for the formation of microbial diversity. Therefore, it is more important for a taxonomist to reveal the genetic and ecological relationships among the species, and based on these analyses, to explain why such diverse mi-

crobial species exist and how they have evolved. In recent decades, using pure cultures as well as culture-independent approaches, microbiologists and ecologists have begun to study the biogeography of microbial life on earth comprehensively (Martiny et al., 2006; O'Malley, 2007). Remarkably, genome sequences are providing detailed information about the complete genetic composition of microbes in a defined ecological niche, and offering an uncommon opportunity to search for evolutionary traces left in the genome and to reveal what causes the ecological diversity of the microbiota.

A natural criterion to identify microbiota with evolutionary significance is to characterize ecological features that may distinguish them from their close relatives. Historically, pathogens were classified as different species based on their ability to cause a distinctive disease (Fraser et al., 2007) and these are now often relegated to "pathovar" status. Mapping of prokaryotic diversity onto environmental resources indicates that closely related groups of micro-organisms might be ecologically divergent (Hunt et al., 2008). Although the traditional approaches concerning the definition of species and molecular microbial ecology hints at the concept of ecological species (Cohan, 2002), they are intrinsically poor in resolution and power for understanding the ecological diversity of microbes, and thus limited in their implication to special purpose classifications, in this case, ecology. Nevertheless, environmental factors are important not only for shaping different species but also for their role in the evolution of microbes. Particularly, when the cryptic lineage of one species has formed and readies itself to adapt to different niches, periodic selective sweeps could, in theory lead to a new species boundary. Therefore, it is a reasonable hypothesis that the genetic diversity of prokaryotes correspond to some kinds of ecological diversity, which in turn, are likely the natural force to generate new genetic diversity.

Understanding microbial speciation

The genomes of micro-organisms have been continuously evolving ever since the origination of life. As the key dynamic force of molecular evolution, most of the single nucleotide mutations were neutral when they occurred but then either lost or fixed in a population via genetic drift (Kimura, 1968; Reeves, 1992) and/or wiped out by periodic selection (Cohan and Perry, 2007). Over longer evolutionary time, those "neutral" variations could also have contributed to speciation, especially for accumulating the divergence of genomes. On the other hand, adapting to various and complicated ecological niches, populations were under natural selection which eventually may favor genes encoding new functions that are seemingly unnecessary or even hazardous or genetic elements being silenced or reduced (Arber, 2000; Caporale, 2003). Actually, the history of evolution was recorded not only by mutations in genetic sequences known as single nucleotide variations (SNVs) or polymorphisms (SNPs), but also by various types of more complex DNA alterations, which sculpted prokaryotic chromosomes more significantly (Doolittle and Zhaxybayeva, 2009). Based on the type of genetic material transferred, the genome could have evolved via vertical and/or lateral inheritances.

Speciation of prokaryotes occurs in two forms. Vertical inheritance occurs according to the Darwinian-Mendelian model of parent-to-offspring gene flow, i.e. an organism receives genetic material from its ancestor, the species from which it has evolved. However, this has been severely challenged by the quantitative and qualitative importance of genetic transfers between lineages, i.e. horizontal gene transfer (HGT) (Charlebois et al., 2003). Hence, the paradigmatic shift from a monistic to a pluralistic understanding of the evolutionary processes is illustrated by a graph-theoretical shift, from trees (i.e. connected acyclic graphs) to networks (i.e. connected graphs which may contain reticulations) (Bapteste et al., 2009). However, this kind of evolutionary network seems to be complex particularly at lower levels, because HGT occurs frequently among closely related individuals and species, and more rarely between genealogically distant relatives (Andam and Gogarten, 2011). Actually, HGT could be an important element in shaping the population structure of bacterial species, and it indicates that bacterial speciation might present a reticulate evolutionary history (Cardazzo et al., 2008). In addition, the similar habitat and the close geographical distance between the donor and the recipient for their genetic material exchange seem to be another requirement for the occurrence of HGT (Pearson et al., 2009; Luo et al., 2011). In fact, even when the complication brought by lateral inheritance was excluded, the phylogenetic incongruence of orthologous genes implied that they probably have a different evolutionary history (Bapteste et al., 2005). Indeed, employing different tree reconstruction methods may give rise to a non-negligible statistically significant incongruence (Jeffroy et al., 2006).

By using traditional chemotaxonomic approaches, which provided merely phenotypic evidence to distinguish the subject from other references, it is difficult to reveal and understand the speciation of prokaryotes or to answer the question about what caused these differences. On the other hand, the molecular phylogeny of an organism is typically based on a single gene, usually the 16S rRNA gene, or recruiting other housekeeping genes, while the effect of the environmental pressure related HGTs are usually ignored. Although a statistically well-supported tree would be obtained, we still

need to better understand what happened to result in the two bifurcated branches.

Integrating the extensive biological knowledge of taxa

Answers of what exactly happens when species diverge from their ancestor, and what the similarities or differences of the biological characteristics are among the diverse species are important for a complete taxonomic study. The genomic diversity contained in pan-genome of a species may be substantial and these data must be summarized within a species description for the purpose of taxonomy. In practice, all genes belonging to the pan-genome can be divided into two parts: one contains orthologous genes (including paralogous genes); another contains specific genes of one strain or a group (not all) of strains. To summarize the shared genes within the description for a species, the orthologous and paralogous genes should be identified by bioinformatic tools firstly, and then mapped in different phenotypic (metabolism and physiology, etc.) pathways and even networks (see Barona-Gómez et al., 2012). Therefore, the ever-increasing genomic information will be helpful not only for delineating a more accurate and precise phylogenetic relationship of a species, but also for truly understanding the biology concealed in the prokaryotic systematics.

In the era of chemotaxonomy, chemical characteristics of metabolism, growth, differentiation and cellular components such as cell wall and membrane constituents were commonly used phenotypes for bacterial identification, notably in Gram-stain-positive actinomycetes. However, analysis and comparison of these chemical indices makes it an inefficient and onerous task in many cases. With the assistance of whole genome sequence information, it is becoming realistic to gradually correlate the chemotaxonomic phenotypes with the molecular genotypes of the corresponding taxa, particularly, species and subspecies (Sutcliffe, 2010; Zhao et al., 2010). Of course, at this moment, it is still difficult to achieve this worthy aim in practice, because the gene expression profiles for some (maybe most of) the phenotypes heavily rely on the related signal transduction and regulatory systems both encoded by their corresponding genes and depends on their interactions with environmental signals. For those phenotypes determined by multiple genes and their interactions under different environmental conditions, although comparative and functional genomic analyses and epigenetic studies are designed to elucidate the molecular mechanism in order to understand and further control the processes in a quantitative manner, it is not easy to develop a precise and stable correlation as a criterion for taxonomy at this moment. However, on one hand, these relationships eventually will be established with increasing accuracy and precision. On the other hand, most of the phenotypes included in polyphasic taxonomy are relatively stable and qualitative and therefore, their exact relationship with genotypes should be able to be established via efforts combining bioinformatic annotation and experimental proof. Finally, via integration of genomic analysis and genetic studies with the effort of reanalyzing the physiological characteristics critical for taxonomy, new criteria may arise and even ~~to~~ replace some of the old criteria. In that direction, taxonomy may function not only in describing the species as defined by its original scope, but also provides fundamental research schema to facilitate the study of physiology and ecology related to the subject taxa.

One successful example is the correlation of bacterial serotypes with their genetic make-ups. Classically, serology was used to name pathogens, such as ~1500 "species" of *Salmonella* in the early editions of *Bergey's Manual*. Although by 1984; the species number had been reduced to ~6, serotypes still maintained as the criteria not only for *Salmonella* but also for the identification of *Escherichia* clinical isolates (e.g. O157 or O164 of the Enterohemorrhagic *Escherichia coli*/EHEC) in the study of epidemiology for surveillance (Beutin et al., 2007; Switt et al., 2009). Due to its tedious testing procedure and the numerous special reagents required, it is exciting progress in developing a simple and convenient method for rapid bacterial serotyping via genetic analysis based on genomic information, which correlates the gene clusters (or genes) to the synthesis of corresponding bacterial surface antigen (Liu et al., 2008). Recently, serotypes of several bacteria like *Vibrio parahemolyticus*, *Proteus* and *Cronobacter sakazakii* were shown to be identified by using this approach as well (Okura et al., 2008; Wang et al., 2010; Sun et al., 2011).

As we have emphasized, prokaryotic taxonomy is an essential subdiscipline of microbiology. Although its taxa should be organized in a phylogenetic network, the biological characteristics encoded by the genome may not be revealed unless comparative and functional genomic analyses are executed that match with their phenotypes. This kind of biological knowledge, integrated into the phylogenomic backbone of taxonomy may further facilitate biology research in general, including those originally impossible cases of uncultured bacteria with their genome sequence derived from metagenomic sequencing.

Approaching the ultimate aim of prokaryotic taxonomy

In summary, prokaryotic taxonomy is a subject fundamental

for exploring microbial world on Earth, and answering the questions about what they are, how diverse they are, and what relationships exist among them. Taxonomy shares intricate relationships with other disciplines in microbiology and even other fields of biology. This essence makes the boundaries between taxonomy and other disciplines ambiguous sometimes, which, in our opinion, is good for the development of taxonomy. For instance, although ecological investigations usually go beyond the scope of taxonomy, prokaryotic taxonomy is the inevitable basis for ecology with regard to defining the microbial diversity in one kind of unexploited environment via the description of taxonomic positions of prokaryotes detected by any method. While the population genetic study of one bacterial species, either a pathogen or a commensal relative to human beings, not only presents a potential route of microevolution (e.g. Stukenbrock et al., 2011; Takuno et al., 2011), but also provides valuable information for the understanding of speciation (e.g. Luo et al., 2011; Zhang et al., 2010). The relationship of this kind of genetic study with taxonomy might be reflected at the level of speciation, which was usually ignored by traditional taxonomy. Philosophically, there are two elementary substances in the theoretic system that discriminates taxonomy from other subdisciplines of microbiology. One is the element (i.e. species or population) that makes up the system; while the other is the connection, referring in particular to the evolutionary relationships, among the elements. In fact, all taxonomic studies circumfuse this theoretic system, to which all practical areas (nomenclature, identification, and classification) of taxonomy offer their services to solve the scientific questions about these two objects. Therefore, the ultimate aim of prokaryotic taxonomy is to establish a rational and hierarchical system to accommodate the micro-organisms with huge biological diversity, which may facilitate research aiming at answering the above questions. In turn, the results of these studies will provide further information for understanding and improving the system, and prompting other subdisciplines in microbiology.

The present taxonomic system has been organized as a book, dictionary or encyclopedia, such as *Bergey's Manual of Systematic Bacteriology*, 2nd edition (Boone and Castenholz, 2001; Brenner et al., 2005; De Vos et al., 2009; Krieg et al., 2010; Goodfellow et al., 2012), whose framework relies mainly on the phylogeny of the 16S rRNA gene. Up to date, it has successfully managed to include all prokaryotic microbes. However, several issues have arisen to perturb the current system. Firstly, the information included in the traditional taxonomic book is limited due to narrow range of gene information (16S RNA and DNA–DNA hybridization) that was used to construct the phylogenetic framework. Secondly, in many cases, the phenotypes used for describing different taxa are yet to be correlated with their encoding genotypes. Finally, the seeming segregation of phylogeny from their biological traits of taxa has made it more and more complex in description rather than rational in their natural history. Therefore, we believe it is important to emphasize to understand the nature of species and why they exist, not only what the differences are among them. For recognizing a single micro-organism (a population actually), we should move our sight into the cell and observe what happens there and understand why, by using more and more genomic and epigenomic information associated with their ambient environmental conditions. Along with our increasing knowledge learned from phylogenomic analysis of the species via large scale sequencing efforts, more important or critical biological traits (phenotypes) revealed by genomic, functional genomic and/or proteomic analyses and related experimental studies should be involved in the criteria for taxonomy.

For approaching the ultimate goal of prokaryotic taxonomy and making it integrative and comprehensive rather than limited in both the phylogeny and biological knowledge of taxa, we are proposing a web-based open source "Molecular Systematics Library of Prokaryotes" to accommodate all species supported by the backbones of their genomic phylogeny and enriched by their related biological and ecological knowledge. Actually, this Library could have a great impact to the renovation of the current classification system. It recruits more information (genes) from genome, e.g. central metabolic pathway related genes in addition to genes involved in DNA replication, recombination and repair, to establish the backbone for classification, and thus, will be more comprehensive with regarding to the knowledge of biodiversity.

At this moment, although this proposal is rather imaginative than practical due to limitations of both the availability of enough genome sequences targeting most species that are broadly distributed among the current prokaryotic taxa and accurate genotype–phenotype annotations at all levels of biology, ranging from phylogeny to physiology. We recognize that the annotations for genes of genomes currently available often lead to confused or incorrect inferences regarding the role of specific gene products. Therefore, both challenges and opportunities are present for today's prokaryotic taxonomists. First, a genome-scale metabolic network should be reconstructed with respect to the taxonomic, particularly the chemotaxonomic, characteristics of taxa. The genotypes bioinformatically annotated should be verified and translated into taxonomic phenotypes. In addition, when some of the phenotypes cannot be accurately correlated with the genotypes directly, tools of functional genomics should be

employed to study the relationship at the epigenomics level. Second, all shared genes (orthologs) amongst different species under one superior taxon could be used to establish a new framework (a theoretic system), which should be repeatedly verified by additional genome sequences and biological information/knowledge. Finally, with the aid of bioinformatics, systems biology and phylogenomics will continuously enrich our biological and evolutionary knowledge of the subject species, which, to a certain extent, might be transformed to "novel" phenotypes influencing the criteria for taxonomic classification and identification and thus, further enriching or improving the Library. In summary, we strongly believe that accurate annotation of function(s) of gene(s) of the genomes will be one of the major tasks of future "genomic taxonomy". This emerging discipline should attract more bioinformaticians, genomics scientists and microbial geneticists to work closely with the taxonomists to establish the Molecular Systematics Library for Prokaryotes. We also strongly believe that this effort will benefit not only the taxonomy but also the biological research for microbes in general.

Four decades ago, Cowan (1970) said, "Too much attention has been given to nomenclature and too little to the bacteria themselves, their characters, and what they do." Although the situation has been changing ever since, there still is a long distance to the ideal destination. At this moment, we may take advantage of genomics and the genome information it provides to change the status quo of "Too much attention has been given to the difference of microbial characters and too little to what they do either in the cell or in the environment." We believe that prokaryotic taxonomy will truly become an essential discipline of biology again as it was in the dawn of modern science.

Rise of the Phoenix, Born of Fire.

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Intent of the nomenclatural Code and recommendations about naming new species based on genomic sequences

William B. Whitman

The International Code of Nomenclature of Prokaryotes was a major achievement of the last century. By establishing a clear set of rules, it allowed biologists with many different interests and backgrounds to communicate effectively. Furthermore, it made prokaryotic systematics straightforward and accessible. While the Code itself only governs nomenclature and not systematics or taxonomy, many of the minimal standards for descriptions of taxonomic groups proposed by the Ad Hoc Subcommittees far exceed the requirements of the Code and are contrary to both its explicit intent and spirit. They are exclusionary and prevent biologists with serious interests in prokaryotes from describing novel species. The likely consequences of the widespread implementation of these standards are that many biologists will no longer validate the names of newly described prokaryotes and the literature will once again be full of names of uncertain meaning. Within this context, the application of minimal standards for the evaluation of taxonomic descriptions by journals is an editorial decision and not authorized by the Code. With the availability of inexpensive DNA sequencing, prokaryotic species could be routinely described based upon their genomic sequences. These descriptions would fulfil all the requirements of the Code and provide a wealth of insight into the nature of prokaryotes. For these reasons, the genomic sequence should be included in the initial description of novel species whenever practical.

The development of the International Code of Nomenclature of Prokaryotes (formerly the International Code of Nomenclature of Bacteria), hereafter called the Code, was a major achievement in the study of prokaryotes. First published in 1948 (Buchanan et al., 1948), it resulted from a keen appreciation of the importance of nomenclature. By establishing a clear set of rules, it allowed biologists with many different interests and backgrounds to communicate effectively. It made prokaryotic systematics straightforward and accessible to all biologists. One no longer had to be a specialist to characterize and describe new micro-organisms.

Historical background for writing the Code

The fact that nomenclature seems unimportant today is probably a direct consequence of the success of the Code, and many of the problems it was designed to address no longer exist. However, early in the last century the situation was entirely different. The perspective at that time was clearly described by Robert Breed in his presidential address to the Society of American Bacteriologists in 1927 (Breed, 1928). Breed was one of the authors of the original code and served as Chairman of Bergey's Manual Trust from 1937 to 1956. Therefore, he was very knowledgeable about the nomenclature of prokaryotes. He points out that early biologists such as Linné (1774) grouped the prokaryotes in the genus *Chaos*, reflecting the nearly complete ignorance of their properties. Even by the 1900s, the situation was not much improved. Robert Buchanan, also a co-author of the original Code and Chairman of Bergey's Manual Trust from 1957 to1973, stated that "the classification of bacteria is in a chaotic condition" (Buchanan, 1916). At that time, many still argued that prokaryotes did not possess stable characteristics and could not be grouped into species in the same way that plants and animals were classified (Breed, 1928). Rules taken for granted today, such as the "law of priority" were not widely recognized. Key publications were frequently unavailable, either because they were published in unfamiliar languages, not properly cited, or in journals with very limited circulation. The concept of nomenclatural types was not widely applied. Similarly, the descriptions of many prokaryotes were often so vague that they could refer to multiple micro-organisms.

The absence of a nomenclatural system caused these enormous difficulties. For instance, the law of priority associates the name with the first published description of the taxon. Without this law, it is possible to use a name to mean another taxon or create another name for the same taxon, a practice which used to be commonplace. For instance, the

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genus *Bacterium* was proposed multiple times to refer to very different micro-organisms (Breed, 1928). First proposed by Ehrenberg in 1838 to designate various species of rods, subsequent workers were unable to figure out exactly what Ehrenberg described. In 1896, Lehmann and Neumann then used the term for nonsporeforming rods, and in 1900 Migula used it to refer to sporeforming rods. In 1920, Winslow et al. used it for the colon group with *B. coli* as the type and listed as synomyms *Tyrothrix*, *Actinobacter* (in part), *Klebsiella* (in part), *Gliscrobacterium*, *Aerobacter*, *Salmonella*, *Pyobacillus*, and a few others. Given this confusion, the name had little value and was eventually discarded.

The application of priority is possible only when there is consensus as to what constitutes a publication. For this reason, the Bacteriological Code (1990 Revision) (Lapage et al., 1992) explicitly states that a publication for the purposes of nomenclature must be "…generally available, by sale or distribution, to the scientific community, printed material for the purpose of providing a permanent record." (Rule 25a). It also explicitly excludes meeting minutes and abstracts, catalogs of collections, newspaper, and unpublished manuscripts (Rule 25b), practices which were also common prior to institution of the Code.

The requirement for valid publication also follows from the law of priority. Names appearing before 1978 must appear on the Approved Lists of Bacterial Names to be validly published. After that date, names must be published in the *International Journal of Systematic Bacteriology* (IJSB) or (after 1999) the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM) (Rule 27). Validation requires that descriptions of new taxa published outside the IJSEM appear on the Validation Lists in that journal. This rule ensures that the descriptions of new names can be found and not unintentionally used again. At one time, it was common for many universities or regional associations to publish their own journals. By one estimate, there were over 800 publications including papers of interest to nomenclature (Skerman, 1989). This caused enormous problems. For instance, Yasuke Terasaki's superb descriptions of spirilla were published in the *Bulletin of the Suzugamine Women's College* (Terasaki, 1972, 1973). Only an expert on these micro-organisms would likely be aware of these or similar publications outside the mainstream literature. Fortunately, in this case descriptions of his major taxonomic findings were also published in the IJSB (Terasaki, 1979).

Nomenclatural types were originally controversial and only gradually introduced into common practice. This system requires that every name be associated with an example or "type". Breed credits Buchanan (Buchanan, 1917a, 1917b, 1918a, 1918b, 1918c) as the first to apply the concept of nomenclatural type species consistently in the descriptions of genera (Breed, 1928). The 1st edition of *Bergey's Manual* may have been the first comprehensive classification of prokaryotes to designate type species (Bergey et al., 1923). Associating names with types stabilized their usage. Historical descriptions of many taxa were frequently too vague to distinguish similar prokaryotes. As more sophisticated tests and new taxonomic theories were developed, it became clear that the descriptions could be applied to many different species. Now the names remained associated with those prokaryotes similar to the type species. The creation of type culture collections to make type strains widely available followed logically from this idea. Now it was possible to fix species to specific micro-organisms or samples, which further reduced the ambiguity. Type strains were not consistently available prior to the Approved Lists of Bacterial Names in 1980. This list included largely names with nomenclatural type strains, thus removing nomenclatural standing and priority of tens of thousands of names (Sneath and Brenner, 1992). The small number of species without type strains was limited to species with distinctive morphologies or other characteristics, such as *Planctomyces bekefii*, and well known symbionts, such as *Pasteuria ramosa*. Thus, only after this time, was it possible to consistently associate names with actual biological entities, further stabilizing nomenclature.

Although we recognize today that the Code's system of nomenclature was essential for the development of microbiology, its implementation took decades. The formation of the first Nomenclatural Committee for the International Society for Microbiology, which later became the International Union of Microbiological Societies (or IUMS), was first authorized in 1930. The first Code appeared in 1948 (Buchanan et al., 1948). The Approved Lists of Bacterial Names was not completed until 1980 (Skerman et al., 1989). The Code also underwent a major revision in 1990 and has been updated periodically (Lapage et al., 1992). For instance, with the discovery of the *Archaea*, the Code was renamed the International Code of Nomenclature of Prokaryotes, and the committee charged with its implementation was renamed the International Committee on Systematics of Prokaryotes (ICSP) (Labeda, 2000).

Intent of the Code

The Code is comprised of Principles, Rules, and Recommendations (Buchanan et al., 1948; Lapage et al., 1992). The Principles form the basis of the Code. The Rules are designed to implement the Principles and are binding on nomenclature. Thus, names that are not consistent with the

Rules have no standing in the Code and are not protected by the law of priority. The Recommendations deal with subsidiary points and are intended to serve as guides. They are not binding, and names contrary to the Recommendations cannot be rejected for this reason and can have priority. The preamble to the Code also contains some General Considerations, which describe the intention of the Code.

Importantly, the Code is for nomenclature and not systematics or taxonomy. This intent is clearly stated in General Consideration 4: "Rules of nomenclature do not govern the delimitation of taxa nor determine their relations. The Rules are primarily for assessing the correctness of names…" (Lapage et al., 1992). Likewise, Principle 1 states that the essential points in nomenclature are stability of names, rejection of names that cause confusion, and avoidance of the useless creation of names. A fourth point was recently added [Principle 1(4)]: "Nothing in this Code may be construed to restrict the freedom of taxonomic thought or action" (De Vos and Trüper, 2000). Likewise, it is important that Minimal Standards are a Recommendation and not a Rule. Recommendation 30b states that the "examination and description (of a new species) should conform at least to the **minimal standards** (if available) required for the relevant taxon of prokaryotes." However, the purpose of the minimal standards is restricted "to include tests for the establishment of generic identity and for the **diagnosis** of the species, i.e., an indication of the characters which would distinguish species from others." To emphasize this point, Note b was added which states: "It is the aim of minimal standards to provide guidance on the description of taxa for taxonomists seeking such advice. However, these standards are not to be applied in such a way as to contradict Principle 1(4)" (De Vos and Truper, 2000). Thus, they are recommended practices and cannot serve as the basis for not validating a new name. Within this context, the application of minimal standards for the evaluation of taxonomic descriptions by the IJSEM or any other journal is an editorial decision. It is not authorized by the Code, which does not deal with the correctness of names but only the way in which they are formed (Tindall, 1999).

Minimal standards

Unfortunately, the minimal standards for many taxonomic groups far exceed the requirements and intent of the Code. As I know from personal experience, the phenotypic characters included in the minimal standards for methanogens, which I co-authored, is nearly useless in assigning methanogens to genera or distinguishing species. All I can say is that it seemed like a good idea at the time, and I ascribe this failure to the inherent biological properties of these highly specialized lithotrophs, which are extremely difficult to distinguish on phenotypic grounds, and not to a lack of effort by me and my colleagues. More serious are recommendations for standards that require highly specialized and expensive methodologies that are found in only a few laboratories (Tindall et al., 2010). These standards miss the point. Standards should be prepared for the users of systematics, which includes biologists with a wide range of interests, and not just experts in taxonomy. Clinical microbiologists who identify new pathogens must be able to name them. Microbial physiologists who isolate novel micro-organisms with unique metabolic capabilities must be able to name them. Microbial ecologists who isolate micro-organisms important in special habitats or environmental processes must be able to name them. These names are needed to communicate effectively about these micro-organisms and the processes which involve them. The goal of descriptions of new species is to make public to the scientific community the discovery of new organisms. The Code only requires evidence of novelty. Once the rather limited demands of the Code are met, the descriptions should be allowed to include the properties that made that micro-organism interesting to the investigators who discovered them.

Most importantly, in the current system, there are no major consequences of mistakes. Names are not treasures but educated guesses or hypotheses about the nature of the microbial world. They are always open for re-examination on both methodological and theoretical grounds. Because the type strains are now always available, the creation of species which inadvertently duplicate established species (or subjective synonyms) can easily be removed from any taxonomic scheme. This is not sloppy science. Instead, it is the normal process of discovery, where new information is continually re-evaluated.

Minimal standards that are excessive are exclusionary and prevent biologists with serious interests in prokaryotes from describing novel species. They drive scientists out of the field and seriously damage prokaryotic systematics. The likely consequence of their widespread adaption is that many biologists will no longer validate the names of newly described prokaryotes. When that happens, the literature will once again be full of names of uncertain meaning, and the difficult work of several generations of microbial systematists will be undone. Moreover, excessive standards directly contradict the spirit of the Code, which invites participation by all and provides guarantees through the rules of priority that everyone's contributions will be recognized. Again, the process of naming a new taxon should be straightforward and easy.

The role of genomic sequencing

With the availability of inexpensive DNA sequencing, it is now possible to routinely describe prokaryotic species based upon their genomic sequences. Moreover, the Phylogenomic Species Concept provides strong theoretical support for this approach (Staley, 2006, 2009). Such species will meet all the criteria of the Code. Type strains will be uniquely and unambiguously identified, and redundancy of nomenclature will be impossible. Moreover, the genomic sequences will more than satisfy Recommendation 30b for minimal standards. Not only will they establish the generic identity, but they will provide a diagnosis of the species with a precision unimaginable at the time the Code was written. This principle was recognized 25 years ago in the Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics, which stated that "the complete DNA sequence would be the reference standard to determine phylogeny and that phylogeny should determine taxonomy. Furthermore, nomenclature should agree with (and reflect) genomic information." (Wayne et al., 1987). From this perspective, genomic sequencing may be the preferred approach for describing new species in the future. Although our knowledge of species defined by genomic sequence will be different from our current phenotypic systems, it will not be less. Instead of knowing their metabolic properties through enzyme activities and growth substrates, we will know their major metabolic pathways through their gene content. We will know which vitamins and amino acids they can make and which must be provided. We will know their nutrients by identification of transport systems. We will know whether they are oligotrophs, specialists, generalists, or symbionts from their genome complexity. We will know about their quorum-sensing systems, stress responses, and regulatory systems. We will know if they possess gas vesicles, flagella, or pili. We will know if their cell envelopes contain peptidoglycan, outer membranes, and surface layer proteins. Many of our observations will be ambiguous. By determining genes, we are only determining potentials. However, this is not much different from systems based upon phenotypic observations, which are limited by our inability to find appropriate laboratory conditions. Under the very artificial conditions of the laboratory, the behavior of prokaryotes is very different from what happens in the environment. After all, how often are colonies observed outside of the laboratory? Like genomics, phenotypic-based systems provide little direct information about the true nature of prokaryotes. They also require sophisticated interpretation to understand the biological entities being studied. Basing species on genomic sequences is different from what we have done, but it is not wrong and does not contradict the Code.

Prokaryotes are the dominant life form on earth (Whitman, 2009; Whitman et al., 1998). Will we know less about them when a million prokaryotic species are described solely on the basis of their genomic sequences? If we know the genomic sequence of 10,000 of the most common prokaryotic commensals of humans, will we know less about human health, nutrition, and evolution than we do now? If we know the genomic sequence of 10,000 of the most common prokaryotic commensals for each of our domestic animals, will we know less about their health and how to care for them? If we know the genomic sequence of 100,000 of the most common prokaryotes in soil, will we know less about soil fertility, nutrient cycling, and biochemistry? Will we be less able to feed ourselves or less able to create sustainable agricultural systems with less reliance on fertilizers and pesticides? If we know the genomic sequence of a million prokaryotic species that dominate the oceans, the lakes, rivers and streams, the swamps, marshes and bogs, hydrothermal vents, and deep subsurface, will we have less understanding of life's diversity, the evolutionary processes taking place all around us, the history of life and how the biosphere formed, or the roles of prokaryotes in our world? Will we be less able to understand and anticipate climate change? When this happens, will we have fewer antibiotics, fewer anticancer drugs, fewer novel enzymes for biotechnology, and fewer metabolic pathways for synthetic biology?

The widespread application of the Phylogenomic Species Concept will provide vast new insights into the biology of prokaryotes and numerous valuable tools for biotechnology. Thus, recent efforts by the DMSZ and Joint Genomics Institute to sequence the genomes of all the deposited type strains provide a great service to biology (Klenk and Goker, 2010). Additional efforts to include genomic sequences in the initial description of novel species should be encouraged whenever practical. For instance, it would be a relatively simple matter for the large national centers to sequence and annotate genomes upon presentation of the Certification of Availability from at least one international culture collection. This Certification is required for the valid description of all novel species and ensures that the culture will be available to other investigators.

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Professor Dr Dr h.c. mult. Otto Kandler: distinguished botanist and microbiologist

Karl-Heinz Schleifer

It is a great privilege to write a short biography of my PhD supervisor and long-time mentor, Otto Kandler (Figure 1).

Difficult path to a remarkable career

Otto Kandler was born on 23 October 1920 in Deggendorf, Bavaria. His father was a professional gardener. Growing up and helping in his father's market garden, Otto Kandler became interested in plant life and biology in general. He had read about Darwin when he was twelve years old and mentioned it to a catholic priest. The priest punished him with two strikes on his hands with a rod. This memorable incident may have been the reason for Otto Kandler's continuous interest in the origin and evolution of life (Kandler, 1979, 1987).

As a very young man he wanted to follow in the footsteps of his dad but the Second World War interfered with his intentions. He had to join the German army as a radio reporter on the Russian Front. At the end of the war he was in Austria and escaped by bicycle to the Western Front to avoid capture by the Russians. After spending a few months in an American prison camp he was allowed to return home. By then, he was eager to study biology but in the post-war confusion it was difficult for students. Much of the university of Munich had been bombed and the students had to help to remove rubble from the ruins. There were no scholarships available and students had to find other ways to pay for their education. Nevertheless, in 1946 he enrolled in botany, zoology, chemistry, and physics at the Ludwig-Maximilians-University in Munich and financed his studies by growing and selling cabbage and flowers in Deggendorf. He majored in botany and, in order to study metabolism and the effect of auxin, he started to grow plant tissue cultures, at that time a rather new field (Kandler, 1948; 1950). He received his doctor's degree with honors in 1949 (Kandler, 1950a). From 1949 till 1957 he was Assistant Professor of botany at the University in Munich and finished his "Habilitation" in 1953. Plant physiology was his major research topic but – already at that time - he showed some interest in bacteria. In particular, the presence or absence of cell walls in bacteria caught his interest. He convinced a young student, Gertraud Schäfer, to do her doctorate under his supervision on L-form bacteria and pleuro-pneumonia-like organisms (PPLOs). The PPLOs, which are wall-less, penicillin-resistant bacteria, are now classified as mycoplasmas. Gertraud finished her doctorate with great success, and soon became his wife and mother of three daughters. The two of them published cutting-edge papers on the proliferation of PPLOs and L-form bacteria by a budding process (Kandler and Kandler, 1954, 1955; 1956; Kandler et al., 1954). Even now, after more than 50 years, these publications are still of interest for recent research projects (Leaver et al., 2009). Gertraud and Otto Kandler have been happily married for almost 60 years.

Figure 1. Otto Kandler in 1976.

Plant physiology

Otto Kandler was very interested in plant-growth-promoting factors (Kandler, 1952) and photosynthesis (Kandler, 1950b). He presented for the first time experimental

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evidence of photophosphorylation using *Chlorella* cells (Kandler, 1950b, 1954, 1955) and this was the reason he was offered a Rockefeller Fellowship (1956–1957). At that time, radioactively labeled compounds were not available in Germany. Therefore, he decided to join the laboratories of Martin Gibbs and Melvin Calvin in Berkeley, where he was able to use labeled compounds for his experiments. He enjoyed working in California. He told me once that the blue skies and the pleasant ambient temperature were ideal to work in the laboratory. Together with Martin Gibbs he published two important papers on the formation of sugars as subsequent products of the Calvin Cycle (Kandler and Gibbs, 1956; Gibbs and Kandler, 1957). Later on, he and his group studied the biosynthesis and physiological function of plant-specific oligosaccharides such as apiose, hamamelose, stachyose, umbelliferose, selaginose and verbascose (Kandler and Hopf, 1980, 1982). He continued his research on photosynthesis (Tanner et al., 1969; Klob et al., 1972).

At the end of his career he was a fierce opponent of the so-called "Waldsterben" (forest dieback), initiated through a hysterical discussion that started in Germany in the early 1980s (Kandler, 1990). There was an ongoing, quite emotional discussion about the state of the German forest. He had been publically discredited and even personally insulted despite his convincing scientific arguments that forest diebacks had already occurred in former times. Therefore, he was quite pleased that the world-known philosopher Karl Popper supported his critical attitude towards the "green saga of Waldsterben" (Popper's phrase) in an interview with the German magazine *Spiegel* in 1990.

Professional career

Upon his return from the United States he was dissatisfied with the poor laboratory conditions at the university and was glad to find an opportunity to become director of the Bacteriological Institute of the South German Dairy Research Center in Freising-Weihenstephan in 1957. This was actually a brave decision since he had no inkling of dairy microbiology but soon he developed an interest in applied microbiology and biotechnology that he maintained throughout his career.

In 1960, he was appointed Full professor and Head of the Department of Botany of the Technical University Munich and simultaneously maintained his position as director of the Bacteriological Institute in Freising. It was convenient for me since I was living in Freising and did not have to take the train to Munich to do my diploma and doctoral thesis under the supervision of Otto Kandler. The disadvantage, however, was that the only time to meet him and discuss scientific matters was on weekends, mainly on late on Saturday afternoons or Sunday mornings.

Figure 2. Otto and Gertraud Kandler during the conferral ceremony of the honorary doctorate at the Technical University of Munich (1985).

In 1968, he was appointed Full professor and Head of the Department of Botany of the Ludwig-Maximilians University and stayed there until his retirement in 1985. He was an honorary member of the German Society of Hygiene and Microbiology. He was both Dean of the Faculty of General Sciences (TUM 1962) and of the Faculty of Biology (LMU 1973). From 1969 to 1976 he was a member of the Senate of the German Science Foundation (DFG). From 1983 to 1988 he was Chairman of the Scientific Advisory Board of the National Research Center of Biotechnology in Braunschweig. He was also the prime mover for the foundation of the German Collection of Microorganisms and the founder as well as the Editor-in-Chief of *Systematic* and *Applied Microbiology* and served for a long time on the editorial board of Archives of Microbiology and Zeitschrift für Pflanzenphysiologie. He was elected to the German National Academy of Sciences (Leopoldina) in 1970 and to the Bavarian Academy of Sciences in 1982. He received the Bergey Award in 1982 and the Ferdinand Cohn Medal of the German Society of Hygiene and Microbiology (DGHM) in 1989. He received honorary doctoral degrees from the University Ghent (Belgium) in 1981 and from the Technical University Munich in 1985 (Figure 2).

He is an honorary member of the Association of General and Applied Microbiology, the largest microbiological society in Germany. He had several opportunities to move to other positions outside of Bavaria but he never accepted. He

is tightly connected to his homeland and the following quotation may come close to his attitude: *Extra Bavariam nulla vita est, et si est vita, non est ita* (There is no life outside of Bavaria, and if yes, not this one).

Otto Kandler's professional profile creates the impression that his major interests are botany. However, his scientific activities were much broader and certainly had an influence on his teaching activities. He called himself a biologist. Therefore, he taught, not only general botany and plant physiology, but also ecology, food microbiology and bacterial taxonomy (Figure 3). His broad scientific interests can be easily judged from the titles of his 400 publications. The topics range from plant tissue cultures, plant physiology, photosynthesis, plant oligosaccharides, bacterial cell walls, physiology of bacteria, applied microbiology to "Waldsterben" and origin of life and evolution. However, it has to be mentioned that about two thirds of his publications deal with microbiological aspects. Kandler's broad scientific activities are also reflected by the fact that four of his former PhD students or coworkers were appointed as full professors of botany and 13 as microbiologists (H.H. Martin, H.J. Kutzner, R. Plapp, A. Böck, K.H. Schleifer, M. Teuber, K.-O. Stetter, W. Hammes, W. Holzapfel, F. Fiedler, H. König, J. Winter, and R. Hensel).

The chemical composition of bacterial cell walls

Otto Kandler was one of the first scientists who studied the cell wall of bacteria. Already in 1958 he published a paper in *Nature* on the cell-wall composition of *Proteus vulgaris* (Kandler et al., 1958) and of other bacteria (Kandler and Hund, 1959). Later on he investigated the inhibiting effects of penicillin and cycloserine on the biosynthesis of bacterial cell walls (Rau-Hund and Kandler, 1962; Plapp and Kandler, 1965a, 1965b). Subsequent to the design and development of a simple method for the determination of the primary structure of peptidoglycan (Schleifer and Kandler, 1967), most of the currently known peptidoglycan types were described by Kandler's group (see Schleifer and Kandler, 1972). They also studied the exogeneous and endogenous effects on the modification of peptidoglycan (Schleifer et al., 1975) and the biosynthesis of peptidoglycan (Hammes and Kandler, 1976). In 1977, Kandler and Hippe described the lack of peptidoglycan in *Methanosarcina barkeri.* This was the beginning of his interest in archaeal research (see below).

Bacterial physiology

Kandler's interest in plant biochemistry and physiology also had an impact on his bacteriological studies. Already in 1956, he determined the amino acid composition of hydrolysates of bacteria by paper chromatography (Kandler and Zehender, 1956). At the same time, he carried out comparative studies on the metabolism of nucleic acid and respiration of *Proteus vulgaris* and pleuropneumonia-like organisms (Kandler et al., 1956) as well as studies on the physiology of *Caulobacter* (Hund and Kandler, 1956). In 1961, he published in Nature a study on the biosynthesis of acetoin by *Leuconostoc citrovorum.* (Kandler and Busse, 1961). He was also interested in the carbohydrate metabolism of lactic acid bacteria (Weiss et al., 1968, Stetter and Kandler, 1973, Kandler, 1983), cellulomonas (Stackebrandt and Kandler, 1980) and bifidobacteria (Lauer and Kandler, 1976).

Figure 3. Digging up a soil profile for the students.

Taxonomy of bacteria

Otto Kandler had a strong interest in the systematics of plants and especially of bacteria (Kandler and Schleifer, 1980). Already in 1959 he proposed that the amino acid composition of the cell walls of bacteria may be used for the differentiation of bacteria in dairy products (Kandler et al., 1959). Subsequently, he dealt with the classification of staphylococci and lactobacilli (Abo-Elnaga and Kandler, 1965; Kandler, 1967a). He described new species of lactobacilli (Lauer and Kandler, 1980; Weiß et al., 1981; Kandler et al., 1983a, 1983b; Kandler and Kunath, 1983), streptococci (Collins et al., 1983, 1984), cellulomonas (Stackebrandt and Kandler 1979, 1980), acetomicrobia (Soutschek et al., 1984) and bacilli (Scholz et al., 1987). He also made important contributions to the taxonomy of bifidobacteria (Kandler and Lauer, 1974; Lauer and Kandler, 1983). In 1967, he recognized that the amino acid sequence of the peptidoglycan (murein) of the bacterial cell wall is a valuable chemotaxonomic marker (Kandler, 1967b). A little later, the different peptidoglycan types and their taxonomic implication were described in a

review in more detail (Schleifer and Kandler, 1972). By the way, this review is a citation classic and one of the most cited papers in bacteriology (more than 2500 times cited). He also wrote a review on the evolution of the systematics of bacteria (Kandler, 1985a) and coauthored a study on the phylogenetic relationship of the genera *Thermus* and *Deinococcus* (Hensel et al., 1986).

Applied microbiology and biotechnology

Louis Pasteur was one of Kandler's scientific heroes. I still can remember two quotes of Pasteur he mentioned to me at several occasions and which are characteristic of his attitude. "Where observation is concerned, chance favors only the prepared mind" and "there are no such things as *applied sciences*, only applications of science". Therefore, he was always full of ideas to exploit his scientific findings. During his tenure as director of the Bacteriological Institute of the South German Dairy Research Center in Freising-Weihenstephan he focused on dairy microbiology. He studied the shelf-life of milk that was heated under different conditions (Kandler, 1960), designed new methods for checking the quality of raw milk (Kandler, 1961), dealt with failures during the production of yoghurt (Kandler and Frank, 1963), studied the effect of bacteria content on the quality of raw milk (Kandler, 1964a) and the utilization of *Lactobacillus acidophilus* as starter culture for dairy products (Kandler, 1964b) as well as the percentage of L(+)- and D(−)-lactic acid in samples of yoghurt (Kunath and Kandler, 1980). He holds different patents on starter cultures for the production of fermented milk and vegetable (Sauerkraut) products. He also proposed methods for successfully combating micro-organisms in cooling water systems (Kandler, 1966). In 1967, he published a review on the taxonomy and technological importance of lactobacilli (Kandler, 1967). Moreover, he investigated conditions for the killing of bacterial spores at ultra-high temperature (Miller and Kandler, 1967). Later on he conducted research on thermophilic methanogens and their ability to produce methane (biogas) from sewage and other waste. For instance, he investigated the efficiency and stability of methane formation of wastes at mesophilic and thermophilic temperatures (Kandler et al., 1983) and wrote a review on archaebacteria and their biotechnological implications (Kandler, 1985b).

The founder of archaebacterial research in Germany

In my opinion, the launching of archaebacterial research in Germany was probably the most successful and far-reaching decision in his outstanding scientific career. He was the first scientist outside the United States who recognized the future importance of archaebacterial research. Based on his interest in bacterial cell walls (see above), he and his group analyzed the cell walls of extreme halophiles (Steber and Schleifer, 1975) and of *Methanosarcina barkeri* (Kandler and Hippe, 1977). Both organisms lacked peptidoglycan, a unique component of the bacterial cell wall. During this time Marvin Bryant was visiting Kandler in Munich. They had planned a joint research project on the cell-wall composition of other methanogenic bacteria. While Marvin Bryant was sitting in Otto Kandler's office, a letter from Ralph Wolfe arrived reporting from Carl Woese's exciting results on the unique phylogenetic position of methanogenic bacteria within prokaryotes. In 1976, Kandler wrote to Wolfe and mentioned that methanogens and halophiles may be "ancient relics" that have branched off from the bulk of the prokaryotes before peptidoglycan had been "invented". He asked Wolfe to send him lyophilized cells of methanogens for analyzing their cell walls. In January 1977, Kandler attended a Gordon Conference on bacterial cell walls in Santa Monica and visited Ralph Wolfe in Urbana on his way back to Germany. It was the first time Kandler met Carl Woese and learned about his results of the comparative cataloguing of 16S rRNA and their phylogenetic implications. Kandler was deeply impressed. When he returned from the United States he was very excited and told his co-workers that he has met "the Darwin of the 20th century" (Figure 4). He was convinced that research on *Archaebacteria* had a great future and he was successful in persuading the German research organization to support special research projects for this group of organisms. This was the beginning of a success story on archaebacterial research in Germany with many scientists involved such as August Böck, Gerhard Gottschalk, Karl Otto Stetter, Ralf Thauer and Wolfram Zillig, just to name a few.

In contrast to the United States, where archaebacterial funding was quite poor, this kind of research expanded quickly in Germany due to Otto Kandler's strenuous efforts. He organized the first meeting on archaebacteria in Munich in 1978 and also the first international workshop on archaebacteria in 1981. Woese's participation in these meetings was an important impulse for further research on archaebacteria in Germany. Twelve professors from across Germany attended the meetings. Woese was met with fanfare and a brass band when he arrived in Munich. Lectures of these meetings were published as special issues of *Systematic and Applied Microbiology*, a journal that was launched by Kandler in 1977. Woese was so impressed by Kandler's activities and efforts that he wrote to Wolfram Zillig in 1979 "Munich will soon be even more famous as the world capital for archaebacterial research than for its beer" and "a bit of my heart still remains in München" (Sapp, 2009).

Figure 4. Carl Woese, Ralph Wolfe and Otto Kandler (from left to right) relaxing after a hike in the Bavarian alps (1981).

A long-lasting friendship developed between Otto Kandler and Carl Woese. Woese called it a "complementary relationship". A close collaboration evolved. The Urbana group analyzed the 16S rRNA gene sequences of bacteria and archaebacteria, whereas the Munich group studied their cell-wall composition. (Kandler and König, 1978). The chemical structure of the unique pseudomurein was elucidated (König and Kandler, 1979a, 1979b; König et al., 1982) and its biosynthesis investigated (König et al., 1989; Hartmann et al., 1989).

To further improve the collaboration with the Urbana group, Erko Stackebrandt, a former PhD student of Kandler, joined Carl Woese's laboratory in 1978 where he spent a very productive year as a postdoc. Upon his return from Urbana, Stackebrandt got a position as a research associate at the Department of Microbiology at the Technical University of Munich and introduced and improved the16S rRNA technology together with Wolfgang Ludwig.

Otto Kandler, Carl Woese and Wolfram Zillig began a persistent mutual discussion about the relationship among the *Archaebacteria*, *Eubacteria* and *Eucarya*. In particular, Zillig's studies on RNA polymerases supported the fundamental uniqueness of archaebacteria (Zillig et al., 1982). In 1990, Kandler recommended shortening the names of the three groups. After long debates Kandler and Woese agreed to name them *Archaea*, *Bacteria* and *Eucarya.* However, there was still no decision about what to call the taxonomic level. Several proposals were considered, e.g. empire, realm, urkingdom or domain. Finally, they agreed on the term domain (Woese et al., 1990). As mentioned before, Otto Kandler was also very interested in the early evolution of life and in one of his last papers he rejected the existence of a common "first cell" (often referred to as LUCA – last universal common ancestor). He postulated that each of three domains of life had its own progenitor cell which originated from a "multiphenotypical population of pre-cells" (Kandler, 1994).

Résumé

Otto Kandler, Professor Emeritus of Botany at the Ludwig-Maximilians-University Munich is an internationally well-known, highly respected scientist and one of the most outstanding microbiologists in Germany. He had a tremendous enthusiasm for research on a wide variety of different topics, both in microbiology and plant physiology. In microbiology, he is particularly well known for his work on bacterial and archaeal cell walls and their implication on taxonomy and phylogeny. He was the first scientist who recognized the importance of cell-wall composition for the differentiation of *Bacteria* from *Archaea* and initiated the successful funding of research on *Archaea* in Germany. Moreover, he was also interested in the physiology and biochemistry of several groups of bacteria, especially lactic acid bacteria as well as food microbiology and biotechnology such as the optimization of biogas production under mesophilic and thermophilic conditions. He was a dedicated and extremely hard-working scientist and had the ability to fill his students with his enthusiasm for research. Therefore, it is not surprising that many of his former students and co-workers advanced to leading positions in the field of microbiology. Much of Otto Kandler's life was shared with his caring wife and former PhD student, Gertraud, who is equally enthusiastic about science as her husband, and their three beloved daughters. Despite his numerous activities he always had an open ear for his family.

Although he was strong-willed, his concern for fairness, thoroughness, and pride in accomplishment governed his behavior in every personal and professional situation. He had exceptionally high standards of performance and his gift for scientific analysis was more than a match for any problem. He was an extremely acute and reliable critic and reporter of both scientific matters and human affairs. It is not often that one has the opportunity to share the joy of experiences of science with someone who feels the same way. I was fortunate to have had Otto Kandler as a mentor and spiritual father. He is a remarkable individual, and he left his mark not only on science, but on all those who interacted with him.

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My life as a microbiologist and naturalist

Richard W. Castenholz

The early years

This article is focused on events in my life that influenced my career as a microbiologist. Except for a few family references and photographs, the personal aspects of my life are excluded. The recollections are mainly in chronological order.

I turned 80 in May of 2011. The thought of retirement is repellent and saddening, but inevitable. I don't want to sit back and enjoy some sort of invented hobby. My hobby is and will continue to be observing and investigating phototrophic micro-organisms in and from strange and sometimes extreme environments. I have had many other avocations, such as jogging, cross-country skiing, backpack camping, and birding, but recently these have suffered as a result of my twin hip replacements.

But identification and classification of various objects has always been a large part of my interests. These interests began early. When six years old I would sit on our porch or front window and identify the makes of cars going by. Fortunately, it was a quiet suburban street, so this happened only occasionally. I suppose I had some book guide, but I kept a notebook since I was able to spell and read. When I was seven my parents and I went on a trip for several weeks to the west coast by rail (Chicago Northwestern/Union Pacific) in which we had a drawing room with bunks for all of us (probably nothing like this exists today). At that time, seeing mountains, desert, and prairie made me decide that eventually I would live in the West, not Chicago or the Midwest.

I was born in Chicago in 1931 and lived in Oak Park, Illinois (a western suburb of Chicago), with my mother, Lillian, and father, William, through infancy and youth up through the four years in Oak Park/River Forest High School. When I was old enough to walk around the block or the neighborhood, I made a census of dog breeds that I could see in yards. Since this was an upscale suburb, I think that most of the dogs were pure breeds, and therefore easy to identify. During winter of 8th grade I walked over the whole suburb and counted all the robin nests in the barren elm trees that lined the streets. I became a birdwatcher in 1945 and kept a record of what species I saw every day until 1949, a year after I started college at the University of Michigan. I had enlisted four of my friends into bird identification. It is interesting to look over these notebooks, because I also noted who was with me on the weekend trips. Almost all of these friends are deceased.

In high school I was definitely a nerd. One friend (Willy) was as enthusiastic as I, and we competed with members of the Chicago Ornithological Society for the most birds sighted, identified, and verified in 1947. However, 60-year-old-plus Amy G. Baldwin won by three species over my list. Her advantage was that she worked all night as a nurse at St Luke's and had the daytime free every day. She was a humorous and gracious person, and during my college days I came back to Chicago regularly and on a couple of occasions took her to the Chicago Symphony. There were many others in the COS who became weekend friends. It was a bit scary to go to the monthly meetings, since they were in the Field Museum at night with the entry almost dark and the Carl Akeley elephants looming.

Although short-lived, I also decided to identify and list all the native trees and shrubs in one of the major forest preserves on the west side of the Chicago suburbs, using a rather detailed book. I was unable to recruit friends for this endeavor, since the summer was very hot and humid.

Another extracurricular activity during the first or second year of high school was a weekly meeting that three of my friends and I held for at least 6 months. This was a "biology club" in which each of us had responsibility for a group of animal phyla or botanical divisions. Each of us would give a "report" or description and initiate a discussion for each animal or botanical group. Although it sounds serious each evening was quite entertaining, and I remember the argument as to who should have the responsibility for "sea squirts".

Because of my acquired expertise in bird identification, I was invited by distant cousins Margaret and Morey to spend the summers of 1947 and 1948 at their place on the Little Spokane River, north of Spokane, mainly to teach them how to identify the birds of the area. We became very close until their deaths about 50 years later and I remain so with their son Bart and his wife Lindell.

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In addition to the bird-watching activity, I had some great teachers in high school: Mr Price, who taught ancient history and looked in profile like one of the Egyptian pharaohs; Miss Hardie, who stimulated my reading of Joseph Conrad and James Fenimore Cooper as well as the required Dickens and others; Miss Muir, who brought Latin to life by her descriptions and slides of Rome where she visited every summer; and Miss Turner, who taught a year of physical geography and geology. Then there was my friend, Mr McMenamin, who taught a whole year of botany. During that year I had already developed an interest in algae and aquatic plants, and was asked by him to illustrate the algal part of his lab manual, which I did.

The interest in natural history developed along with bird watching from my weeks in northern Wisconsin during many summers when I was a teenager. I was invited there (near Sayner) by my father's first cousin Amy and her German husband Louis (a former forester and retired electrical engineer). They were childless and more or less adopted me. They owned 25 acres at the end of the road at Plum Lake, right across from Wilmotte's Island with its tall pines and a large house with a siding of pine bark. Louis was a remarkable man who taught me the art of silently following porcupines, bears, and other animals downwind. He also knew the local birds and we went deer spotting almost every evening. We fished together and every evening he sat with a cigar that he rolled slowly in his mouth as we conversed about life and science. However, it was at this time that I started swimming underwater with a face mask and became fascinated by algae and aquatic plants, an interest that continued throughout my life. As a result, I gave up fishing forever.

The undergraduate years

High school was an interesting time for me in several ways. It was a period of great indecision. Although I was greatly interested in birds and their habits, I thought that the only way to make a living using this skill was to go into the Fish and Wildlife Service. Therefore, my first year at Michigan was as a wildlife management major that was then in the School of Forestry. This meant summer at the university forestry camp with forest mensuration, logging practice, engineering drawing, and other subjects of little interest to me. However, by the end of the first year at Michigan I had made drastic changes and dropped out of forestry school. I had a full year of botany by two great teachers: F.K. Sparrow taught for the introductory semester – it was fascinating how he presented what could have been a very dull course. The second semester was by Elzada Clover, who taught Systematic Botany. She was the first woman to raft the Colorado River through the Grand Canyon. Her enthusiasm convinced me that I should major in botany and make a living by teaching and doing research in that field, even though at the time teaching was a terrifying prospect. This anxiety was somewhat relieved by my oral presentation as a sophomore in an evening seminar in an ornithology course.

Dr Sparrow allowed me, as a sophomore, into his graduate course on aquatic flowering plants in which I thrived, and later into a course on "aquatic fungi". William Randolph Taylor taught a course in algae and lower plants as well as one in freshwater algae. Although he was unofficially voted the most boring lecturer in the Botany Department, I found the subject and his great knowledge of the subject compelling. Not only did I take these courses, but I signed up for undergraduate research with Taylor in order to learn the marine algae of the north Pacific where I was expected to go with the university expedition to the Aleutians. My responsibility was for collecting and preserving the algae. I was included in this expedition by professor Harley Bartlett ("Uncle Harley") who frequently involved undergrads in research. Unfortunately, the Korean War began that summer and the Navy declared the Aleutians off-limits. Consequently I spent a second summer working for the US Forest Service from a pack camp north of Priest River, Idaho, pulling *Ribes* (currants and gooseberries) in a white pine blister control effort. The only reading I had with me was G.M. Smith's *Cryptogamic Botany*, *Vol. I (Algae* and *Fungi)*, which I went over several times. Nowadays I prefer good fiction. Later, during the Korean War I joined the Naval Air Reserve, but was never activated nor advanced above "seaman recruit".

I spent the next summer (1951) at the Michigan biological station at Douglas Lake, and there I reached my true depth in the study of algae, taking two courses on freshwater algae from Gilbert Morgan Smith, who had recently retired from Stanford. In his advanced class with only about six students he ended the day with martinis served in his cottage for all students who wished to partake. His wife was the most active spouse at the station, and she taught swimming for all the children of faculty and students. I believe Smith took a particular liking to me, for after the session, it turned out that he was staying on Plum Lake at a sister's cottage, and I was across the lake at my father's cousin, so I was invited to row over and have cocktails with him and family. Later in the summer I drove with my friend Willy to Mexico, where we identified many new birds, and on the way back via California we stopped at the Smith home in Palo Alto (we had been invited) and had a home-cooked dinner with him and his wife. During that visit he phoned George Papenfuss at Berkeley by way of introduction and the next day I was shown around the herbarium and department by Papenfuss.

In a later year, at one of my early national meetings, G.M. Smith introduced me to several prominent phycologists as a promising young scientist in the field. His death in 1959 was a sad event.

The Graduate School years

After graduating from Michigan in 1952, I was "sent" by Taylor as an MS student to the University of Miami Marine Station (at this time still in Coral Gables) in order to learn marine algae of this area with the intention of coming back to work with Taylor for a PhD a year later. At Miami I was on a fellowship that involved my creating a marine algal herbarium for the institute based almost entirely on my own collections. Besides taking courses in marine ecology, phycology, and oceanography, I had to do a large number of collecting trips on my own in order to service the new herbarium. Every 2–3 weeks I took part in a research trip into the Gulf Stream to study the vertical migration of the "deep scattering layer" (various invertebrates) over diel periods as part of the research program of Dr Hilary Moore. As part of his marine ecology course I did a study on the effects of tidal emergence and submergence times on the growth of the red alga *Bostrychia* sp. on mangrove roots. I "camped" out several weekends for this study, in the then undeveloped Bear Cut on Biscayne Bay, cooking lamb's kidneys and veggies over open fires.

During the winter break, I hitch-hiked from Miami to Marathon, Texas, to meet a fellow phycologist (Vernon Proctor) who was working on his PhD at the University of Missouri. We then got a ride to the Chisos Mountains in Big Bend National Park, camping and collecting algae from ephemeral ponds. We became friends in 1949 when we both worked for Kaniksu National Forest in northern Idaho, and tented at Pelke Camp. Clem Wallace was a very kind and introspective camp boss, and he taught everyone who wished to know all the plants of the forest. On the way to Texas, I had various adventures, the worst being waking up in my sleeping bag next to a large coiled-up diamond back rattlesnake in the woods outside of Chipley, Florida.

Instead of returning to Michigan where I had already been accepted as a grad student, I decided to try elsewhere for a PhD. I was accepted at USC and British Columbia with financial aid, but realized this would mean working on marine algal taxonomy with Dawson or Scagel, respectively, so I decided to accept a teaching fellowship at Washington State University at Pullman, hoping to work on some aspect of freshwater algal ecology. There was another reason, however. I wanted to be close to my "adopted parents" on the Little Spokane. Although there was no one at Washington

Figure 1. Richard Castenholz with mother, Lillian, and father, William, at time of Dick's receiving PhD at Washington State University, Pullman 1957.

State who currently did research in my field, Noe Higinbotham was very knowledgeable and took me on as his PhD student. I will never regret working with him. He took great interest in my research and gave excellent advice. He and his wife became part of my life and that of my first wife, Margit Hollerud, whom I met in Pullman and married in 1954. My work for the degree was a study of the seasonal productivity of sessile diatoms in a series of lakes in the Lower Grand Coulee, ranging from freshwater to saline/meromictic at the end of the 20-mile canyon. This was later published in *Limnology and Oceanography* (e.g. Castenholz, 1960) and favorably described in G.E. Hutchinson's *Treatise in Limnology*, Vol. 3 (1975), published by John Wiley & Sons. Since diatom taxonomy required the expertise of specialists, I spent most of the summer of 1956 at the Academy of Natural Sciences of Philadelphia checking my identifications with Ruth Patrick, then the world expert on diatom taxonomy. At WSU there were several excellent teachers. They included Bob Hungate (microbiologist *par excellence*), Rexford Daubenmire (outstanding plant ecologist), Adolph Hecht, and Marion Ownby. Ownby taught a full year of advanced systematic botany in which all orders of plants worldwide were considered.

At the time of my defense in 1957, there were few post-doc opportunities (Figure 1). Although I applied for a Fulbright Scholarship to work with Heinrichs Skuja in Sweden, the few other opportunities in my field were two temporary aca-

demic positions in the West, and a couple of jobs at state colleges in California, who at that time stated that time for research was not allowed. Therefore, I was extremely fortunate in finding an opening at the University of Oregon, an opening caused by the move of phycologist Al Bernatowitz from Oregon to Hawaii. I was hired as a tenure track instructor that was followed a year later as an assistant professor. My wife and I had visited Oregon a year earlier invited by a friend whom I knew at Michigan, Jake Straus. I am sure that Jake's presence at Oregon influenced the decision to hire me when the field had been narrowed to two. In fact I knew Jake when I was an undergraduate glassware washer at Michigan for his PhD project under Carl D. LaRue, plant ecologist and physiologist from whom I had an excellent course. LaRue, after having had a heart attack still led field trips in his plant ecology class, but was carried by students on a homemade sedan chair.

The late 1950s and the 1960s at Oregon

Thus, in fall of 1957, began the first and only permanent job in my life, a faculty position in the Department of Biology, University of Oregon, that has now lasted 54 years. My teaching was intensive during the first couple of years: courses in general biology, mycology, phycology, and plant morphology. My colleagues then were Jake Straus, Sandy Tepfer, Ralph Huestis, Clarence Clancy, Frank Sipe, Jim Kezer, Bradley Scheer, Arnold Shotwell, Bob Morris, Peter Frank, Leroy Detling, and Bayard McConaughey, the entire biology faculty (all deceased). In 1959 the Institute of Molecular Biology was formed by Aaron Novick and later that year Frank Stahl was added.

By this time I was convinced that studying micro-organisms, whether or not they had medical or commercial importance, was as legitimate as studying any organisms that shared the earth with us. My point of view from then on was that of the micro-organisms themselves and how they managed to survive and thrive in their environments.

In the summer of 1958, I taught the phycology course at O.I.M.B. (Oregon Institute of Marine Biology) in Charleston, Oregon. All told during my first year I earned $5500, a sum that enabled life with an apartment rental. A year later, we bought a house on an acre of land for $10,500 and had our first child, a boy named Reave Lars. In 1963 we adopted a boy born that year, Ralph Alex. During subsequent summers at O.I.M.B. through 1962 I taught the algal part of an NSF institute in marine biology for college teachers who had had no marine experience, while Cadet Hand or Eugene Kozloff in different years did the animal portion (Figure 2).

My research during these early years at Oregon was on the

Figure 2. Richard Castenholz with plankton sample on boat off the Oregon coast, 1960.

ecology of epilithic intertidal marine diatoms and the effect of gastropod grazers on the establishment and maintenance of these diatom biofilms (e.g. Castenholz, 1961). Another study established the effect of directional faces and timing of tides on the epilithic diatom species and their abundance.

In 1959 or 1960, I travelled to Hunter's Hot Springs north of Lakeview, Oregon, and observed the distinct and abrupt zonation of cyanobacteria (then called blue-green algae) associated with the temperature gradient. I was fascinated by this striking picture and thought that these cyanobacteria must be very difficult to culture, since I could not find much in the literature. However, I found that isolation and culturing was relatively easy. Thus, my first PhD student, Jack Peary, began and finished his research on the cyanobacteria of Hunter's Hot Springs. The original small stakes that he placed to measure distances from the source of "Jack's Stream" still exist (i.e. in 2011), in a stream that ranged from ~93°C to about 35°C. At this time or shortly thereafter I shifted in part from marine epilithic diatioms to the physiology and ecology of thermophilic cyanobacteria. It becomes difficult to describe the proliferation of research that occurred after this shift. Tom Brock had already established that ~73°C was the upper temperature limit for cyanobacteria in Yellowstone alkaline springs, and the same limit stood for Hunter's Hot Springs and later, eastern Asia (in all cases, a unicellular *Synechococcus* sp.). PhD student, Jack Meeks, showed in one culture isolate that the growth range of this *Synechococcus* was from 55°C to ~72°C, thus, it was an obligate thermophile (Meeks and Castenholz, 1971).

Later, it was shown that 60–64°C was the upper limit for *Synechococcus* and other cyanobacteria in Iceland, New Zealand, and Europe, simply because the high-temperature species of *Synechococcus* did not exist in those locations (Castenholz, 1969, 1978, 1996). In fact, no thermophilic

Figure 3. Richard Castenholz with mother, Lillian, grandmother, Bertha Wandrey, and Reave, 7 years, and Ralph, 3 years, at the Northern Pacific train depot in Livingston, Montana, 1966.

Figure 4. Richard Castenholz with older son, Reave, and younger son, Ralph, at Big Lake, Oregon, 1967.

species of *Synechococcus* has ever been found in Iceland springs, perhaps because of the relatively recent emergence of the land surface from under a total ice cap. In Hunter's Hot Springs, Peary showed with cultures that there were at least four thermotypes of *Synechococcus*, indistinguishable by morphology that overlapped in temperature range and optima (Peary and Castenholz, 1964). Later, these identical (or similar thermotypes) and the high temperature form were shown by a 16S rDNA-based phylogeny to have evolved from moderate temperature types (Miller and Castenholz, 2000).

In 1959 I participated in the International Limnological Congress in Vienna, my first international meeting. Compared with present-day conferences this one was magnificent. It ran for nearly a week in Vienna. One evening, in a nearby small village, new wine was available gratis at all the cellars. One day there was a field trip to Lake Balaton and to the Esterhazy Palace in Eisenstadt to listen to a Haydn quartet in the venue where Haydn composed and exercised his orchestra. In addition there was a 2-day trip through the lakes of the Salzkammergut with the final sessions of the congress in Salzburg. My presentation was still on diatoms, and during this meeting I met some of the great limnologists and diatomologists.

The work with diatoms continued during my first sabbatical leave in 1963–1964 at the Biologisk Statjon at Espegrend, near Bergen, Norway. There I measured the seasonal periodicity of sessile marine diatoms (Castenholz, 1967). At this time I was well indoctrinated in the taxonomy of diatoms, an "art" learned well from Ruth Patrick in Philadelphia. The venture into diatom ecology on the Norwegian coast was my last for diatoms. Shortly after, in 1965, I was promoted to associate professor with tenure. During these early years my research was supported by NSF, much later by NASA as well.

In 1966, I made my first hot spring exploratory trip to Yellowstone National Park (Figures 3 and 4). There I met Tom Brock who had already begun his extensive work. Together we explored many hot springs, unofficially naming some. Because of his height and great walking pace I had to run sometimes to keep up with him. It was not until 1968 that I seriously began culture isolations and temperature measurements in Yellowstone, together with three PhD students under my direction (Bev Pierson, Jack Meeks, and Larry Halfen) plus two undergrads and one dog (Tiny) and camping gear, all in one standard station wagon.

Late 1960s and the 1970s

I had switched entirely after 1964 to a study of the community ecology and physiological ecology of photosynthetic bacteria of hot springs, primarily cyanobacteria, but also of anoxygenic phototrophic bacteria. These studies eventually resulted in over 30 publications involving the ecology, physiology, and behavior of organisms of Hunter's Hot Springs (e.g. Castenholz, 1969; Ward and Castenholz, 2000; Ward et al., 2012).

Late in the summer of 1969 I flew to Switzerland and presented an annotated list of the cultured cyanobacteria in my collection at a meeting at the limnological station at Kastanienbaum on Lake Lucerne. The host was the remarkable Otto Jaag who translated all the talks in French and German into English for the benefit of Brian Whitton, T.V. Desikachary, and myself. The US moon landing was announced from the cockpit on the outward flight over the Atlantic.

The year 1970 was very eventful. First, there was an international meeting on cyanobacteria in Madras, India hosted by T.V. Desikachary. Many new colleagues were met as well as others already known (e.g. Tom Brock, Brian Whitton). This allowed me to travel and collect from hot springs in the northern state of Himachal Pradesh, hosted by a colleague in Chandigahr. On the return I stopped in Japan, had a privately chauffeured trip by the father of a student in Oregon, covering many hot springs from which several collections and isolates were obtained, including the type genus and species of *Chloroflexus aurantiacus* Pierson and Castenholz.

The year also included a research trip to Yellowstone with students, and a NASA-sponsored 2-week trip by 4-wheel bus and plane to almost all volcanic and thermal spring sites in Iceland, including an intimate view of Mt Hekla erupting. The participants included volcanologists, geochemists, and microbiologists. They also included Tom Brock, and two of my current and past PhD students, Conrad Wickstrom and Richard Sheridan, respectively. Many collections and isolates were made, including some from the recently emergent volcanic island of Surtsey. One of the great pleasures was bathing in warm water in one of the lava fissures near Myvatn.

By 1969, I had reached full professorship and in 1970 was awarded a Guggenheim Fellowship that I used during my second sabbatical, one that took me to the University of Auckland, New Zealand, with Valentine Chapman as my host. My family and I took the slow route to New Zealand, traveling by ship (the *Orsova*), and it enabled me to complete a research paper during the voyage. It was an especially enjoyable trip. From Auckland I frequently visited, the hot spring area of Rotorua, Waimangu, and elsewhere, and much research was accomplished, including the demonstration in the field of sulfide-dependent anoxygenic photosynthesis in a cyanobacterium, a process demonstrated about the same time in a culture by Yehuda Cohen in Israel (e.g. Castenholz, 1976, 1977). In research completed in Norway during a later sabbatical in 1977–1978, the same cyanobacteria from New Zealand were shown in culture to photosynthesize anoxygenically (e.g. Castenholz and Utkilen 1984)

Figure 5. Richard Castenholz, sampling on edge of 60°C, pH 3 Waimangu "Cauldron", New Zealand, 1971.

(Figure 5). The return from New Zealand involved a stop in Norway, then in Iceland where more collecting was done. Eventually I arrived at the Marine Biological Lab, Woods Hole, Massachusetts, where I was one of the instructors in the microbial ecology course, founded that summer by Holger Jannasch. I learned much from Holger and Ralph Wolfe, not least, how to sail, in Holger's catboat. Upon returning to Eugene, I and a couple of colleagues bought a 15 foot open sailboat, and I have sailed ever since, in larger and larger boats, currently a 25 ft Seaward, but only a few times in the open sea. On one unfortunate 7-day/night sailing from Oregon to Vancouver Island the only food the three of us had was chocolate (candy, pudding, cake, etc.)! The reason for this situation is too complex to explain.

The 1970s continued with research on sulfide, but one of the main events was the discovery of *Chloroflexus* and its charcterization by PhD, student Bev Pierson and myself (e.g. Pierson and Castenholz, 1971, 1974a, 1974b). This opened up an entirely new category of anoxygenic phototrophic bacteria that did not fit the mold of any previously described group. This eventually led to the discovery, mainly by other researchers, of other genera and species in this group that is now referred to as the phylum *Chloroflexi* (Boone et al.,

2001). The orange-colored undermat in most alkaline hot springs (up to about 70°C) were thought previously to be heterotrophic "flexibacteria", but only when Pierson recorded an absorption spectrum that went well over 700 nm did we discover that bacteriochlorophyll *c* (~740 nm max) and bacteriochlorophyll *a* (803 and 865 nm max) were present and also that photoheterotrophy was the principal mode of metabolism in *Chloroflexus*.

Other work during the 1970s included a more detailed description of the species pattern in Hunter's Hot Springs along the thermal gradient, and the characterization of thermal ranges of an undescribed thermophilic ostracod, an animal that established by grazing the lower border of *Oscillatoria* and *Chloroflexus* at about 47–48°C, which is also the upper limit for this ostracod that forms dense populations below this temperature. The result was the establishment of ostracod-resistant/ostracod-dependent populations of two other cyanobacterial species below 47°C (e.g. Wickstrom and Castenholz, 1973, 1985).

In 1977–78 I took a third sabbatical, supported in part by a Fulbright award, at the University of Oslo, Blindern, hosted by John Ormerod. There I worked primarily with Hans Utkilen (a PhD student of John's) on the use of sulfide by a species of cyanobacteria isolated earlier from New Zealand. During the summer of 1977 I travelled to Israel for research and a meeting, and in 1978 also gave talks at the Dahlem Conference in Berlin, at Dundee (W.D.P. Stewart, host), and in Göttingen (Norbert Pfennig, host).

During the early 1970s Larry Halfen worked on motility and its mechanism in the gliding cyanobacterium *Oscillatoria princeps*, and came to a description of the mechanism not far from a current theory as proposed by at least one other investigator, but differing greatly from a hypothesis of another group (e.g. Halfen and Castenholz, 1971; Castenholz, 1982).

The 1980s

In the 1980s I branched into a broad variety of research subjects. These included a study of hypersaline cyanobacterial mats in the area of Guerrero Negro, Mexico (Javor and Castenholz 1981), a demonstration of the diel vertical migration of the cyanobacterium, *Oscillatoria terebriformis*, in Hunter's Hot Springs and elsewhere (e.g. Castenholz, 1968; Richardson and Castenholz, 1987). Stephen Giovannoni, a PhD student in my lab, worked on a variety of projects before he finished (Giovannoni et al., 1987b; Pierson et al., 1985), but focused on *Isosphaera pallida* for his thesis. *Isosphaera* (*ex Isocystis*), a common inhabitant of moderate temperatures in numerous hot springs in North America and elsewhere, originally thought to be a cyanobacterium, was shown by Stephen to be an aerobic heterotroph of the *Planctomycetes* with some unusual properties such as gas vesicles and phototaxis (e.g. Giovannoni et al., 1987a).

I spent the summers of 1982 and 1983 teaching in the Microbial Diversity course at MBL, Woods Hole, under the direction of Harlan Halvorson. It was there, working on projects with Tom Schmidt and Ken Noll (students then) that I realized the importance for survival of light-regulated vertical migration of cyanobacteria in mats. This led to several years of research in this field. In 1985 and again in 1989 I spent several weeks at the University of Aarhus, Denmark, with Niels Peter Revsbech and Bo Jørgensen, first to learn how to make and use O_2 microelectrodes and then to look at vertical migrations of cyanobacteria in benthic mats in the Limfjord. In 1986, 4 years after my marriage to Phyllis Larson, I went again to New Zealand on a sabbatical leave, this time to the Taupo Research Lab at the invitation of Warwick Vincent. Phyllis and I travelled extensively, but mainly to the Rotorua region. During this stay I studied the "behavior", including vertical migration, of *Oscillatoria* cf. *boryana* in a sulfide-rich tepid pond. The results led to the conclusion that sulfide-dependent anoxygenic photosynthesis took place in the early hours of light, but was replaced by a shift to oxygenic photosynthesis with increasing light intensity during the day (Castenholz et al., 1991).

During the New Zealand stay I also discovered a sulfide-rich warm stream that harbored a thick mat of thermophilic *Chlorobium*, i.e. a population that occurred up to 57°C (Castenholz et al., 1990). This bacterium was later isolated by Mike Madigan and described by Wahlund et al. (1990), and is now known as *Chlorobaculum tepidum*, a completely sequenced, highly used model for the green sulfur bacteria.

It was in 1985, that Bill Schopf came to Eugene and invited me to become a member of the Precambrian-Paleobiology Research Group (PPRG). As a consequence I was in New Zealand a second time in 1986 with a number of the members of that group, including Dave Des Marais, Bo Jørgensen, John Bauld, Bob Horodyski, and Dave Ward. A large compilation of work done by most members of the PPRG was published as The Proterozoic Biosphere, a Multidisciplinary Study (Schopf and Klein, 1992)

In Oregon, PhD student Doug Nelson worked out the heterotrophic metabolism and behavior of *Beggiatoa* in Hunter's Hot Springs (Nelson and Castenholz, 1981a, 1981b, 1982), and PhD student Toivo Kallas determined in the lab that thermophilic cyanobacteria of the genus *Synechococ-*

Figure 6. Camp at Bratina Island, Antarctica, January 1990. From left to right: Malcolm Downes, Richard Castenholz, and Warwick Vincent.

cus did not attain an optimum pH for growth below about 7, although strains occurred in and were isolated from thermal waters as low as pH 4.5 (e.g. Kallas and Castenholz, 1982). In addition, during the 1980s PhD student Walter Dodds completed a study of a cold-water (4–5°C), nitrogen-limited spring pool that maintained a large population of a huge colony, syctonemin-containing *Nostoc* that was shown to be dependent on a snail species that "cleaned" the colony surface by grazing on "epi-*Nostoc*" diatoms (e.g. Dodds and Castenholz, 1988).

At the end of the 1980s Warwick Vincent invited me to join him for a season in Antarctica (1989–1990) with the New Zealand Antarctic Program. Thus, in December of 1989 I arrived at Scott Base via Christchurch, just in time for a riotous and rather lewd New Year's Eve party. On New Year's day dozens of nude male and female Kiwis dunked through a seal hole into the −2°C seawater and survived. No photos were allowed, but somehow a large number appeared in the Scott Base bar the next day. We were a party of three: Malcolm Downes, Warwick, and myself (Figure 6). After my survival training we helicoptered to Bratina Island on the edge of the Ross (McMurdo) Ice Shelf. We were camped there for most of 6 weeks, but with a 3-day collecting and research trip to Cape Royds (with access to the Shackleton

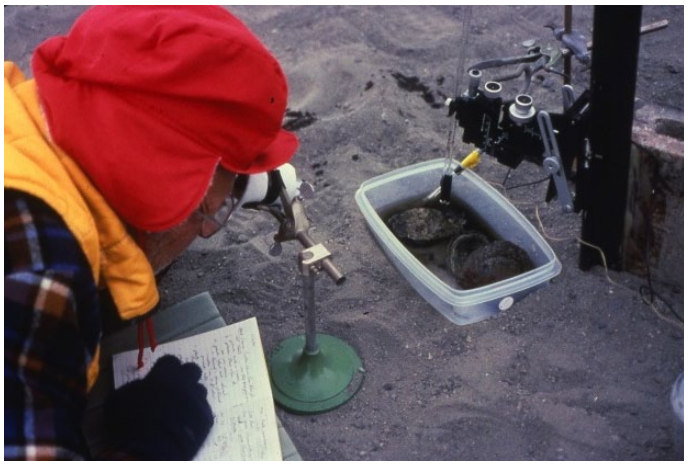

Figure 7. Richard Castenholz measuring O_2 profile in cyanobacterial mat with microelectrode, Bratina Island, Antarctica, January 1990.

hut) and later to the dry valleys, particularly the Taylor and Victoria Valleys. At Bratina we mapped out a route of a few hours over the melt ponds of the ablation moraine of the ice shelf, distinguishing each pond by chemical characteristics and type of cyanobacterial mat that dominated. We named the ponds at that time, but Castenholz Pond eventually became shortened to "Casten Pond". It isn't permanent, in any case, since, as part of the ice shelf, it will eventually end up in the Ross Sea, long after my life span. Not only were cultures established, but photosynthetic measurements and oxygen profiles were made in many of the mats (e.g. Vincent et al., 1993). For the many weeks of camping in Scott Polar tents at Bratina Island we were issued double sleeping bags, enormous coats and warm hats, all of which I found too warm. I was overheated during most of the Antarctic adventure (Figure 7).

During the 1980s I had become an associate member of the Bergey's Manual Trust, and in 1989 the 3rd volume of the *Bergey's Manual of Systematic Bacteriology* was published and this volume covered the cyanobacteria for which my contribution was substantial (Staley et al., 1989).

The 1990s

In the early 1990s PhD student Ferran Garcia-Pichel and I began a long-term project on UV-protection in the cyanobacteria, first with the characterization and protective function of the extra-cellular sheath pigment scytonemin that absorbs mainly in the UVA and UVC spectral regions (e.g. Garcia-Pichel and Castenholz, 1991; Garcia-Pichel, 1992). Later, after the structure of scytonmein was determined (Proteau et al., 1993) work continued with the somewhat dubious function of cytoplasmic mycosporine-like amino

acids (e.g. Garcia-Pichel et al., 1993) (Figure 8). We and others have shown the key role of scytonemin in increasing fitness under conditions of high solar irradiance, especially high UV (e.g. Castenholz and Garcia-Pichel, 2000, 2012). Several papers followed, and later in the 2000s the role of other environmental factors besides UVA in the regulation of scytonemin synthesis in cyanobacteria were revealed by PhD students Jesse Dillon and Erich Fleming (e.g. Dillon and Castenholz, 2003; Fleming and Castenholz, 2007, 2008). During the late 1990s PhD students Jesse Dillon and Scott Miller and MS students Stephan Brenowitz and Chris Wingard conducted photosynthetic studies of cyanobacterial mats in Yellowstone in response to UV radiation and the presence or absence of scytonemin (e.g. Brenowitz and Castenholz, 1997; Dillon et al., 2003).

Besides the investigation of UV-protective compounds, work in the late 1990s showed the detrimental effect of UV radiation in the absence of protective shielding pigments. Scott Miller (PhD student) showed this negative effect in thermophilic *Synechococcus* under field conditions (Miller et al., 1998), and Claudia Kruschel (a researcher from Germany) and Ferran Garcia-Pichel showed the lethal or inhibitory effect of full light and UV on filamentous cyanobacteria that failed to escape by downward migration the high daytime solar radiation in soft microbial mats of hypersaline waters near Guerrero Negro, Mexico (Garcia-Pichel et al., 1994; Kruschel and Castenholz, 1998). Later, this beneficial downward migration was also demonstrated in soft mats of a cold saline pond in Antarctica in 1998, but movement was at a much slower rate (e.g. Nadeau et al., 1999).

Figure 8. Richard Castenholz lifting scytonemin-rich *Lyngbya* mat from intertidal mud flat, Laguna Guerrero Negro, Baja California Sur, Mexico, in the 1990s.

In 1991 I attended my first Bergey's Manual Trust meeting in Prague as a full member. I remained a full member until the compulsory retirement age of 70 in 2001. However, in that year the first volume of the 2nd edition of *Bergey's Manual of Systematic Bacteriology* was published, in which again I contributed a large portion the cyanobacterial section along with several colleagues. Much of it was written or edited during my 5-month stay at the Institut Pasteur in late 1999 and early 2000 with Rosi Rippka and Mike Herdman (Boone et al., 2001). In 2005 I was awarded the Bergey Medal for Distinguished Achievement in Bacterial Taxonomy (Figure 9).

As a result of another invitation I returned to Antarctica, Scott Base, and Bratina Island in the 1997–1998 season. At this time Clive Howard-Williams was my host, again with the New Zealand program. The organization was also able to support the trip of my PhD student, Tracie-Lynn Nadeau, as an assistant. Again, we camped at Bratina Island in tents, but with the addition of a closed outhouse, since a female was camping there for the first time. The results of this research trip was the demonstration, as mentioned above, of vertical migration of *Oscillatoria* in Salt Pond (Ross Ice Shelf), a case where only the lowest light or artificial darkness would bring this subsurface population to the surface (Nadeau et al., 1999). The rates of photosynthesis with respect to temperature and inhibitory UV radiation were also measured in the field and later as cultures in the lab (Nadeau and Castenholz, 1999). One of the surprises was that almost all strains of cyanobacteria from the ponds photosynthesized and grew at maximal rates at 20°C or higher in the field and culture and all very poorly at temperatures normally encountered in nature (i.e. 4°C to rarely10°C). It was very difficult to keep the baths heated in the field experiments; water had to be boiled continuously in the cooking hut before running out and pouring it into the outdoor water baths. The cultures still remain at 12°C and at −80°C in the CCMEE culture collection at Oregon.

My culture collection of cyanobacteria and micro-algae had been established over many years from many locations and habitats, including a large number of isolates from hot springs over much of the globe, from hypersaline waters, endolithic habitats and from Antarctic fresh waters. With the addition of the collection of extreme habitat phototrophs from E. Imre Friedmann in 1999 over 2000 strains are now being maintained in the lab. [The UO Culture Collection of Microorganisms from Extreme Environments (CCMEE): http://cultures.uoregon.edu.]

Figure 9. Participants and spouses at Bergey's Manual Trust meeting on a field trip at Octopus Spring, Yellowstone National Park, 1995. Standing (left–right): Rita Colwell, Joseph Tully, Karl Schleifer, Jan Ursing, Brita Ursing, Noel Krieg, John Holt, Joan Sneath, Marion Murray, Peter Sneath, Gerti Schleifer, Sonja Staley, and Jim Staley; kneeling (left–right): Stan Williams, Robert Murray, Elaine Krichevsky, Micah Krichevsky, and Richard Castenholz. David Boone took the photo.

In the 1990s and 2000s I also became involved in the controversy on what constitutes a species in the cyanobacteria, and how should they be named (i.e. by the International Code of Botanical Nomenclature or the International Code of Nomenclature of Bacteria). I do not wish to go into this now. I realize I am in disagreement with many others, including Aaron Oren and Jiri Komárek. In short, I will be happy for some years to come with simply giving numbers to species or strains of cyanobacteria (e.g. *Synechocystis* PCC 6803), but using morphologically defined names of genera (e.g. Castenholz and Norris, 2005).

The 2000s

In the summer of 2000 I was appointed the first "Visiting Scholar" of the Thermal Biology Institute, Montana State University, Bozeman. During that time I gave a series of ten lectures on hot spring biology and the history of microbial research in Yellowstone. In addition, as was expected, I conducted research, mainly with a post-doc of Tim McDermott's, Tracy Norris. It was a long-term project that showed that cyanobacterial mats in the 45–50°C range that were several weeks or months under UV-shielding filters did not select for opportunistic UV-sensitive cyanobacteria, although this result may have been a consequence of the lack of inoculum of novel thermophilic species in the environment. PCR and denaturing gradient gel electrophoresis (DGGE) was used to be nearly certain of the absence of new species or strains, using 16S rDNA sequences (Norris et al., 2002). I worked out of Bozeman again in the summer of 2001 in a follow-up to this project and the initiation of another project on acidophilic algae.

In 2002, Tracy Norris, having finished her post-doc at Montana State, came to Eugene to teach in microbiology and begin a post-doc under my direction as an NRC Associate. Her choice of research was one in which I had an earlier interest, i.e. of phototrophs in the old travertine (limestone) spring deposits of the Mammoth terraces in Yellowstone and across the Yellowstone River above Gardiner, Montana. Some deposits were from a few thousand to several hundred thousand years ago (Terrace Mountain). Most sites had bands of endolithic cyanobacteria and microalgae one to a few mm below the rock surface, a habitat well-known in sandstones in the dry valleys of Antarctica and several other substrates in various global locations. A large number of these endolithic phototrophs were isolated and cultured. None from the ancient terraces were similar (using 16S rDNA sequences) to the cyanobacteria in the active hot springs (Norris and Castenholz, 2006). These endolithic phototrophs resulted from secondary inoculation and invasion, and were shown to be highly tolerant of desiccation

Figure 10. Richard Castenholz at Nakabusa Hot Springs, Japan, in 2003 at the site where Satoshi Hanada isolated anoxygenic *Roseiflexus castenholzii*, a new genus and species of the *Chloroflexi*.

Figure 11. Former PhD students of Richard Castenholz at meeting of Phototrophic Prokaryotes, Pau, France, 2006. From left to right: Erich Fleming, Ferran Garcia-Pichel, Jack Meeks, Richard Castenholz, Bev Pierson, and Toivo Kallas.

and freezing, both conditions prevalent in Yellowstone. Emily Knowles (MS student) showed that the presence of extracellular polysaccharides from endolithic cyanobacteria could enhance the desiccation tolerance of other endolithic phototrophs that lacked EPS. It was obvious (and later confirmed by others) that the endolithic niche provides protection from high light intensity (and UV radiation), protection from grazing and wind abrasion, but it is likely that growth is very slow in this confined environment even when moist and with probable nutrient limitation (Figures 10 and 11).

In the summer of 2001 Tracy Norris and I began to isolate and culture "cyanidia" (a general term for the three described genera of the rhodophytan order, Cyanidiales), namely *Cyanidium*, *Galdieria*, and *Cyanidioschyzon*. These unicellular algae are known to occur only in acid waters (pH 0–4) and usually at temperatures from about 35°C to 56°C. They constitute the only know phototrophs in such conditions. Their physiology, ecology, and biogeography constitute the subject of most of my current research. During this decade cultures of "cyanidia" were isolated from many sites in Japan (2003), New Zealand (2005 and 2007) and Iceland (2006) and many strains from Yellowstone acidic environments that include streams, acidic thermal soils, rims of steam vents (fumaroles), and narrow bands in moist siliceous rocks in acidic areas (i.e. endoliths). The results amassed by MS student Julie Toplin showed, using the nuclear 18S rDNA and the chloroplastic rbcL gene sequences, that only two genotypes of cyanidia occurred in the Yellowstone collections, one close to *Galdieria sulphuraria* and the most abundant strain nearly identical to *Cyanidioschyzon merolae.* However, it was apparent that this most abundant type had no morphological resemblance to *Cyanidioschyzon merolae* (Toplin et al., 2008). Later phenotypic and genetic evidence suggested that the abundant type from Yellowstone should probably be referred to a new species of the genus *Galdieria* with many "ecotypes". Among several strains differences have been shown in tolerance to arsenite (As III), arsenate (As V), aluminium, mercury, and other potentially toxic substances common in some of the natural acidic environments of Yellowstone. This work is in progress with MS students Christie Lowell and Chelsey Iida. However, the gene sequences of 18S rDNA and *rbcL* were sufficiently different to distinguish many of the strains from Japan, New Zealand, Philippines, and Iceland from those of Yellowstone and from each other (Toplin et al., 2008; Castenholz and McDermott, 2010). Other work in collaboration with Tim McDermott (Professor, Montana State University), Corinne Lehr (former Montana State University post-doc), and Dana Skorupa (PhD, Montana State University) has shown that UV radiation is probably an ecotype-determining environmental factor, and that during summer daytime periods, UV radiation has an inhibitory effect on photosynthesis (Lehr et al., 2007).

Epilogue

One of the main research questions I have asked throughout my career is: how do various micro-organisms tolerate and adapt to environmental extremes? These extremes include high and low temperature, low pH, high salinity and desiccation, freezing, high solar irradiance (especially UV), toxic

levels of sulfide and other potentially poisonous compounds. Another interest throughout my studies was the degree to which endemism occurs among hot spring micro-organisms. The subject of endemism in microbial distributions has been somewhat controversial for many years, but recently many positive examples have been put forward. Since hot spring clusters (especially thermal acidic clusters) appear in many ways as islands often separated by large regional or continental distances it should be expected that geographic endemism should appear in these habitats, if anywhere, especially since difficulties in long-distance dissemination exist for many of these thermophiles. It has now been shown by others and by results of some of my work that endemism exists among thermophiles, at least at continental distances (e.g. Castenholz, 1996; Miller et al., 2007; Ward and Castenholz, 2000; Ward et al., 2012; Toplin et al., 2008) (Figures 12, 13, 14 and 15).

In January 2008 I received an award presented at the NSF RCN/TBI conference, at Mammoth Hot Springs, Yellowstone National Park, "in appreciation for seminal contributions to research in the geothermal systems of Yellowstone National Park". Later in 2009 I received the American Society for Microbiology USFCC/J. Roger Porter Award for studies of microbial diversity. In 1996 I was elected a fellow of the American Academy of Microbiology, and in the 1970s I became a Fellow of the American Association for the Advancement of Science (AAAS).

During my career, entirely at the University of Oregon, I have taught several subjects, including microbiology, freshwater biology, plant morphology and portions of general and core biology. Graduate/senior level courses have included phycology, photosynthetic prokaryotes (with Bill Sistrom), life in extreme environments, and freshwater ecology. The

Figure 12. Richard Castenholz at Octopus Spring, Yellowstone National Park, July 2008.

Figure 13. Richard Castenholz's Freshwater Ecology class at Hunter's Hot Springs, Oregon, in May 2008, plus Professor Brendan Bohannan center in back.

last-named course has been offered in most alternate years since the 1960s and has always been my favorite. In spring 2011 I had one of the best-prepared, interested, and congenial groups of 12 students that I can remember. The Saturday field trips in which the whole class is launched in rafts or boats have sampled physical, chemical, and biological features of freshwater coastal lakes, a stratified mesotrophic lake, an alpine oligotrophic lake, a naturally hyper-eutrophic lake, a saline soda lake, a perennial cold spring pond, and an alkaline hot spring, the last four locations as part of an overnight camping trip to eastern Oregon. Although emphasis these days is on molecular biology, exposure to natural habitats and their organisms is still appreciated by many students.

During all of these years I have been proud to be part of the Biology Department at the University of Oregon and the more recently formed Institute of Ecology and Evolution as well. All members of the faculty, including those I commonly interacted with, for over 50 years have been helpful and congenial, a comment that few faculty members of any institution can make. I have had especially productive and enjoyable collaborations with Michelle Wood and the late Bill Sistrom. I have mentored 21 PhD students, most of whom have tenured academic positions, although a few are retired or deceased. They have been friends as well as students. There have also been many MS students, two of whom are still active in my lab.

I wish to thank the NSF for almost continuous support and also, for a portion of my career, the NASA Astrobiology Program.

Figure 14. Richard Castenholz at Norris Geyser Basin, Yellowstone National Park, in July 2010 with students, left to right: Tyler Roberts, Ashley Pacelli, and Christie Lowell.

Figure 15. Richard Castenholz at Mushroom Spring, Yellowstone National Park in September 2011.

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A little kid from Far Rockaway grows up

Sydney M. Finegold

I was born 12 August 1921 in St Joseph's Hospital, Far Rockaway, a beach community of New York City. We moved to Los Angeles just before my 12th birthday, having lived in New York and New Jersey in the interim. I don't remember much about my life before L.A. except that I enjoyed playing stickball and Kick the Can. My Dad and older brother, Harold, went to L.A. about a year before my Mom, my sister, Pearl,and I joined them. My Dad found some odd jobs and was able to save enough to pay for our trip by Greyhound bus. My Dad was a pharmacist, but lost his drugstore in the Great Depression. He went to L.A., the "land of milk and honey", hoping to find a job. California, at that time, made the Pharmacy examination tough to keep pharmacists from other states from passing it and taking jobs away from California pharmacists.

My Dad was born in Russia, as was my Mom. My Dad had become a Rabbi in Russia, but his heart was not really in it and he gave it up. He worked in a sweat shop for $2 a week in New York, but managed to study and get admitted to the pharmacy school at Columbia University. He did well and was automatically admitted to medical school at Columbia but turned it down because it had been very hard for him financially during his earlier school years and he was trying to save enough money to bring his family over from Russia, which he ultimately did. I never appreciated it earlier in my life but I eventually came to realize that he was a terrific role model. My mother was a role model as well. She was a pharmacist in Russia but never pursued that vocation here. She was a beautiful woman, very warm and friendly, a great cook, and just a good all-around person. When times were really bad for us, she loaded up a couple of valises with notions (needles, thread, tissues, etc.) and walked from door to door selling what she could. It was very tough work and demeaning, but she never complained. My brother, Harold, was warm and friendly also, never could find work that interested him and didn't want to study, but he would give you the shirt off his back. He was a good left-handed tennis player with a hop serve that never bounced the way you expected it to. He died of asthma at age 70. My sister, Pearl,

a beautiful young woman, was a dietitian (UCLA graduate) who died tragically at the age of 23 in an auto–train wreck where there was no grade crossing or gates. She was on her way to work at a local hospital with a colleague driving. She was my closest friend at that time. I was away at medical school when this happened. I cried openly for hours when I heard about it, much more than I had ever cried all my life. Pearl was a lefty also and a very good tennis player.I shared my family's hard times, of course. I had various odd jobs throughout junior high school and high school – helping my father in his gardening jobs, and working for a chemist who manufactured various food items in his garage. I made 10 cents an hour and it really made me feel good. The food coloring he made was not exciting but his faux maple syrup and food seasoning (very much like Lawry's salt) were delicious. Despite such snacks, I was a very skinny little kid. My parents eventually went on relief, with great reluctance. My siblings and I were pleased. We got a large salt pork roast which my parents refused to eat but we loved it. And I got

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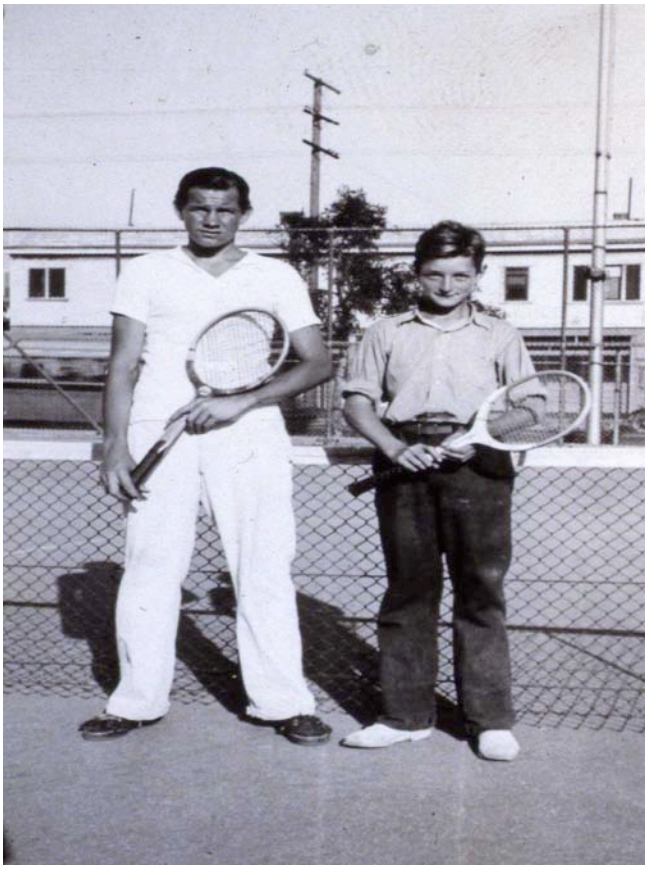
Sydney Finegold's parents, Sam and Jennie, in their 30s.

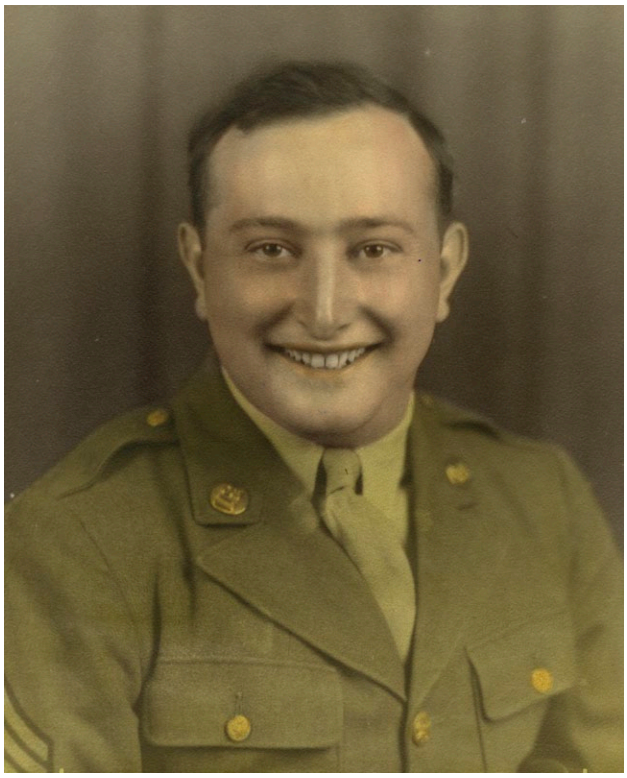
Sydney Finegold's sister, Pearl, in 1944, aged 20.

a brand new pair of corduroys; they were a weird orange color, but I was delighted with them. Nevertheless, my parents decided we should struggle along without help from the relief program after just a week or so on it. I remember some unusual incidents from my high school days. In the first, I encountered a man who asked if I would like to join his church. When I saw the really nice-looking gray uniform I would get, I said, "Sure". When I got home with my uniform, my parents quickly (but nicely) squashed that idea. Another incident came up after my parents had a major argument. My mother (uncharacteristically) tried to point out that my father was a bad man by showing me a newspaper clipping about him serving jail time for beating up a bus driver. When I asked my dad about it, he told me that the bus driver was bugging him because of his Jewish accent. My mom's idea backfired because I was really proud of my dad for what he did. A third incident came up in connection with a kids' basketball team I played on for the local Temple (my only affiliation with the Temple was this kids' club with lots of sports! I am pretty much secular). I didn't get to play much basketball because I was the shortest kid in the club, bur one day we were supposed to play a club in Long Beach and no one had access to a car. When they asked me if I had a car, I told them I could borrow my dad's flatbed truck—an old Star model with a pull-out ignition switch instead of a key. I had a driver's license even though I was only 13 because in those days if you had to help drive the family around you could get licensed. I didn't tell my dad that I was doing this. We just piled all the kids on the flat bed in back and took off. As we drove through the city of Bell, we were pulled over by a motorcycle policeman. I hadn't remembered to take my driver's license and the truck had no registration slip in the glove compartment. So they hauled us into jail. When I was asked if I had my dad's permission to take the truck, I said, "Of course". Well they called my dad and, bless his heart, he said yes when asked about letting me use the truck. They made him come out to pick us up and to bring his registration certificate with him. So, we spent 2 hours or so in jail and never got to Long Beach to play the game. Later, at home, I got a lecture about how dangerous it was to carry a bunch of young kids on the flatbed of the truck with nothing to hold on to.

I was only 4ft, 10 1/2 in tall when I entered high school, but gained exactly one foot in height during my 3 years there. The highlight of my high school period was playing on the tennis team and earning my letter two of the three years I played. My parents had bought me a tennis racket for my 13th birthday, a very big day for Jewish boys. We were very poor still at that point, and I'm sure they skimped on many a meal to be able to pay for the racket. I still regard this as the very best present I have received in my entire life. As I learned more about tennis, I realized that that racket was a pretty cheap one. But I loved it dearly and it got me into tennis head over heels. I also realized later that my folks took careful note of my spending weekends at the nearby



Sydney Finegold (right) with high school tennis doubles partner Frank Hite.



Sydney Finegold's brother, Harold, in 1941.

Exposition Park where Pancho Gonzales was playing as a teenager and beating all the adults there. I never had had lessons but I watched Pancho and others playing and picked up a lot about the game that way. (This became a way of life for me; I realized that I could learn a lot by just watching, and listening to, people.)

I had always heard a lot about UCLA and dreamed of going there someday. I applied to only that one school and luckily was admitted. I passed the admittance English exam, which most people did not; that boosted my confidence considerably and saved me from needing to take remedial English instead of something more interesting. I met with a counselor soon after I started; I was asked what I planned to do with my life. I told her that I didn't know, but I loved science and teaching (and also medicine, but that was out of the question because I would never be able to afford it). She said there was a big need for botany teachers and that should fill the bill nicely for me. I was dubious about her choice, but agreed to it. I hated it from the beginning, although I have a few good memories. The entire class loved to joke about the department chair whose first name was Florabelle and who always wore a dark green smock; we called her "chlorophyll". In my second year, I took a course in general bacteriology, as part of the Botany major. I fell in love with it immediately and promptly changed my major to bacteriology. I was in heaven and for the first time in my life I began to study.

As a freshman, I had a part-time job in an experimental horticulture orchard. I had no idea what research was, I am ashamed to say, but the oranges were very large and delicious and I was still a starving kid, so I kept eating them until they caught me one day and fired me. I dearly hope that I didn't ruin too many experiments. When I became a bacteriology major, we made our own media and I soon learned that beef extract was delicious on crackers, or even by itself. And, talk about luck (which has followed me throughout my life), I was invited to take a part-time job testing the swimming pool water for coliform counts, etc., in the Girls' Gym!! I stayed out of school for 1 year after my freshman year to help with my finances. I lived at home and there was no tuition (just an Incidental Fee of $18.50 per year for a gym locker, towels, football tickets, etc.), but I was ashamed of the few clothes I had and needed snack money, movie money, etc. I earned $65 a month. I bought my first suit (second-hand) and my first watch [also second-hand (pun not intended)], but with a green crystal), and a lot of second-hand furniture for my parents. I took philosophy, which was a very stimulating subject, and psychology both semesters

in the UCLA Extension Division in the evenings during that year so that I would keep my tie-in with learning and be sure to return to school after 1 year, which I did. Later, at UCLA, I got a really classy job typing manuscripts for a famous chemistry professor, Max Dunn. It was an NYA (National Youth Administration) job and paid 25 cents an hour (big time stuff)! I played tennis at UCLA; I felt that I was an ordinary player, but I tried hard (especially against USC) and earned my frosh numerals and two varsity letters. One of the more exciting things to happen to me in my life was when I went up to the Athletic Office to pick up my frosh numerals (42, for 1942, when I would have graduated if I had not stayed out to work that 1 year). As I came out of the office, grinning from ear to ear, whom should I meet but Jackie Robinson, one of the finest athletes and finest persons to ever live. He not only lettered every year in all four sports in which he participated (baseball, football, basketball, and track) but he was one of each team's star players; he went on to break the color line in major league baseball. He beamed at me and said, "Hey, that's terrific, kid. Congratulations!". I was so excited I almost fell down the stairs. I certainly got some perspective after that chance encounter.

Of interest is the fact that I heard very little about anaerobic bacteria or anaerobic infections even though I was a bacteriology major at a first-class university. We didn't see (or smell) them in any of the labs. and they were discussed only in terms of the major clostridial infections or intoxications (tetanus, botulism, and gas gangrene), and briefly at that. Theodore Beckwith (chair) and Meridian Ball were inspiring teachers and they, and the program generally for bacteriology majors, made me know that I made the right decision to adopt that major.

I was lucky enough to win a $100 California State scholarship one year; that was a real godsend and made a huge difference in my life.

I joined the Marine Corps reserve in order to stay in school and avoid being drafted into the military. I knew about the legendary feats of the U.S. Marines but didn't really have a clue as to how they fit into things and exactly what they did. After finishing boot camp (in which I worked my head off, and gained some 20 pounds of muscle), I transferred to the Navy because I had learned that the Navy provided medical services for the Marines. My first assignment in the Navy was as a Navy Corpsman and I was put in the clinical laboratory of the US Naval Hospital in Long Beach, CA. There I met an inspiring pathologist, Leon Rosove, MD (whose son, Michael, is an outstanding hematologist on the medical faculty at UCLA). Leon Rosove did all the autopsies and I watched him do the dissections on virtually all of them, and soaked up his discussions of the disease and the cause of death. In the absence of an Infectious Disease physician (the specialty was not established for another 15 years or so), Dr Rosove, a real role model, was the Attending Physician for three medical wards (a pneumonia ward, a rheumatic fever ward, and a ward with patients with miscellaneous infections) in addition to his pathology duties. This was 1943 and 1944 and World War II was ongoing. Penicillin had just been discovered and virtually all of it was reserved for the military. I had what I considered a very prestigious job; I was to collect all the urine from all patients treated with penicillin in order that the drug might be recovered for use in additional patients (the manufacturing process was still not up to speed). I had pneumonia in 1943 (type unknown) and had failed therapy with sulfonamides. They started me on penicillin at what would now be considered pediatric dosage – 20,000 units every 8 hours. And they would check on me after each dose because they were not yet sure what side effects might be encountered. I responded dramatically. There was another case that intrigued me – a young dental officer who had a ruptured appendix and then developed gas gangrene of the abdominal wall. All of the medical officers conferred on this patient and agreed the prognosis was probably hopeless, but they clearly needed to drain an associated abscess and debride all necrotic tissue. They hoped that with penicillin, they might possibly save him. I knew this young man because he played tennis (he was Captain of the team at UCLA). He made what everyone considered a miraculous recovery. I heard about him playing in a big tennis tournament just a year or two ago and felt very happy about it.

I was fortunate to be appointed to the Navy Independent Duty School in Norfolk, Virginia. This was a 3-month course, quite intensive, on all aspects of medicine, other than major surgery, that might be encountered in a battle such as a beach assault or other situations in the military when a physician was not available. I thoroughly enjoyed this program and it was to prove very useful to me later. Following this, I was reassigned to the Naval Hospital in Long Beach, California.

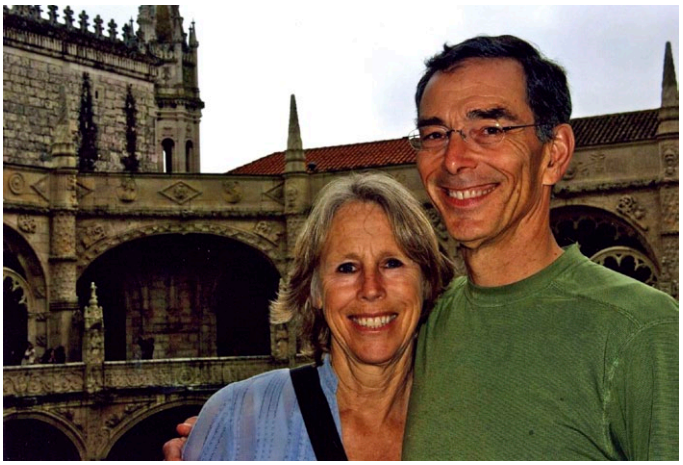
I was happy to see a notice on the bulletin board one day that the Navy was going to select candidates for medical school through the V-12 program and requesting seamen who were interested and qualified to apply. I was not a pre-med major in college but my bacteriology major did qualify me, luckily. We had to list our top three choices and mine were Cal, Stanford, and Wisconsin (which had, and still has, an excellent microbiology program). Eventually I heard that I was selected and should await orders to proceed to Galveston, Texas. However, when I received military orders not long after that they were to serve in lieu of a physician on an LST

Sydney and Mary Finegold with their two eldest children, Joe and Pat, in 1952.

(Landing Ship Tank). I was crushed to lose the opportunity of medical school, but the following day those orders were superseded by orders for medical school! Once again, my luck held out.

I knew nothing about Galveston beforehand, but I found the city, the state and the people wonderful (except for the climate – oppressive humidity). It is where I met my wife and mother of my three children, Mary Louise Saunders, a beautiful and most wonderful person, in my freshman class. We enjoyed discussing our classes and projects, and our jobs. Mary was as poor as I was and we both worked while going through med school. Luckily, I had the so-called GI Bill because of my prior military service and we could get by OK with our two small part-time jobs. Mary worked for Dr Morris Pollard doing mouse studies of poliomyelitis and I worked for Dr Edgar Poth, a surgeon who had introduced the so-called intestinal sulfonamides for preoperative preparation of the bowel prior to colonic surgery. Mary and I married between semesters of our second year and had a 1-weekend honeymoon in Houston.

Our first child, Joe, was born in Galveston near the end of our internship there at the US Marine Hospital (a hospital for merchant seamen). Joe's middle name is Gregory in honor of Raymond Gregory, then Chair of Internal Medicine and a most inspiring internist and teacher. Joe attended UCLA,



Sid's son Joe with wife, Kim.

and obtained a Master's degree in automotive engineering, and built the first working hydrogen car. He subsequently decided on a career in business and obtained an MBA (with honors) from Harvard. He and his wife Kim, a nurse practitioner, live near me – in Santa Barbara. Their two children, Chris and Brie, graduated from the University of California, San Diego. Brie has worked for various non-profit companies providing assistance to downtrodden people, mostly in developing countries and has travelled and lived extensively in Africa and Latin America. She was a good athlete and could take her old Saab car apart and put it back together again! Joe had taught her how, of course. Chris just obtained his MBA from the University of California, Berkeley, and is now working for Google. At UC San Diego, he was an Academic All American water polo player and the star of the team.

Dr Poth was an accomplished surgeon and something of an engineer. He had pecan trees in his backyard and invented a machine to automatically shell them with whole nuts as the end product. The apparatus he designed was a moving belt on which pecans were automatically "fed" one at a time with the same exact distance between the nuts. At one point, as they moved along, a little drill came down and drilled a small hole in the shell. Further down the line, acetylene gas was injected into this hole and finally a spark was directed at the hole and the shell blew off leaving an undamaged pecan. He designed and built a similar assembly apparatus for automatically feeding dogs meatballs, containing one or another drug he was testing, to the dogs every 4 or 6 hours. The apparatus consisted of a number of cages in two rows, with one dog per cage. Each cage had a metal tube with holes on each side so that he could place a series of medicated meatballs in the tubes, each meatball separated from the next one in the tube by a metal pin that was connected to a chain that moved at just the right speed to drop one

Sid with granddaughter Brie, daughter Pat, and her husband, Michael.

meatball into each cage at exactly the proper interval (4 or 6 hours). An animal technician would insert a polished hollow glass tube equipped with a suction bulb into the rectum of the dogs to obtain fecal specimens for microbiologic studies (my job). We used a variety of media designed to select out particular groups of bacteria. On the whole, the media were appropriate and organisms recovered on the plate media were identified and counted. The one flaw in the setup was the one used for recovery of anaerobic bacteria. Dr Poth used Brewer plates with the lid dipping down into the bottom of the dish to minimize the air space above the Brewer thioglycollate agar that was used. It was not recognized that most so-called aerobes in humans are facultative and grow at least as well anaerobically (or better) than aerobically and there was no setup for subculture in different atmospheres. Anything that grew on the Brewer plates was called an anaerobe (and not further classified). (I recognized the flaw in later years when I began to work on anaerobes and bowel flora in serious fashion.)

While in medical school, I decided it would be important to study what impact antimicrobial agents had on the bowel flora. My wife and I and another classmate all took various antimicrobials and provided stool specimens for study. There were no IRBs in those days and no one cleared projects or followed them for problems. Luckily, we didn't encounter any side effects of note and finally reported our results in the *Texas Reports on Biology and Medicine*. Even today, the FDA still does not insist that pharmaceutical companies dealing with drugs that are not absorbed after oral administration provide pertinent pharmacologic and microbiologic data.

I talked to various of my medical school professors about what opportunities there might be in academic medicine that would permit me to use my microbiology training. They all said that there were no specific disciplines for that background except in microbiology *per se*, but that I should find someone who was doing research on microbes and join that team for a residency in internal medicine. I had heard of Louis Weinstein in Boston and liked what I had read of his papers so I made an appointment to meet with him, using most of our meager savings to make that trip. When I got there, I found out that Dr Weinstein was busy and could not see me, but sent one of his Fellows to meet with me. The Fellow was very nice, but I was really put off to not even get to meet Weinstein, so I dropped that program from my list. Next I went to see Dr Wesley Spink at the University of Minnesota and his younger colleague, Dr Wendell Hall at the Minneapolis Veterans Affairs (VA Hospital). They were both friendly people, clearly very bright and productive, and most enthusiastic. These were qualities I had found important in my young career. So, I ended up accepting a position there, driving up there from Galveston when Joe was 3 weeks old. I worked primarily at the VA where Dr Spink made rounds with Dr Hall in a program that was a medical residency, but they used the words "infectious diseases" and made rounds once or twice weekly on patients with these entities. So, in essence, it was a combined program although it was not labeled as such. My first year there was purely clinical, with both internal medicine and some infectious disease problems. At the beginning of my second year, I was offered a position as head of infectious diseases at the Minneapolis General Hospital, a county facility with a contagious disease unit where patients with that type of infectious diseases were isolated and which received patients from around the city. I was given the Faculty title of Instructor of Medicine and was given a lab with a technician and a dishwasher, both of whom earned more than I did! I did not have a clue as to how I should go about setting up my laboratory. Dr Burton (Bud) Waisbren, who preceded me at "the General" very kindly spent much of the weekend before he left with me, teaching me something about contagious diseases, how to set up a lab, and what might be an interesting focus for my first venture on my own into formal research (antibiotic susceptibility testing is what he recommended). I would have been totally lost if Bud had not interrupted his packing, etc., to coach me as much as he could for the good part of that weekend.

My Minneapolis experience was notable in three respects in particular. First, our daughter Patricia (Pat) was born there in 1951; secondly, we had a major outbreak of poliomyelitis in Minneapolis that year with many bulbar cases; and

thirdly, I saw an interesting case of pleural empyema that was to affect my subsequent career.

Pat was, and still is, a pure delight—warm and friendly and very bright. She is currently Professor of French at Dalhousie University in Halifax (and was Departmental Chair on two occasions and has won several University honors). Pat and her husband, Michael, a Professor of Canadian History at Dalhousie, have two children also. Misty obtained a Master's degree at the University of Toronto and became an expert archivist. She is currently archiving important historical documents at a museum in Winnipeg. Andy has not yet settled on a career, but has shown excellent talent in the fields of music (playing the cello and electric guitar in a band that he organized) and in theater arts. He obtained a Canadian government grant to write and perform a play that was well received in Montreal where he now lives.

The polio cases from all over the city were referred to our Contagious Disease Unit at Minneapolis General Hospital. At that time "iron lungs" were used for patients with respiratory paralysis; they overflowed the rooms and crowded all the hallways. Now, of course, we can use the small bedside respirators for such cases and care of that type of patient is much simpler as a result. If the bulbar polio patient required a tracheotomy to permit easier and safer handling of secretions, it was very difficult to make the required connection to the iron lung (this was before intubation was used except for the operating room and it would not have been suitable for long-term airway management anyway).

The case of empyema was a puzzle. Frank pus that was foul-smelling was obtained on thoracentesis of the pleural space. There were numerous polymorphonuclear leukocytes on Gram stain, but no recognizable bacterial forms; there were bizarre Gram-negative staining pleomorphic forms. I presented the case to our monthly statewide Infectious Diseases Seminar and no one in the audience knew what was going on in the patient, who was clearly quite ill. The attendees at the conference were from the State Health Department, the University of Minnesota infectious disease specialists (not yet called that as it still was not an established specialty) and microbiologists, and miscellaneous hospitals, including the Minneapolis General Hospital. Finally, since no one could offer any plausible diagnoses or plan of action, one of my fellow residents in internal medicine timidly suggested that maybe it was an anaerobic infection. Earlier, he had been a student at Johns Hopkins School of Medicine. He said he remembered one of the faculty there talking about how difficult anaerobic infections were to diagnose on occasion and that they often produced a foul odor and the organisms might be difficult to recognize on Gram stain and difficult to grow. No one knew how to respond to these comments.

Mary and Sydney Finegold some time in the 1960s.

I should also comment on the Minneapolis weather. The winters were tough, especially since I bought a house, from a departing trainee, which was on a corner (therefore, twice as much snow to shovel) and which had a driveway that went down steeply to our garage. It was on a street that the snow plow visited regularly and every time it went by we had a huge accumulation of snow in the driveway!

Soon after I started my third year in Minneapolis, I was called back into military service because I had no active duty after going to medical school (for one semester under the Navy V-12 program) even though I had over 2 years of active duty before I received that appointment. This time I was allowed to transfer to the Army where I was a Regimental Surgeon (this called for a rank of Major, but all ranks were frozen so I went in as a First Lieutenant). I was with the 34th Infantry Regiment in the Second Infantry Division. After training at Fort Sam Houston in San Antonio, Texas, where Mary's family lived, my regiment was shipped to Gotemba, Japan, at the foot of Mt Fuji which I could see in all its splendor from the window of my barracks. I was Commander of the Medical Company of the 34th Regiment. I set up a physical training program and an educational program to prepare for service in the field in Korea as a backup to another Infantry unit that was already there. Although we did not have many patients, I set up a military field hospital to have that experience. There were only three interesting patients during the time we were in Japan – a young boy who was brought in by his father after drowning in a small body of water near their home (unfortunately, he was dead on arrival and we were unable to resuscitate him); a patient who had renal shutdown after being given a bottle of distilled water intra-

venously instead of normal saline (we air-evacuated him to a larger facility where he made an uneventful recovery); and me in whom I diagnosed German measles! Another interesting experience I had was driving military jeeps and a two-ton truck on the opposite side of the road from our custom in the village of Gotemba and to Yokohama (I did this just for fun; it was not in my job description).

We then shipped out to Korea where we were stationed in Pusan, at that time a small city which was very poor and with poor sanitation; most of the houses had flattened tin cans for roofs. Soon we moved out into position behind the other infantry unit. The artillery fire from the enemy was really loud and each shell shook the ground; it was very scary, but we did not suffer any casualties and eventually (after 13 months altogether overseas) my tour of duty was up and I was sent home and discharged. When I got home, Joe and Pat repeatedly said, "Drive fast, Daddy, like Mommy does!" (I drove pretty slowly in Japan, on the left side of the road).

Instead of finishing up my training in Minneapolis, we decided to move to Los Angeles where my family lived. The UCLA Medical School was being built and their faculty was housed at the VA Hospital in the Western part of Los Angeles, a 10-minute drive from where the UCLA Medical Center was to be built. I started at the VA in December 1953 and have been here ever since. I trained with William (Bill) Hewitt, a prince of a man and an expert clinical infectious diseases person. He was very articulate; I loved to hear his formal presentations and lectures. I learned a good deal about life and how to be a gentleman, as well as infectious diseases, from him. He was a good tennis player and we had many fun matches. After 1 year of training in internal medicine/infectious diseases, I was offered a job as Instructor of Medicine at UCLA. The salary was low so they permitted me to have a half-time job at the VA Hospital (then known as the Wadsworth VA, now VA Hospital West Los Angeles). I worked in the Emergency Room from 12 noon Saturday to 8 am Sunday every weekend (a 20-hour shift and therefore half-time). Some weekends I wouldn't get much sleep, but I would catch up on Sunday. It was a great experience, seeing the patient before anyone else and often they were quite ill. I had a crackerjack nurse named Johnny who would screen patients for me when things got busy. She had tremendous clinical judgment and I very quickly learned that if she whispered "Drop that patient and come here quickly" that I better do that! Surgeons, radiologists, and other specialists took calls from home, but were always available on short notice.

After 2 years, I switched over to the VA full-time as Chief of the Infectious Diseases Section and the Pulmonary Disease Section. After some 3 years or so, a full-time Chief was selected for the Pulmonary Disease Section and I stayed with the Infectious Diseases Section. Various people around the country were naming their units Infectious Disease Section or Division, etc. Finally, Max Finland, informally known as the "Father of Infectious Diseases", at the Boston City Hospital, Ed Kass and others decided it was time to organize an official national society, the Infectious Diseases Society of America. An organizational meeting was set up in 1961 and Bill Hewitt and I were invited to join in the organizational activities. The Society came into being in 1962. Formal training programs came along later and specific criteria for certification of training programs and of Fellows was later still.

Not long after I became Chief of Infectious Diseases at the VA, we had a patient very reminiscent of the one I had encountered in Minneapolis. It was a case of putrid pleural empyema with no growth on (aerobic) cultures and questionable pleomorphic bacteria on Gram stain. I discussed the case with Dr Vera Sutter, head of the Clinical Microbiology Lab. at the VA and told her what my fellow trainee in Minneapolis said about it possibly being anaerobic.

Vera said she was sure that she had an old Brewer anaerobic jar somewhere in the hospital basement. She found it, cleaned it up, and we finally figured out how to use it, with ordinary line gas. In many areas, line gas (natural gas) is toxic to anaerobes, but luckily it was not in L.A. We had a funny-looking setup, but we were able to grow several anaerobes from the empyema fluid of this patient. That started a concerted effort to look for anaerobes in all kinds of specimens and we ultimately learned a good bit about anaerobic bacteriology and clinical aspects of anaerobic infections. When oral neomycin was first introduced, we studied it because of my previous interest in preoperative bowel "preps" while working with Dr Poth in Galveston. We set up various culture media in plates and we also used a series of fluid thioglycolate broths, with serial ten-fold dilutions of stool and various concentrations of neomycin. It was a major disappointment to find no growth on any of our plates. The broths were turbid, but Gram stain showed funny-looking debris and nothing grew on subcultures onto plates. It (finally!) dawned on me that these broths were probably growing anaerobes and this was quickly verified by subculture onto plates incubated in the Brewer jar.

There were not many people interested in anaerobes in the U.S. at that time and not much literature that was helpful. David T. Smith of Duke certainly knew about them and was doing various studies in animals. He died before I thought to try to contact him (now, of course, everyone feels free to e-mail with any questions about anaerobes or anaerobic

Sydney Finegold (third from the right on the back row) surrounded by his laboratory crew in the 1970s.

infections, but it just didn't seem proper to write or phone in those days). William Altemeier, a distinguished surgeon, wrote about anaerobes in surgical infections and was interested in the so-called *Bacteroides melaninogenicus* (later expanded to several genera and multiple species). Edwin Pulaski was another surgeon interested in anaerobes. There were dentists, notably Sigmund Socransky, at the Forsyth Dental Infirmary in Boston, involved with these organisms. Louis DeSpain Smith and later Theodor Rosebury wrote texts on the nonsporeforming anaerobes and the indigenous microflora, including anaerobes. I met Dr L. DS Smith and had a very enjoyable conversation with him. The French, and to a lesser extent, Germans had articles on anaerobes in the 1890s and the British a little later. I was absolutely amazed on how much was known back then about both anaerobic bacteriology and also anaerobic infections. In my early days at the VA Hospital (then known as Wadsworth VA), I was caught up in running the Infectious Disease and Pulmonary Disease Sections and steering a Hospital Infection Committee.

I finally realized that I was overlooking a gold mine of information by not studying anaerobic infections and bowel flora intensively, and mended my ways. And I got brash and made efforts to meet more people who had been working with anaerobes, many in various meetings, and some that I made a point to visit during travels. I was warmly welcomed by Henri Beerens of Lille, France, who had written a nice monograph on anaerobic bacteria with some clinical information. I was privileged to meet Andre Prévot, the master from Paris, who wrote countless articles, monographs, and books. He was in ill health then and did not wish to speak English although he knew it quite well, so I struggled with my rudimentary French. He was very pleasant. It is interesting to note that this leader in anaerobic bacteriology never isolated any of the pigmented Gram-stain-negative anaerobic bacilli because he did not use blood or hemoglobin in his media. I met Trevor Willis of the UK whose book, *Clostridia of Wound Infection* is a classic. Others from the UK who I have met in more recent years include Brian Duerden, Haroun Shah, and Saheer Gharbia, all leaders in the field of anaerobes (plus many others too numerous to mention). Ivan Hall, who described *Clostridium difficile* and many other clostridia, contacted me regarding his wife who had advanced cancer and disturbing foul-smelling discharges from decubitus ulcers. He felt she had secondary infection with anaerobes but was unable to isolate any clostridia. I suggested that she might have infection with nonsporeforming anaerobes and that the odor might be controlled by the use of metronidazole. I had the opportunity to meet him not long after that and enjoyed his company a great deal. He was elderly at that time, but he was quite tall and had perfect posture, a most imposing man.

Over the course of my experience at Wadsworth, I participated in training of some 85 clinical infectious disease Fellows, microbiologists, and visiting scientists. Included was one obstetrics/gynecology faculty member, a general surgeon, a head and neck surgeon, and a pediatrician.

Sid (second from left) with cousin Sylvia, Mary, his Dad, and brother Harold. Photo taken in 1975.

Sydney Finegold at the VA Middleton Award ceremony in Washington, D.C., in 1984. Front row: Joyce (son Mike's wife), Sid and Mary; back row: sons Joe and Mike, daughter Pat and her husband, Michael.

The bulk of my research dealt with anaerobic bacteria and anaerobic infections and the makeup of the bowel flora and its role in disease. The following summarizes some of the projects we have undertaken in my laboratory.

Early on, when John Bartlett was a Fellow in my group, we did a lot of work on the role of anaerobes in various pleuropulmonary infections – aspiration pneumonia, lung abscess, and pleural empyema and clearly established the importance of a variety of anaerobes in these entities. The empyema patients were no problem in terms of specimen collection (just thoracentesis), but for the others John Bartlett, who was very adept at percutaneous transtracheal aspiration and was willing to come see patients at any hour, accumulated a great deal of good clinical and microbiologic data on aspiration pneumonia and lung abscess, as well as empyema.

Our group, with collaborators, has published a laboratory manual for clinical anaerobic bacteriology, now in its sixth edition. Our studies, together with Dr Robert Bennion, have included extensive analysis of the bacteriology of appendicitis. In the case of gangrenous and perforated appendicitis, we found an average of 12 organisms (9 anaerobic and 3 aerobic) per specimen – many more than previous investigators had noted. We also studied the antimicrobial susceptibility of the isolates and the clinical outcome, evaluated the response to various antibacterial regimens and compared the microbiology of acute and complicated appendicitis. In these studies, we demonstrated the superiority of culture of the appendix tissue over culture of peritoneal fluid.

During the studies on appendicitis, we encountered a novel species of anaerobic Gram-stain-negative bacillus which we named *Bilophila wadsworthia*. This organism was the third most common anaerobe we encountered in gangrenous and perforated appendicitis despite being a relatively minor component of the indigenous bowel flora, suggesting that it has significant virulence. We and others have subsequently encountered this organism frequently in a wide variety of clinically significant infections, many of which were quite serious (e.g. bacteremia, empyema, purulent arthritis). Of interest, in the appendicitis study, our research laboratory (in 1989) was unable to identify fully certain anaerobic Gram-stain-negative and Gram-stain-positive rods occurring in almost one-third of patients, using only phenotypic tests.

We have also collaborated with Dr Bennion, Dr David Talan, and others on a study of the bacteriology of skin and soft tissue infections in intravenous drug users (86 specimens) and others with no such history (74 specimens); 70% of these infections were closed lesions that were sampled by aspiration with a syringe and needle. *Staphylococcus aureus* and the "*Streptococcus milleri* group" were prominent aerobes in both groups of patients; coagulase-negative staphylococci and *Streptococcus pyogenes* were also prominent in the non-IVDU group. The anaerobic flora differed in the two groups as well. The IVDU group most commonly had *Fusobacterium nucleatum*, pigmented *Prevotella* species, *Peptostreptococcus micros*, *Actinomyces odontolyticus*, and *Veillonella* species, whereas the non-IVDU group had primarily *Peptostreptococcus* species, *Bacteroides* species, and Gram-stain-positive anaerobic bacilli. In the overall patient population, 64% of subjects had one or more β-lactamase-producing organism.

We have studied the flora of 13 wound infections following head and neck cancer surgery. Preoperative aerobic cultures of the oropharynx and skin were of no value in predicting likely infecting organisms postoperatively. Postoperatively, α-hemolytic streptococci of various types, *Staphylococcus*

The Finegold laboratory crew ca. 1990. Seated, left to right: Chengxu Liu, Yuli Song, and Maureen McTeague; standing, left to right: Paula Summanen Carlson, Sydney Finegold, Tom Tomzynski, Hana Wexler, and Denise Molitoris.

aureus and *Staphylococcus epidermidis* were the principal isolates among the aerobes. Principal among the large group of anaerobes recovered were several species of *Peptostreptococcus* (most of which have subsequently been placed in other genera), *Fusobacterium nucleatum*, and several species of *Bacteroides* (also mostly reclassified as *Prevotella* or *Porphyromonas* subsequently), including *Bacteroides melaninogenicus* and *Bacteroides oralis*. One of the *Peptostreptococcus* species was renamed *Finegoldia magna* by a British group and therein lies a tale. My cousin (and close friend), Milton Finegold, is a highly recognized pediatric pathologist at Texas Children's Hospital and Baylor University School of Medicine in Houston where he was formerly Chairman of the Department. On one occasion when I was giving a lecture at his medical school, the Dean (who really likes Milton and is a close friend of his) in introducing me mentioned that I had this bacterium *Finegoldia magna* named after me and looked over at Milton and said, "How come you are only 'Finegoldia minima'"? Milton and I were both embarrassed by this, but it was all in good fun.

A paper from our laboratory covered a multicenter study of the bacteriology of chronic bacterial maxillary sinusitis in 114 evaluable adults. There were 70 aerobic and 64 anaerobic pathogens recovered. The prevalent anaerobes were *Prevotella* spp., anaerobic streptococci, and *Fusobacterium* spp. Recurrences of disease following antimicrobial therapy were twice as frequent with anaerobes as with aerobes when counts of anaerobes were 10^3/ml or greater. A pathogenic role for *Granulicatella* in chronic sinusitis was documented for the first time.

A novel species that we have described is *Sutterella wadsworthensis* which was "split out" of the *Bacteroides* (now *Campylobacter*) *gracilis* "group"; it was suspected to be a novel species because of its unique resistance to metronidazole.

We studied what we are calling the *Clostridium clostridioforme* "group". In the course of studies we are carrying out in relation to the possible role of intestinal microflora in autism, we encountered an organism that we identified as *Clostridium clostridioforme* in autistic subjects' fecal flora that was not present in control children's fecal flora (later, molecular studies proved that it was also present in the controls, but in significantly smaller numbers). Careful studies on this organism demonstrated certain phenotypic differences from *Clostridium clostridioforme* and 16S rDNA studies suggested a possible novel species that was confirmed by DNA–DNA reassociation studies. We have named this organism *Clostridium bolteae*; we have also encountered it in bacteremia and in intra-abdominal abscess secondary to perforated appendicitis. Recently, a former Post-Doctoral Fellow in the UCLA-VA Clinical Microbiology Fellowship Program called my attention to a fatal case of sepsis due to *Clostridium clostridioforme*; we asked to study the organism recovered, and 16S rDNA studies proved it to be, in-

UCLA Clinical Laboratory group ca. 2005. From left to right: Sydney Finegold, Jim Miller, Liz Wagar, Matt Goetz, Dave Bruckner, Maurice White, and Marie-Claire Rowlinson.

stead, a related organism recently described from the UK as *Clostridium hathewayi*. So, here are two different virulent organisms that can readily be confused with *Clostridium clostridioforme* that were confirmed as different by 16S rDNA studies. The *Clostridium clostridioforme* "group" is a unique group, the members of which all show Gram-stain-negative relatively thin rods with tapered ends and colonies which show a crystalline internal structure (*Clostridium clostridioforme* was classified as an anaerobic Gram-stain-negative rod related to *Fusobacterium* in the older literature). All three major species in this group are significant pathogens, having been recovered from a number of serious infections, including bacteremia. The source of all these organisms appears to be the lower bowel.

In the past few years, we have learned or developed various molecular methods, such as 16S rDNA sequencing, multiplex PCR, spacer-region (SR)-PCR pattern analysis, Rep-PCR, DGGE, and real-time PCR, for rapid and accurate identification of clinically important anaerobic bacteria. In one of our recent studies on Gram-positive anaerobic cocci (GPAC), we evaluated the utility of 16S rDNA sequencing as a means of identifying clinically important GPAC by sequencing 13 type strains of established GPAC species and 156 clinical isolates which had been studied only by phenotypic tests. Based on the sequences of the 13 type strains obtained in this study, 84% (131/156) of clinical isolates were accurately identified to species level, with the other 25 clinical strains revealing nine unique sequences that may represent eight novel species. This is in contrast to phenotypic identification, by which only 56% of isolates were correctly identified to species level. We have done 16S rDNA sequence analysis on more than 1000 clinically important anaerobic bacteria (either from our clinical isolate collection or our type strain collection), and compared the sequence data with those in public databases. We have contributed our clean sequence data to public databases, and we are establishing a 16S rRNA gene sequence database for anaerobic bacteria for our own studies. Furthermore, based on the sequence information, two multiplex PCR schemes were developed for rapid and accurate identification of ten *Bacteroides fragilis* group species and 14 GPAC species originally in the genus *Peptostreptococcus*, respectively. In one study, we reported a rapid and reliable two-step multiplex PCR assay to identify the then ten *Bacteroides fragilis* group species. The primers were designed from nucleotide sequences of the 16S rRNA, the 16S–23S rRNA intergenic spacer region, and part of the 23S rRNA gene. The established two-step multiplex PCR identification scheme was applied to the identification of 155 clinical isolates of the *Bacteroides fragilis* group that were previously identified to species level by phenotypic tests. The new scheme was more accurate than phenotypic identification, which was accurate only 84.5% of the time. In another study, we reported a two-step multiplex PCR assay to identify 14 GPAC species originally in the genus *Peptostreptococcus*. The identification obtained from multiplex PCR assays showed 100% agreement with 16S rDNA sequencing identification, but only 65% (123/190) agreed with identification obtained by phenotypic tests. These multiplex PCR schemes that we have established are simple, rapid, and reliable methods for the identification of these two groups of anaerobes. They will permit more accurate assessment of the role of various *Bacteroides fragilis* group and GPAC members in infection and of the degree of antimicrobial resistance in each of the group members.

Our group has had a long-standing interest in bowel flora.

Sid with one of his former Fellows, Ellie Goldstein.

Sid with one of his special Fellow trainees, John Bartlett, who is currently Professor at Johns Hopkins (2008).

Our studies have been published in various peer-reviewed journals at different times and then summarized in depth in a chapter published in 2009 (in *Encyclopedia of Microbiology*, Elsevier, pp. 422–443). We have also published a summary of our own data and a literature review on clostridia in the human gut flora (Chapter 1 of *Clostridia in Gastrointestinal Disease*, edited by S.P. Borriello, 1985) and wrote the section on *Clostridium* in the 1st edition of *Bergey's Manual of Systematic Bacteriology*. We wrote the *Bacteroides*, *Alistipes*, and *Porphyromonas* sections for the 2nd edition of *Bergey's Manual of Systematic Bacteriology*, and the "*Clostridium*" and "Anaerobic Gram-positive Cocci" sections for the 9th edition of the *Manual of Clinical Microbiology* and the "Anaerobic Gram-positive Cocci" for the 10th edition. We have developed an excellent selective medium for clostridia and have been involved in a number of studies of *Clostridium difficile*-induced antimicrobial-agent related pseudomembranous colitis, including the toxins of this organism, and bacterial interference between this bacterium and normal fecal flora. Other studies include the importance of anaerobic bacteria in blind loop syndrome and in D-lactic acidosis.

Our group had the opportunity to study two adult patients with D-lactic acidosis. The conditions predisposing to this entity were extensive small bowel resection following mesenteric infarction in one patient, and jejunoileal bypass in the second. In addition to acidosis, patients with this problem manifest significant psychiatric and neurologic abnormalities. Stool cultures on these two patients showed high counts and a marked predominance of Gram-stain-positive, nonsporeforming bacteria, principally bifidobacteria, lactobacilli,and *Eubacterium*. In sharp contrast with the normal situation, neither patient had *Bacteroides* present in counts above 10^8/g of stool. D-Lactate was produced from appropriate media in significant amounts by many of the bacteria isolated from the patients, but not from any bacteria recovered from normal control patients. The patients had a good clinical response and a normalization of their stool flora on oral vancomycin therapy.

We have done extensive studies on the gut bacteriology of patients with jejunoileal bypass used for the treatment of morbid obesity. Eight patients had studies at the time of the surgery, but before the bypass procedure was done, by aspiration of bowel contents. No antibiotics were used in preparation of the patients for surgery. In five patients, jejunal specimens were sterile and the other three had low counts of a predominantly aerobic flora. Ileal contents yielded variable but usually higher counts than in the jejunum and there were similar numbers of aerobes and anaerobes. In four patients whose bypass had been established and who required a subsequent surgery for complications of the bypass procedure, it was possible to obtain specimens again for comparison. Contents of the functioning small bowel in three of the patients showed counts of $10^{5–7.6}$/ml and the flora resembled that of feces. Four specimens from the excluded loops were colonized with fecal organisms and the counts ranged from $10^{6.4}$ to $10^{9.7}$/ml. Thus, in jejunoileal bypass both the functioning small bowel and the excluded loop become colonized with colonic flora. The enteropathy and systemic complications, including hepatosteatosis, improved on metronidazole, an antimicrobial active virtually only against anaerobic bacteria.

In 1998, Bolte published an intriguing hypothesis that *Clostridium tetani* (or other bacteria in the gut) might play a role in late-onset autism. On the basis of this hypothesis, an open-label trial of oral vancomycin was contemplated. Dr Richard Sandler, a pediatric gastroenterologist in Chicago,

was working with Ellen Bolte at that time; he called me to see if I would work with them on this proposed study to provide suggestions re. antimicrobials and to do bacteriologic studies on the stools of the patients before and after treatment. In this study, patients ranged from 43 to 84 months in age. By the Childhood Autism Rating Scale, six children met the criteria for severe autism, two for moderate autism, and three for mild autism. Vancomycin was given orally for 8 weeks. There was good evidence of a clinical response in 8 of 10 evaluable children with late-onset autism. The response involved improved behavior, communication, and social skills. Behavior and communication analog rating scales were not done in blinded fashion, but paired videotapes (30 minutes in a playroom environment) made before and during therapy were evaluated in blinded fashion by a clinical child psychologist who had no personal contact with the children. Patients relapsed to their pre-treatment state within a few weeks of discontinuation of the vancomycin.

The second study that we completed on autistic children was entirely microbiologic in nature. There were two parts to this study: (1) fecal studies (using pre-treatment specimens from the above study plus normal controls) with cultures designed primarily to recover clostridia, and (2) studies of the upper gastrointestinal contents. In both parts of the study, both autistic children and control children (age- and sex-matched) contributed specimens. In the case of the fecal flora studies, the decision was made to focus on clostridia because of the enormous amount of work involved in doing total flora studies by conventional microbiologic methods. Identification of isolates was done both by conventional phenotypic tests and by 16S rRNA gene sequencing. We found 23 species of *Clostridium* in the stools of 13 autistic children and 15 in the stools of the eight control children studied. The geometric mean count of clostridia was 1 log higher in the stools of autistic children than in the controls. Altogether, there were 25 different species of clostridia encountered.

In the upper GI part of the study, specimens from seven children with autism and four control children were studied. The cultures were set up to identify any bacterium or yeast that was present. The gastric pH was elevated in two of four children with autism for whom gastric pH was measured; these children had not been on H_2 blockers or proton pump inhibitors. The two children with hypochlorhydria had the most profuse microbial flora present. The most striking microbiologic result was that nonsporeforming anaerobic and microaerophilic bacteria were totally absent from specimens from the control children. In contrast, of the four children with autism whose specimens yielded growth, such organisms were present; two children's specimens had 7–9 different species. Among the three subjects with autism who had few or no organisms recovered from their specimens, one had never had diarrhea or constipation and a second had had multiple courses of antimicrobials for recurrent sinusitis, but none in the month prior to the sampling.

The above fecal specimens were tested subsequently using real-time PCR (RT-PCR); we found that cell count differences of *Clostridium bolteae* and *Clostridium* clusters I and XI, but not cluster XIV ab, were statistically significant between autistic and age- and sex-matched control children (higher bacterial counts in autistic children).

We made a big step forward when we joined with Scot Dowd of Lubbock, Texas, to employ pyrosequencing, a rapid-throughput method that permits examining many more samples in a given time than with cultural methods. Like other molecular methods such as real-time PCR, the organism can be identified by matching the DNA with readily available databases, but it cannot be recovered for other studies such as virulence for animals and antimicrobial susceptibility testing. However, it identifies many of the organisms that are uncultivable, gives a fairly rough quantification, and with the proper analysis provides a much broader picture than is feasible with cultural techniques. We were able to determine that the fecal flora of autistic subjects was abnormal when compared to normal controls.

Comparing the principal phyla detected by pyrosequencing, we note that the normal controls had 64% *Firmicutes* and 30% *Bacteroidetes* whereas the corresponding figures were reversed for the autistic subjects – 37% for the *Firmicutes* and 52% for the *Bacteroidetes*. This is a striking example of an abnormal fecal flora. Siblings were in between, suggesting that they may have acquired some abnormality of their flora from their association with their autistic sibling. This, if documented, would have profound epidemiological significance.Finally, from this pyrosequencing study, we were able to suspect that *Desulfovibrio* and *Bacteroides vulgatus* might play a key role in regressive autism because they were statistically significantly more prevalent in autistic subjects than in controls. We proceeded to do selective culture and real-time PCR for these two types of organisms on all of the stools studied previously by pyrosequencing. *Bacteroides vulgatus* turned out not to show any significant difference between the stool samples from both groups, but the genus *Desulfovibrio* and its five species found in the human gut showed statistically significant differences, indicating that *Desulfovibrio* may be a key bug in regressive autism.

A recent paper of ours, accepted for publication as part of a symposium, provides information on susceptibility of *Desulfovibrio* species to various antimicrobials. These data are

Marilyn Lerner (5'0") with (from left) Sydney's son Mike, son Joe, and grandson Chris (ranging in height from 6'4½" to 6'7").

Marilyn and Sid in 2007.

of interest in terms of potential therapy of regressive autism and also in terms of the pathogenesis of the disease and possibilities for prevention. The data must be interpreted with respect to levels of the various drugs obtained in the bowel. Penicillins and cephalosporins, as well as trimethoprim/sulfamethoxazole (and fluoroquinolones which should not be used in young children anyway beause of potential toxicity) may well predispose to regressive autism in consideration of the resistance of *Desulfovibrio* to them and their impact on the bowel flora which would permit this organism to grow to larger numbers as the competition is reduced. The data also indicate other drugs that might be used to treat ear infections, for example, with less risk of major bowel flora changes of the type discussed; included would be drugs such as the macrolides and tetracyclines. Aztreonam, a monobactam antibiotic that has been used for decades and was supplanted by newer drugs is now being used again in selected situations where Gram-stain-negative bacilli are resistant to many or most other drugs. This drug is not supposed to be active against anaerobes but it is moderately active against *Desulfovibrio.* It is susceptible to destruction by β-lactamases (which *Desulfovibrio* produces itself) but otherwise has a number of good features for this setting such as not being absorbed to any extent when given orally, and it is well tolerated. Combining it with sulbactam, a β-lactamase inhibitor, provides synergistic activity while eliminating the threat posed by the common β-lactamases.

Other notable events during my term at the Wadsworth VA (now VA Medical Center WLA and other names!) include the birth of my third child, Mike, in 1958. He was due around New Year's Day, but Mary worked hard in our garden hoping we could get another income tax deduction. As it turned out, he was born at 1:45 a.m. on 1 January. He was the first baby born in the new year for our entire Medical Center and we won a free playpen as a result! Mike, like Joe and Pat, has been a real blessing. He is down to earth, really funny and punny, and lives nearby so that we play tennis doubles, as partners, almost every Sunday. I also get to see him a lot otherwise because he's so close. He is a teacher and tennis coach at Venice High School, which is a short walk from my home in Marina Del Rey. As tennis gets more difficult for me, Mike really helps out because he covers the court so well. About the third set, I get loosened up and become more effective. Mike works for me during school vacation periods, entering data into my database, getting hard to find articles from the UCLA library, and even doing errands, which saves me a lot of time. He has a heart the size of his head (not literally!); he is always doing things for people. Although he and his wife, Joyce (who also is very nice to me) have no children, Mike is like a father to many of his students, some of whose parents are divorced or who have to work two or three jobs to get by and don't have as much time for their children as other parents. He is available any time for anybody who needs help. One day he received a call around 4 a.m. from one of his students who lived in a bad part of town because she felt she needed help to deal with her parents. He went over and straightened everything out.

My wife developed pancreatic cancer and managed to survive for 13 months because she was such a fighter. She was playing tennis while wearing her chemotherapy infusion pump! She died in 1994. Because I enjoyed married life with Mary so much, I rushed into another marriage relative-

ly soon after she died and it turned out to be a bad mistake. We separated after some 3 years and I have just divorced her. Fortunately, I met a lovely and wonderful woman, Marilyn Lerner, not long after the separation. We have a lot of fun together, playing tennis, going to movies and the theater and musical performances as well as UCLA basketball games, and traveling. She is a nurse, is very bright and has broad interests. She volunteers for the Venice homeless people's clinic, plays the violin, and is involved in golf and roller skating. She is an excellent dancer, but we don't mesh well with that. Her husband, a psychiatrist, died just about the same time that Mary did. Neither of us wants to remarry, so we just enjoy life together.

I "retired" in 2000. I cut way back on my clinical work but continued it for 2–3 more years before restricting myself to research, with occasional lectures. I have enjoyed this a great deal, especially since research was the principal leg of the "academic three-legged stool" that suffered when one gets very busy with clinical work and teaching. I still do miss the clinical side, however.

The research is fun and exciting, but what makes it very special is the bright young people who have worked with me and taught me so much. Three people deserve special praise as they have worked for me on and off for approximately 25 years – Paula Summanen Carlson, Julie Downes, and Denise Molitoris. Others who deserve special credit for their contributions to the laboratory include Vera Sutter, Hannah Wexler, Hannele Jousimies-Somer, Yuli Song, Chengxu Liu, Ellen Jo Baron, and many Infectious Disease Fellows (notably John Bartlett, Ellie J.C. Goldstein, Lance George, Godfrey Harding, and Itzhak Brook). I am also indebted to the many outstanding collaborators I have been blessed to work with over the years, many too many to mention. They have added immeasurably to the work of the laboratory and they have enriched my life. It is remarkable that in academia one can have a personal first family such as I have and have had and, in addition, a second family of mentors, students, and colleagues.

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Isolation of novel natural products from un- or underexplored marine actinomycetes

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Microbial systematics is pivotal to meeting some of the major challenges facing humankind, as exemplified by the need for novel natural product medicines. A systematics-guided bioprospecting investigation of bioactive compounds from unique, untapped, ecological niches, including the construction of high-quality libraries of marine microbes and their crude extracts, has become an efficient approach to drive drug discovery. The criteria for strain selection include: the uniqueness and richness of biodiversity of the ecolological niches from which the strains are isolated (Hayakawa et al., 2004), symbiosis with medicinal plants (i.e. endophytes), morphological and taxonomical distinctiveness, and a bias towards the study of actinomycetes for their proven track record as prolific sources of natural products. We optimize natural product production for the selected strains, prepare extracts and subject them to high-throughput (HTP) screening (both cell-based and target-based), followed up by LC-MS dereplication, and prioritize the strains for fermentation scale-up, isolate and determine the structures of the novel natural products, and produce and assemble the library for screening. Our ecology- and taxonomy-based approach to strain selection for novel natural product discovery contrasts with traditional natural product discovery programs, most of which rely heavily on the designs of the HTP assays to screen often randomly collected micro-organisms.

Introduction

New drugs especially antibiotics are urgently needed to thwart pathogens (Talbot et al., 2006) and life-threatening diseases (Salas et al., 2009). Bacterial marine natural products are important sources of novel natural products for drug discovery (Blunt et al., 2003; 2004; Faulkner, 2000; Haefner, 2003). Marine bacteria have a common mechanism to incorporate bromine or other halogens into organic compounds that can potentially lead to enhanced bioactivities. Historically, the isolation of new metabolites from marine organisms began in 1960s. In 1966, the first marine bacterial metabolite reported was the highly brominated antibiotic 2,3,4-tribromo-5-(l′-hydroxy-2′,4′-dibromophenyl) pyrrole. This compound was isolated from an *Alteromonas* species obtained from the surface of the Caribbean seagrass *Thalassia* by Burkholder and co-workers (Salas et al., 2009). In 1977 the same group isolated an antibiotic-producing *Pseudomonas* from a tidepool in La Jolla, California. Isolation and purification of the metabolites yielded 6-bromo-indole carboxaldehyde, its debromo analog, and a mixture of 2-n-pentyl- and 2-n-heptylquinolinol. The most interesting of these was 2-n-pentylquinolinol, which had potent activity against *Staphylococcus aureus*. The unique metabolite 6-bromo-indole carboxaldehyde lacked antimicrobial properties (Fenical, 1993). The isolation of these highly brominated compounds illustrates a unique property that distinguishes marine from terrestrial bacteria. Since then, a broad spectrum of biological activities has been detected, for instance, antibiotic, antifungal, toxic, cytotoxic, neurotoxic, antimitotic, antiviral, antineoplastic, and cardiovascular (CV) activities.

In more recent years, new targets have been added for general screening, including: AIDS, immunosuppressive, anti-inflammation, Alzheimer's disease, ageing processes, and some tropical diseases (Kelecom, 2002). Although research on metabolites from marine micro-organisms is growing rapidly, a key problem for the scientists in pharmaceutical industries and academic sectors is to effectively discover active marine microbes and obtain their natural products.

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Fortunately, advances in microbial systematics and analytical techniques in constructing high-quality natural product libraries and high-throughput screening methods have facilitated the development of novel natural products discovery. Among marine microbes, the actinomycetes group is a rich source of drug candidates as new secondary metabolites can be discovered by screening dereplicated, novel actinomycetes (Goodfellow and Fiedler, 2010) or by re-screening known actinomycetes directed by genomics (Hertweck et al., 2011) through systematics technology. This mini-review is based upon actinobacterial systematics which is used to direct novel natural products discovery by two separate routes: isolating un-explored marine actinomycetes and re-screening under-explored marine actinomycetes.

Systematics leads to new natural products investigations

Discovery of novel actinomycetes from marine sources

Actinomycetes are widely distributed in different marine ecosystems and have evolved great genomic and metabolic diversity. There is a tremendous potential for the isolation of novel secondary metabolites from marine actinomycetes. The probability of discovering new secondary metabolites from novel actinomycetes is much higher than from "old" or common actinomycetes. Thus, isolating novel actinomycete strains is one of the best approaches to discovering novel natural products. Therefore, maximizing the types of samples and developing isolation and culturing strategies will enhance contributions (Zhang et al., 2003). The predicted values of actinobacterial diversity shows that as many as 1353 taxa exist in marine habitats, and approximately 90% of these taxa represent novel species and genera-based on 16S rRNA gene clone library data. Recent data from culture-dependent studies have revealed that the genera *Dietzia*, *Rhodococcus*, *Streptomyces*, *Salinispora*, *Marinophilus*, *Solwaraspora*, *Salinibacterium*, *Aeromicrobium*, *Williamsia*, and *Verrucosispora* exist in marine environments (Lam, 2006). Specifically the genera *Salinispora* and *Verrucosispora* promise to be the most exciting finding because they produced the novel bioactive series of compounds, the salinisporamycins and abyssomicins, respectively (Fenical et al., 2005; Sussmuth et al., 2004).

Investigations for the isolation of novel actinomycetes from marine environments continue, including enrichment techniques, new selection methods and media (Jensen et al., 2005; Magarvey et al., 2004) (Tables 1 and 2). For instance, it is important to provide suitable growth conditions with proper nutrients (carbon, nitrogen, trace metals) or the correct temperature, aeration, and time of cultivation; media supplemented with signaling molecules, such as pyruvate, cyclic adenosine monophosphate, and homoserine lactones; use of "harsh" conditions or pre-treatments to increase the number and diversity of rare actinomycetes; in addition, different antibiotics are used to reduce the growth of unwanted fungi and Gram-stain-negative bacteria.

Genomics combined with systematics

Although great progress has been made, strategies towards the isolation of new marine-derived actinomycetes are still lacking (Lam, 2006). The advance of genome sequencing has revived an interest in rescreening actiomycetes for bioactive compounds. The massive surge in genome-sequencing projects has revealed the great biosynthetic potential and metabolic diversity of micro-organisms. To date, whole-genome sequences of over 126 actinomycetes are published on the website (http://www.genomesonline.org/). The "phylogenomics" approach, first proposed by Jonathan A. Eisen and Philip C. Hanawalt, combines genome sequence information and phylogenetic studies into a composite analysis (Eisen and Hanawalt, 1999). The Genomic Encyclopedia of *Bacteria* and *Archaea* (GEBA) project chose typical micro-organisms for sequencing to reveal the evolutionary relationships. The project produced many benefits including the reconstruction of phylogenetic history, the discovery of new protein families and biological properties, and the prediction of functions for known genes from other organisms (Eisen et al., 2009). The phylogenomics and GEBA project indicate a new era in actinobacterial systematics and improve the prospects of natural product discovery.

The development of phylogenomics brings several novel research methods to infer the evolutionary history of *Actinobacteria* on the basis of their genome analysis. The two primary standard phylogenomic reconstruction methods are sequence-based methods and methods that are based on whole-genome features with rare genomic changes (RGCs) (Philippe et al., 2005). Composition vector (CV) is one of the acknowledged methods which has been used in archaeal taxonomy (Hao et al., 2010). Novel taxa such as suborders *Actinopolysporineae* and *Kineosporiineae*, families *Actinopolysporaceae*, *Kineosporiaceae*, and *Beutenbergiaceae*, and genus *Quadrisphaera* were proposed by the RGCs method (Li et al., 2009). Using whole-genome analysis to overcome the limitation of poorly resolved phylogenies is becoming a promising method. Different universally conserved protein-encoding genes mined from the genome were applied to resolve this problem (Breitling et al., 2010). Theoretically, species having a close relationship implies that they have the same gene order and may produce the same kind of metabolites. Thus, it is very important to determine the most accurate evolutionary position of actinomycetes.

Table 1. Commonly used pretreatment methods

Pretreatment method	Reference
Filter method	Hirsch and Christensen (1983)
Dispersion and differential centrifugation (DDC)	Hopkins et al. (1991)
Radiation method	Zotchev et al. (2007)
Stamping method	Fenical et al. (2002)
Dilution and heat-shock method	Fenical et al. (2002)

Table 2. Commonly used isolation media

Selective medium	Selective agent	Target taxa	Reference
Arginine-vitamins agar	None	*Microbispora*, *Streptosporangium* spp.	Nonomura and Ohara (1969)
Colloidal chitin agar	None	Actinomycetes	Hsu and Lockwood (1975)
Humic acid-salts-vitamin agar	None	*Dactylosporangium*, *Microbispora*, *Micromonospora*, *Streptosporangium*, *Thermomonospora* spp.	Hayakawa and Nonomura (1987)
Humic acid-gellan gum	None	*Microbispora*	Suzuki et al. (1998)
Marine agar	None	*Kocuria marina*	Kim et al. (2004)
		Salinibacterium amurskyense	Han et al. (2003)
		Serinicoccus marinus	Chun et al. (2004)
Starch-casein-mineral salts agar	Cycloheximide nystatin	*Streptomyces* spp.	Kuester and Williams (1964)
Diagnostic sensitivity test agar	Demethylchlortetracycline	*Nocardia asteroides*	Orchard and Goodfellow (1974)
	Demethylchlortetracycline, chlortetracycline		Orchard et al. (1977)
	Methacycline		
Inorganic mineral salts agar	Cycloheximide nystatin	*Rhodococcus* spp.	Rowbotham and Cross (1977)
Glucose yeast extract	Rifampicin	*Actinomadura* spp., *Streptomyces albus*	Athalye et al. (1985)
		Thermomonospora chromogena	
Raffinose histidine agar	Cycloheximide, nystatin	*Streptomyces chromofuscus*	Vickers et al. (1984)
Starch-casein-seawater agar	Actidione	*Streptomyces* spp.	Goodfellow and Haynes (1984)
Starch-casein agar plus 0.5%NaCl	Cycloheximide, nalidixic acid, nystatin	*Actinoplanes* spp.	Takizawa et al. (1993)
Glucose yeast extract agar	Cycloheximide	*Gordonia* and *Tsukamurella* spp.	Goodfellow et al. (1991)
SM3 agar	Novobiocin, nalidixic acid	*Amycolatopsis* spp.	Tan (2002)
M1-M5	Cycloheximide, rifampicin	*Salinispora* spp.	Fenical et al. (2002)
Starch casein agar	Cycloheximide, nystatin	*Streptacidiphilus* spp.	Kim et al. (2003)
Humic acid-vitamin agar	Cycloheximide	*Streptomyces violaceusniger*	Hayakawa et al. (2004)

Research on actinobacterial whole-genome sequences is an effective method to study actinobacterial physiological differentiation, regulation of metabolic networks, prediction of natural products, taxonomy and evolutionary relations, etc. A recent study contains several aspects of actinobacterial genomics and natural product isolation. These include whole-genome sequence mining, genome scanning, metagenomics, and heterologous expression (Shen and Van Lanen, 2006).

High-quality actinobacterial natural product libraries

The inherent superiority of marine actinobacterial isolates and the development of high-throughput screening technology make a Marine Microbe Natural Product Library (MMNPL) attractive for drug discovery (Bugni et al., 2008; Ganju et al., 2008; Koehn, 2008). To make the MMNPL more convenient for new screening patterns and to shorten the bioactive marine natural products discovery pipeline, we have characterized an MMNPL paradigm guided by microbial systematics (Zhang et al., 2007) (Figure 1).

Biodiversity- and taxonomy-guided library construction

Chemical diversity is coupled to the novel biodiversity of marine actinomycetes (Koehn and Carter, 2005; Singh and Pelaez, 2008). A major goal for constructing a MMNPL is to increase the diversity of the natural products while decreasing their redundancy. Techniques including selective isolation, phenotype analysis and quick identification based on taxonomy are most commonly used in discovering marine actinomycetes (Amann and Ludwig, 2000; Anderson et al., 2005; Hentschel et al., 2001; Keller and Zengler, 2004; Larsen et al., 2005; Muller et al., 2008). By using these strategies, we have isolated more than 10,000 marine actinobacterial strains, which covered most genera of actinomycetes. After the analysis of the genetic diversity of PKS and NRPS biosynthetic systems in these strains, the systematic alteration of easy accessible fermentation parameters (media composition, pH value, temperature, addition of enzyme inhibitors, oxygen supply, culture vessel) were employed to stimulate the novel compounds produced from these gene clusters based on our high-throughput bioassays (Zeeck et al., 2002). Many of the extracts from our isolates showed potential bioactivities (Ashforth et al., 2010). These data demonstrated the dogma (novel isolates→novel gene clusters→novel compounds) and the value of taxonomy-guided strain selection and biosynthesis gene cluster analysis.

Dereplication of actinomycetes and their products

Another important part of an MMNPL is decreasing redundancy – in order to reduce screening costs and increase the success rate, dereplication approaches are urgently needed.

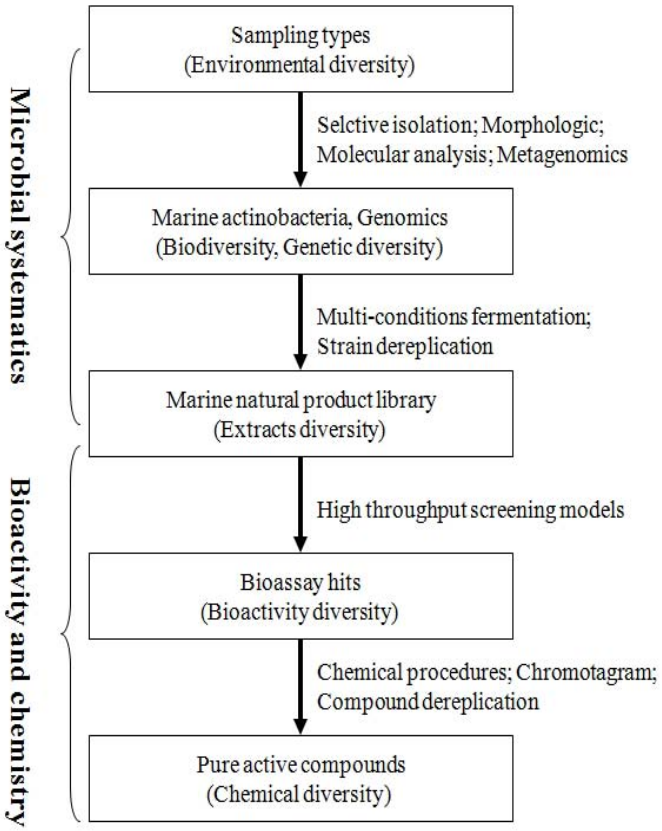

Figure 1. Building a Marine Microbe Natural Product Library in the laboratory.

The redundancy of actinomycetes strains is the major issue for high-quality natural products library construction. The selection of media and pre-treatment methods are important for isolation of actinomycetes from marine sources. But further dereplication of genetically identical actinomycete strains is still necessary before natural products library construction as they may contain the potential for producing the same metabolites.

The dereplication of strains can be fully achieved by actinobacterial taxonomy. These methods are widely used in novel strain identification. Dereplication of strains becomes much easier with the taxonomy methods – phenotypic profiling, chemical analysis, and genotypic profiling. Morphology discrimination such as spore formation, aerial mycelia, strain colonies and diffusible pigment by the naked eye or microscopy is the most rapid way to dereplicate strains. Colony color groups reflect the taxonomic diversity of cultivable actinomycetes in marine environment on oatmeal agar directly. Chemical analyses are also commonly used in analyzing whole-cell sugar, polar lipids or diaminopimelic

acid (DAP) type (Maldonado et al., 2009) to assign strains to different groups. Integrated with the genotypic data such as Restriction Fragment Length Polymorphism (RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE) of the 16S/18S genes, the dereplication work can be fully achieved. The latest method is to use the polyketide synthase (PKS) gene probe to detect strains. The results indicate that strains that had similar band patterns harbored similar PKS genes. For example, Ayuso-Sacido and Genilloud (2005) designed new PCR primers targeted to specifically amplify NRPS and PKS-I gene sequences from actinomycetes. The sequence analysis of amplified products cloned from two model systems and used to validate these molecular tools has shown the extreme richness of NRPS or PKS-I-like sequences in the actinomycete genome. When these PCR primers were tested on a large collection of 210 reference strains encompassing all major families and genera of the actinomycetes, it was observed that the wide distribution of these genes in the well-known productive *Streptomyces* species is also extended to other minor lineages where in some cases very few bioactive compounds have been identified to date. Using the PKS genes for direct dereplication work is more convenient for bioactive compound screening. Dereplication is the basic procedure to be used for differentiating phenotypically ambiguous strains in order to facilitate the follow-up screening strategies and thereby minimize costs and time in sorting large collections of isolates.

Following the actinomycetes dereplication, the chemical dereplication procedure also plays an important role in MMNPL. For example, Brandao et al. developed a pyrolysis mass spectrometry (PyMS) method (Brandao et al., 2002). This technique is a whole-cell fingerprinting technique that enables the rapid and reproducible sorting of micro-organisms, which uses small samples and has the advantage of being fully automated. Moreover, PyMS analysis revealed significant variation within pyrogroups that contained strains with the same genotypic (PRS) characteristics, thus emphasizing its discriminatory capacity at the interspecies level. For the sake of the accelerated dereplication of natural products (Munro et al., 2008), advances in modern spectroscopic methods have allowed the development of tandem analytical techniques, such as MS/MS/MS (Konishi et al., 2007), GC-EI/MS (Timmermann et al., 2006), HPLC-SPE-NMR (Jaroszewski et al., 2005), LC-MS-MS (Wolfender et al., 2000), and LC-NMR (Bobzin et al., 2000; Henkel et al., 2007). All of these techniques increase the chemical diversity of MMNPL.

Prediction of the relation between taxonomy and secondary metabolite production

Streptomycin was the first class of antibiotics derived from the actinomycetes. Since then, large groups of antibiotics have been isolated from actinomycetes, and *Streptomyces* became the main source of antibiotic-producing strains. But when the redundancy problem of repeatedly isolating the same compounds from *Streptomyces* became more and more serious, isolating novel rare actinomycetes became the goal of drug discovery researchers. Unfortunately, no definite rule of "which genus of actinomycetes produces which groups of compounds" has been discovered or published to date. We may occasionally predict the metabolites based on previously reported data. Ying Huang et al. (2011) screened 1068 actinomycetes using degenerate primers designed according to the conserved regions of polyether epoxidases and discovered a group of strains that have the potential of producing polyether ionophores. The genera having the ability to produce polyether ionophores are *Streptomyces*, *Actinomadura*, *Dactylosporangium*, and *Micromonospora* (Huang et al., 2011). The final results of these experiments revealed 13 positive isolates that belonged to five genera that were able to produce polyether ionophores as predicted. In addition, two new analogs were found.

Prospects and conclusions

The marine environment is a prolific resource for the isolation of less exploited microorganisms. Marine microbiology with a distinct focus on bioactive compounds is developing strongly in several countries. Furthermore, it has been reiterated that natural product research and discovery in marine actinobacteria shows exceptional promise. Hence, there is an increasing need to discover more novel marine actinobacteria and their novel biosynthesis gene clusters. Furthermore, constructing a library of marine actinobacteria guided by microbial systematics is also amenable to the discovery of biological compounds.

Dedication

We would like to dedicate this paper to the more than 370 participants from 27 countries at the Inaugural Conference of Bergey's International Society for Microbial Systematics in Beijing.

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CVTrees support the Bergey's Systematics and provide high resolution at species levels and below

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CV stands for Composition Vector. CVTree is an alignment-free method for inferring phylogenetic trees from prokaryote whole genomes by using CVs after a subtraction procedure. CVTree is the name of a public domain Web Server that implements the method. The results obtained by this method or by the Web Server are also sometimes called CVTrees. The branchings in CVTrees are verified by direct comparison with the Bergey's Systematics and agree well with the latter at all taxonomic ranks from phylum down to species with a few exceptions, which in most cases correspond to long-debated problems and may hint on possible taxonomic revisions. Moreover, CVTrees provide higher strain resolution at species level and below. In this mini-review the CVTree method will be described and some of its results will be shown.

Introduction

The 2nd edition of *Bergey's Manual of Systematic Bacteriology* follows "a phylogenetic framework based on analysis of the nucleotide sequence of the small ribosomal subunit RNA, rather than a phenotypic structure" (Garrity's Preface). The successful and impressive "congruence" of prokaryotic phylogeny and taxonomy on the basis of 16S rRNA gene sequence analysis raises the problem of independent justification of the latter. The CVTree approach to infer prokaryotic phylogeny, introduced a few years ago (Qi et al., 2004a, 2004b; Hao et al., 2004), distinguishes itself from the 16S rRNA analysis both in input data (whole genomes) and in methodology (alignment-free). It is remarkable that the CVTree results support the 16S rRNA phylogeny and Bergey's Systematics in a majority of branches from phyla down to genera and species. Moreover, at the species level and below where 16S rRNA analysis lacks sharp resolution, CVTree provides a definite and reasonable branching scheme.

As the cost of sequencing a prokaryote genome keeps dropping and automatic annotation of bacterial genomes has reached a more or less ripe stage as compared to that for *Eukarya*, we expect that CVTree will become a convenient tool in microbial systematics to obtain a rapid approximation of the classification of a newly sequenced organism and for a more effective design of phenotyping experiments.

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Feasibility of using whole genomes

Since the first two bacterial genomes were published in 1995 (Fleischmann et al., 1995; Fraser et al., 1995) the number of sequenced prokaryotic genomes has kept rapidly growing. At the moment of writing these lines, more than 1450 well-annotated genomes have been released and there are more than 7200 on-going sequencing projects. Although the selection of species to be sequenced in most cases is based on practical needs other than for phylogenetic or taxonomic considerations (the GEBA project being an exception, see Wu et al., 2009), the sequenced genomes now cover a wide taxonomic range. As of 2 July 2011 the prokaryotic genomes released at the NCBI FTP site came from 29 (6) phyla, 52 (10) classes, 111 (11) orders, 213 (33) families, 466 (62) genera, 1032 species and 1411 strains (numbers in parentheses indicate uncertain taxa including those labeled as *Incertae sedis* or *Candidatus*). The coverage is much wider than the collection of about 400 16S rRNA gene sequences when Carl Woese and coworkers proposed a phylogenetic definition of the major eubacterial taxa (Woese et al., 1985).

An essential advantage of using whole genomes consists in circumventing the lateral gene transfer (LGT) problem as both LGT and lineage-dependent gene loss are merely mechanisms of genome evolution. One avoids as well the tedious task of finding orthologous genes because anyone

who believes in Charles Darwin must admit that all genomes are orthologs.

Necessity of being alignment-free

Prokaryotic genomes are extremely diverse in their size and gene content. A small genome of *Mycoplasma genitalium* has only less than half a million basepairs and less than 500 genes, not to mention the tiny genomes of a few endosymbiont bacteria, e.g. *Candidatus* Hodgkinia cicadicola. The largest genome sequenced so far, that of *Sorangium cellulosum*, has more than 13 million basepairs and 12,000 genes. It is very difficult, if not impossible, to "align" such genomes or to identify orthologous genes shared by all species.

Our alignment-free approach starts by counting the number of appearances of all possible (overlapping) K-peptides in proteins encoded in a genome. A "raw" CV with 20^K components arranged in lexicographic order of all possible K-peptides is formed for each species. The direct count of a K-peptide is put in the corresponding component. Then an expected count calculated from the numbers of (K-1)- and (K-2)-peptides by using a (K-2)-th order Markov prediction formula is subtracted from the original count to suppress the effect of neutral mutations and highlight the role of natural selection. The recalculated CV is used to represent a species and pairwise distances between species are calculated to produce a distance/dissimilarity matrix. Then a tree is constructed by using the standard Neighbor-Joining program in the PHYLIP package (Felsenstein, 2008). The subtraction procedure is crucial for the success of CVTree. For derivation of the Markov prediction formula and other details please see Qi et al. (2004) and Hao et al. (2004). Being a newly proposed method that differs from traditional approaches in many aspects, we have solved a few foundation problems which led to interesting mathematics and some remaining problems call for further scrutiny (Li et al., 2010). The CVTree method has been applied to *Bacteria* and *Archaea* (Qi et al., 2004; Hao et al., 2004; Gao et al., 2007; Li et al., 2010; Sun et al., 2010), fungi (Wang et al., 2009), chloroplasts (Chu et al., 2004), and viruses (Gao et al., 2003, 2007).

Being alignment-free renders the CVTree method parameter-free, because alignment of amino acid sequences involves many parameters via scoring matrices, gap penalties, etc. Though the peptide length K looks like a parameter, it is actually not. We never adjust K and the same K is used for all genomes. K's role is more like the knob in an optical microscope that controls the resolution. Longer Ks put emphasis on species-specificity, but shorter Ks take into account common features shared by many species. In fact, we construct CVTrees for all K=3 to 7 and watch the "convergence" of the branchings with K increasing. For a quick overview of the whole phylogeny based on many available genomes it is sufficient to run a single job for K=5 or 6 and there is no need to use K greater than 7. We omit the technical reasons supporting the above statement.

Although the basic idea of CVTree is quite simple, its implementation is complicated by the necessity of dealing with very long CVs and huge dissimilarity matrices. Therefore, for practitioners in microbiology we designed a public-domain software which has been published twice in the Web Server issues of Nucleic Acids Research (Qi et al., 2004b, Xu et al., 2009). We invite interested colleagues to play with CVTree in their Internet browser at http://tlife.fudan.edu.cn/cvtree/ where an online help file and its printable version is available.

We note in passing that all examples to be given below are drawn from the same set of CVTrees for K=3 to 7 based on a dataset of 1411 prokaryotic genomes, after excluding tiny genomes of a few endosymbiont bacteria.

Direct comparison with taxonomy

Phylogeny and taxonomy are not synonyms, but, ideally, a faithful phylogeny and a reasonable taxonomy should agree with each other in basic topologies. Traditionally, phylogenetic trees are verified by statistical resampling such as bootstrap or jackknife. This kind of test tells at most the stability and self-consistency of the trees, by far not the objective correctness of the results. We advocate the viewpoint that a phylogenetic tree should be checked by direct comparison with taxonomy which is not based on the same kind of phylogeny. We note that CVTrees are supported by both jackknife and bootstrap tests as well (Zuo et al., 2010). It is remarkable that this was impossible even a decade ago, as whether prokaryote proteins contain phylogenetic signal was questioned then (Teichmann et al., 1999) and whole-genome based phylogeny was unable to "resolve the major branchings of the Bacteria" (Huynen et al., 1999).

In comparing CVTrees with prokaryote taxonomy our guiding principle is monophyleticity of the branches and a useful notion is a "collapsed tree". Whenever all genomes in the dataset from a certain taxon appear in one and the same branch and no genomes from other taxa occur in that branch, we have full agreement of the phylogeny and taxonomy at that level. Then the branch may be collapsed to a single leaf labeled by the name of the taxon. For example, there are 40 "*Cyanobacteria*" genomes in our dataset and they always form a monophyletic branch; we can collapse the branch to a single leaf labeled for all the *Cyanobacteria*{40}.

Convergence at different taxonomic ranks

To give an idea on the comparison of CVTree results with taxonomy we illustrate this at different taxonomic levels. For example, at the phylum level we have:

Acidobacteria{5}	K3K4K5K6K7
Aquificae{9}	K3--K5K6K7
Chlamydiae{21}	K3K4K5K6K7
Chlorobi{11}	K3K4K5K6K7
Crenarchaeota{33}	--------K7
Cyanobacteria{40}	--K4K5K6K7
Deferribacteres{4}	K3K4K5K6K7
Deinococcus-Thermus{13}	K3--K5K6K7
Dictyoglomi{2}	K3K4K5K6K7
Elusimicrobia{2}	----K5K6K7
Fusobacteria{5}	--K4K5K6K7
Planctomycetes{5}	K3K4K5K6K7
Synergistetes{2}	K3K4K5K6K7
Thaumarchaeota{2}	K3K4K5K6K7
Thermodesulfobacteria{2}	K3K4K5K6K7
Thermotogae{12}	K3--K5K6K7
Verrucomicrobia{4}	----K5K6K7

Here and below K3K4K5K6K7 means the corresponding phylum forms a monophyletic branch in all five trees constructed for K=3 to 7, though the topology may not be exactly the same. A missing K value like K3—K5K6K7 indicates that the phylum does not appear as a monophyletic branch at K=4.

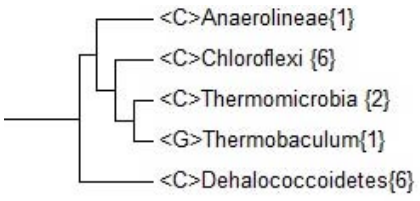
The non-convergence of "*Crenarchaeota*" at K<7 is caused by *Thermofilum* joining "*Candidatus* Koarchaeum" outside but close to the monophyletic cluster.

Phyla absent in the above list do not form monophyletic branches in CVTrees. For example, the phylum *Proteobacteria* is represented by 616 sequenced genomes for the time being, the most among all phyla. Three of its five classes (the *Alphaproteobacteria*, *Betaproteobacteria*, and *Epsilonproteobacteria*) do form monophyletic branches in CVTrees as seen in the partial list of convergent classes shown below:

Acidobacteria{4}	----K5K6K7
Alphaproteobacteria{147}	--K4K5K6K7
Aquificae{9}	K3--K5K6K7
Archaeoglobi{4}	K3K4K5K6K7
Bacteroidia{17}	----K5K6K7
Betaproteobacteria{98}	----K5K6K7
Chlamydiae{21}	K3K4K5K6K7
Chlorobia{11}	K3K4K5K6K7
Chloroflexi{6}	K3K4K5K6K7
Deferribacteres{4}	K3K4K5K6K7
Dehalococcoidetes{6}	K3K4K5K6K7
Deinococci{13}	K3--K5K6K7
Dictyoglomia{2}	K3K4K5K6K7
Elusimicrobia{2}	----K5K6K7
Epsilonproteobacteria{37}	K3K4K5K6K7
Fusobacteria{5}	--K4K5K6K7
Methanobacteria{8}	K3K4K5K6K7
Methanococci{15}	K3K4K5K6K7

The fact that *Betaproteobacteria* is intermixed with the *Gammaproteobacteria*, observed first in 16S rRNA gene sequence analysis (Woese et al., 2000) happens also in CVTrees. If we take Beta/Gamma as a single group as suggested by Woese, then the only class which does not form a monophyletic cluster in CVTrees is *Deltaproteobacteria*. This has been observed in some conserved marker gene studies (Ludwig, 2010). In particular, the "class" splits into several clusters with *Bdellovibrio*, a deltaproteobacterium forming a sister group with *Leptospira* from the phylum *Spirochaetes*, an inexplicable finding that needs to be confirmed by further taxonomic studies.

In the above list there is also a highly convergent order "*Dehalococcoidetes*"{6} that has not been listed in *Bergey's Manual* for the time being. It is this order that caused the otherwise convergent phylum "*Chloroflexi*" to drop out from the list of convergent phyla. The order "*Dehalococcoidetes*" is located not far from "*Chloroflexi*", as shown below (where <C> and <G> indicate Class and Genus):



The branching scheme shown above may be used to test the predictive power of CVTree. The class *Thermomicrobia* was originally defined as a phylum and a class with only one species, *Thermomicrobium roseum*, in volume 1 of *Bergey's Manual of Systematic Bacteriology*. Later on it was proposed to transfer another species from the phylum *Actinobacteria* to this class and to transfer the entire class to the phylum "*Chloroflexi*" (Hugenholtz et al., 2004).

Thermobaculum was not listed in the *Bergey's Manual* Outlines and it was assigned a status of undefined phylum in NCBI taxonomy. However, a recent comparison of gene order in full genome data places it in the phylum "*Chloroflexi*" (Kunisawa, 2011). Therefore, all 16 genomes shown above, which form a monophyletic branch, may correspond to a redefined phylum "Chloroflexi". We anticipate this revision in future editions of *Bergey's Manual*. The fact that "*Chloroflexi*" did not appear in the list of convergent phyla was caused by lagging taxonomic information at NCBI, on which the CVTree collapsing procedure depends.

As a detailed taxon by taxon comparison of CVTree phylogeny with prokaryote taxonomy will be published elsewhere at a later time we omit similar but much longer lists of order, family, genus, and species convergence and touch on an advanced feature of CVTree that goes beyond the reach of 16S rRNA analysis, namely, its high resolving power for strains.

Limitations of 16S rRNA analysis

"Although 16S phylogeny is arguably excellent for classification of *Bacteria* and *Archaea* from the domain level down to the family or genus, it lacks resolution below that level" (Staley, 2006; Fox et al., 1992). "The single category for which SSU sequence divergences cannot provide a sharp resolution is species" (Yarza et al., 2008). Fortunately, whole-genome based CVTrees are able to provide high resolution at the species and infrasubspecific levels which are urgently needed in ecological, environmental, clinical, and pharmaceutical studies.

Let us first look at some excerpts from the genus convergence list:

Edwardsiella{2}	K3K4K5K6K7
Eggerthella{2}	K3K4K5K6K7
Ehrlichia{5}	K3K4K5K6K7
Erwinia{5}	----K5K6K7
Escherichia{30}	K3--------
Exiguobacterium{2}	K3K4K5K6K7
Flavobacterium{2}	K3K4K5K6K7
Francisella{10}	K3K4K5K6K7
Frankia{5}	--K4K5K6K7
… … … … … …	
Salinibacter{2}	K3K4K5K6K7
Salinispora{2}	K3K4K5K6K7
Salmonella{17}	K3K4K5K6K7
Serratia{3}	K3K4K5K6K7
Shewanella{20}	K3K4K5K6K7
Shigella{7}	K3K4------
Sinorhizobium{4}	K3K4K5K6K7

(where "… … …" denotes omitted lines in a long list.)

What caused the "bad" convergence of the genera *Escherichia*{30} and *Shigella*{7}? By going to the species level, we see

Escherichia coli{29}	K3K4K5K6K7
Shigella boydii{2}	K3K4K5K6K7
Shigella flexneri{3}	--K4K5K6K7

all form well-defined species. However, in two papers, published 10 years apart (Pupo et al., 2000; Zhou et al., 2010), the *Shigella* strains were shown by different methods to mixed with *E. coli* strains, thus violating the monophyleticity of the latter. The CVTree results may help to shed light on whether to put *Shigella* within the species *Escherichia coli* or just in the genus *Escherichia* as sister species to *Escherichia coli.*

Another example comes from the *Yersinia pestis* and *Yersinia pseudotuberculosis* problem. Although DNA–DNA hybridization method could not distinguish them as two genomespecies, a proposal to change the classification "was rejected by the Judicial Commission because of possible danger to public health if there was confusion regarding *Yersinia pestis*, the plague bacillus" (Brenner et al., 2003).

However, in CVTrees there is no confusion at all. At the present time there are 14 genomes belonging to the genus *Yersinia*, distributed in three species. They all form monophyletic clusters:

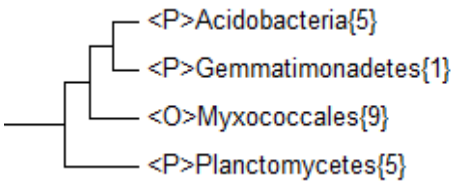
Yersinia{14}	K3K4K5K6K7
Yersinia pestis{8}	K3K4K5K6K7
Yersinia peudotuberculosis{4}	K3K4K5K6K7
Yersinia enterocolitica{2}	----K5K6K7

Therefore, the genus and the species are all well defined; *Yersinia pestis* and *Yersinia peudotuberculosis* are distinguishable species. There is no need for the Judicial Commission to be concerned.

On 1 October 2009, during a short visit to Bielefeld, the author used CVTree to obtain a quick confirmation of Alfred Phueler's conjecture that *Rhizobium lupini* H13-3 is actually an *Agrobacterium* strain very closely related to the strain C58 (Wibberg et al., 2011). This was just another episode of the *Agrobacterium–Rhizobium* debate (Young et al., 2001; Farrand et al., 2003; Young et al., 2003). In the 1404-genome CVTrees, 10 genomes of *Agrobacterium* and *Rhizobium* species, taken together, do form a monophyletic cluster, but the monophyleticity of *Agrobacterium* is violated by a monophyletic group of five *Rhizobium* strains.

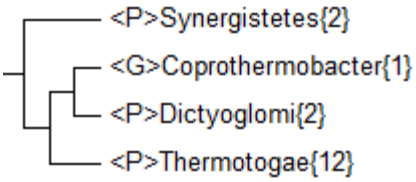
Possible new higher rank taxa

CVTrees may also provide clues about the interrelationships among higher rank taxa. We draw a few examples. As mentioned before, the class *Deltaproteobacteria* does not form a monophyletic branch in CVTrees. One of its orders, *Myxococcales*, represented by nine genomes at this time, escapes from the class as a whole and becomes juxtaposed with some other phyla as shown in the following branching scheme, cut out from a 1404-genome CVtree collapsed to the phylum level (where <P> and <O> indicate Phylum and Order):



Therefore, the *Myxococcales* may comprise a separate phylum.

As another example, we note that the genus *Coprothermobacter*, listed under *Firmicutes* in *Bergey's Manual of Systematic Bacteriology*, 2nd edition, vol. 3, earns itself a position at the phylum rank in CVTrees as follows:



This placement was supported by a 16S rRNA survey, denoting *Coprothermobacter* as an "established" phylum (Schloss et al., 2004).

Instead of continuing with this type of example to be verified by taxonomists in the future, we refer to some CVTree predictions that have been confirmed in the past few years.

Retrospective verification of CVTrees

In the CVTree approach there is no parameter adjustment or sequence selection, the only "change" consists in trees getting larger and larger with new genomes released. Over the years we have noticed that, as a rule, taxonomic revisions lead to better agreement with CVTree. This can be considered as retrospective justification of CVTrees. A few examples follow.

The genus *Oceanobacillus* was listed under *Gammaproteobacteria* (Garrity et al., 2002) and we had to admit this "cross-phylum discrepancy" in our manuscript (Hao et al., 2004) as it appeared in all CVTrees in the *Firmicutes* branch. Fortunately enough, by the time of proof-reading, we noticed that in the next release of the Taxonomic Outline (Garrity et al., 2003) it was moved to *Bacillaceae*.

The two genomes from the genus *Thiomicrospira* did not come together in CVTrees at all K values. While *Thiomicrospira crunogena* remains within *Gammaproteobacteria*, *Thiomicrospira denitrificans* joins *Epsilonproteobacteria* in all CVTrees (Sun et al., 2007). This species was renamed *Sulfurimonas denitrificans* and moved to the Epsilon group in TOBA Rel. 7.7 (Garrity et al., 2007).

In all CVTrees since 2004, the class *Mollicutes*, a monophyletic cluster of its own, never joins other classes of *Firmicutes*. We were encouraged to see that this class has been removed from the *Firmicutes* to become a separate phylum, *Tenericutes*, in *Bergey's Manual of Systematic Bacteriology*, 2nd edition, vol. 4. By the way, the genus *Erysipelothrix* assigned to a new class in the *Firmicutes* in the above volume may also belong to *Tenericutes* according to CVTrees.

Perhaps this is a suitable place to note that in a number of cases the CVTree phylogeny agrees with 16S rRNA analysis (e.g. Yarza et al., 2008) but both differ from the current classification. Besides the Beta group mixing with the Gamma group of the *Proteobacteria*, a phenomenon already mentioned, we see that *Ureaplasma* becomes inserted into *Mycoplasma*, and *Pedicoccus pentosaceus* becomes inserted into *Lactobacillus*, violating the monophyleticity of the latter two genera.

Conclusion and discussion

CVTree is clearly an effort "en route to a genome-based classification of *Archaea* and *Bacteria*" (Klenk et al., 2010). Using whole genomes is both a merit and a demerit of the approach. It is a merit in light of the genomic era when thousands of prokaryotic genomes will be available in the not-too-distant future and a genome contains the maximal amount of phylogenetic information at the molecular level. It is a demerit inasmuch as the number of sequenced genomes can never catch up with that of the 16S rRNA gene sequences. However, CVTree may serve as a complement to 16S rRNA and other phylogenies, helping place microbial systematics on a sound basis. When genome sequences become commonplace in microbial laboratories, it costs no more to use CVTree: just submit a genome to the Web Server and see where the sequence places the taxon. Then, if necessary, one can design more delicate phenotyping experiments to pursue a more in-depth study of phylogeny and taxonomy.

Regarding the relation of CVTree to other whole-genome based methods, we have mentioned the gene order study in connection with the undefined taxon *Thermobaculum*. Another topic worth mentioning is DNA–DNA hybridization performed by the program JSpecies (Richter et al., 2009) or the GGDC Web Server (Auch et al., 2010). We mention two cases of comparing the electronic DNA–DNA hybridization results with CVTree predictions. The first concerns a newly released genome of *Cellvibrio gilvus*, assigned to *Gammaproteobacteria* at NCBI; in CVTrees it joins the genus *Cellulomonas* with the phylum *Actinobacteria*. GGDC supports the CVTree placement. The second example is *Burkholderia* JV3 released in August 2011 at NCBI. CVTree and GGDC both indicate that it is actually another *Stenotrophomonas maltophilia* strain, not a *Burkholderia* at all. We note, however, both JSpecies and GGDC are not alignment-free, as they make use of BLAST results.

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