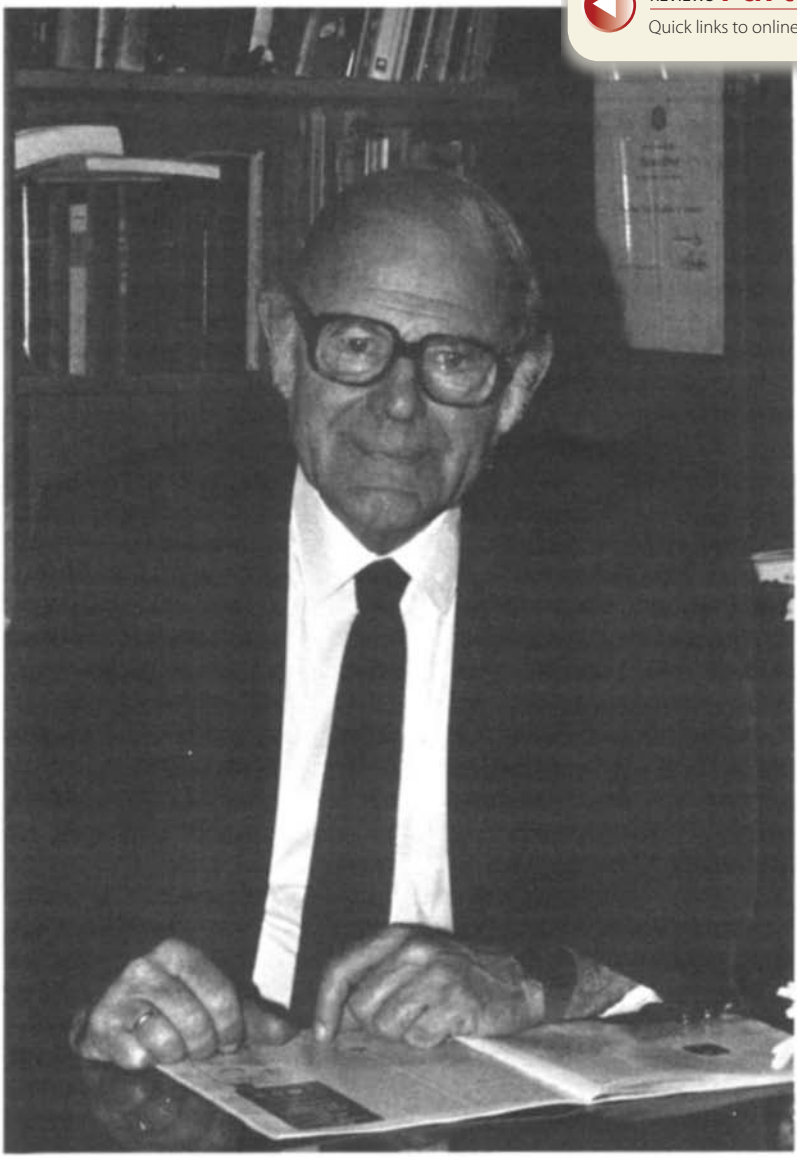

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MY LIFE WITH YEASTS

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To Marinka, 1916–1985

EARLY DAYS IN THE NETHERLANDS

I was born on May 30, 1913 in the small city of Winschoten in the northeastern province of Groningen where I attended grammar school and high school. My mother came from a farming family and my father was one of five sons of my grandfather who operated a fruit winery and vinegar factory. When he retired one of his sons took over the vinegar and distilled beverage operation while my father and his brother Jacob started and developed a separate business for the production of fruit wines, mainly from apples and berries. My

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father was interested in the trade and sales aspects and my uncle Jacob handled the technical and production end of the business. Both of my parents died at an early age while I was still in the beginning of the five-year high school curriculum. My brother and I were very fortunate to be adopted by my uncle Jacob's family.

I was happy in my new environment, which had a profound influence on my later life. I thoroughly enjoyed observing and participating in the operations of the winery, especially during the crushing, pressing, and fermentation stages. Long before I received my first formal training in microbiology, I learned how to use a microscope (a monocular, of course, in those days of the mid 1920s), to observe the budding and growing of the yeast cells, and to determine the alcohol content of the fermenting fruit juices. I also witnessed for the first time the spontaneous and massive development of acetic acid bacteria in a batch of berry juice pressed from overripe fruit that had begun to ferment by the indigenous yeast flora on the berries. Through microscopic examination of the spoiled juice I observed the coexistence of yeast and acetic acid bacteria.

My high school years also contributed in a major way to another aspect of my life, namely my interest in music. After I unsuccessfully attempted to study piano our family doctor, who was very active in the local symphony orchestra, suggested that I learn to play the cello as there was always a shortage of cellists in the orchestra. Fortunately, there was an excellent teacher in my hometown whose major instrument was the cello. I soon fell in love with my new activity and often practiced several hours each day. I have maintained this interest up to the present and have played in many symphony orchestras and numerous chamber groups. I might add that through musical activities I have probably made a greater diversity of friends than through scientific contacts.

The Dutch high school system with its five-year curriculum was considerably more rigorous in its requirements than its American counterpart, as I learned later on. Five years of German and French and four years of English were mandatory (there were no elective courses). These studies, combined with in-depth training in mathematics, chemistry, and physics and a modest amount of biology, culminated in a nationally administered final examination consisting of both written and oral parts. Passing of this examination automatically conferred eligibility to enter any of the national universities of the Netherlands.

In retrospect, considering my later interest in mycology, I would have had more useful training had I attended the local gymnasium with its six-year curriculum. This system offered, in addition to the subjects taught in high school, in-depth training in Latin and Greek. These languages would have been useful to me because new taxa of yeasts and fungi (falling under the Botanical Code) require descriptions in Latin for their publication to be valid.

At any rate, when in 1932 the time came to go to college I chose the Technical University of Delft, which offered various curricula in engineering. I majored in chemical engineering, mainly because of the eminence of A. J. Kluyver, Professor of General and Applied Microbiology, which was at that time a subdivision of chemical engineering. Kluyver's prominence and that of his famous predecessor Martinus Beyerinck had already come to my attention. Their reputations, combined with the possibility that I might later enter the family winery, made the training offered at Delft seem particularly appropriate. It was not, however, until I had taken numerous courses in chemistry, mathematics, physics, and engineering that I was allowed to choose an area of specialization within the field of chemical engineering. I chose technical microbiology so that I could benefit from Kluyver's leadership in that field.

After being advanced to candidacy at the end of four years of study, each student was required to carry out a research project and prepare a thesis (somewhat comparable to the American MS thesis). I worked on the elaboration of extracellular pectin-hydrolyzing enzymes by fungi. This subject was of interest to me because such enzymes (of commercial origin) were used in my family's winery to obtain higher juice yields during pressing and to facilitate filtration as a result of hydrolysis of the viscous fruit pectins. Although the scientific results of my study were limited, the work was important in that it stimulated my scientific curiosity. A significant factor was the direct personal interest Kluyver took in the research of all of his students and postdoctoral visitors. I also had the privilege of becoming acquainted with Charles Clifton, Robert Starkey, and Ben Volcani, who were guests in the laboratory at that time.

DEPARTURE TO CALIFORNIA

Kluyver was a great admirer of microbiological research carried out in the United States; his enthusiasm and my own curiosity about life in that country were instrumental in my decision to do postgraduate work there. The Division of Fruit Products (later known as the Department of Food Science and Technology) at the University of California, Berkeley appeared to be most appropriate for my area of interest and was willing to accept me as a visitor. Kluyver suggested that someone from Delft, a well-known center for research on the systematics and physiology of yeast, better obtain some training in the taxonomy of yeasts before going to California, so I spent several months in the fall of 1938, after having earned my degree of Chemical Engineer, learning something about the principles and practical aspects of identifying yeasts.

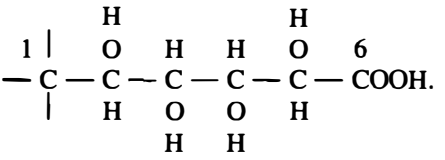
Upon my arrival in Berkeley in early 1939, I met Professors Cruess, Mrak, and Joslyn and other faculty in the department and I began participating in

some of the ongoing research projects. Maynard Joslyn soon suggested that to get the most out of my stay at the University of California I should continue my formal education and obtain a PhD in microbiology to supplement my earlier training in chemistry. I was fortunate to have entered the United States with an immigration visa, which was easy to obtain at that time, because the Second World War broke out the same year and the Netherlands was occupied a year later. I was able therefore to remain in California and to continue my education in the fall semester of 1939. Most of my supplementary course work was in biology and biochemistry, subjects in which my previous training had been deficient.

During my graduate years I had the privilege of becoming Emil Mrak's research assistant. The position was funded by the Agricultural Experiment Station, of which the department was a part. We isolated and identified the yeasts responsible for the spoilage of figs and dates in orchards of central and southern California (27, 28). This was my first foray in the area of yeast ecology and taxonomy.

University regulations stipulated at that time that Experiment Station research done under a research assistantship could not be credited for the PhD dissertation, so I chose Maynard Joslyn to guide my thesis research, which dealt with the elaboration of pectic enzymes by *Penicillium chrysogenum*. Midway in the research Professor H. A. Barker became my mentor because of Joslyn's absence during his service in the Army. "Nook," as he was affectionately called by his friends, became one of my lifelong friends. He had also spent a year in Kluyver's laboratory, but before I began my research there.

My thesis research showed that *P. chrysogenum* excreted in the medium two inducible hydrolytic enzymes required for the breakdown of pectin; pectin esterase (PE) removed methanol from pectin and polygalacturonase (PG) cleaved the glycosidic linkages of the polygalacturonic acid chain formed by the esterase. For their synthesis both enzymes responded simultaneously to a small number of inducers that included pectin, polygalacturonic acid, D-galacturonic acid, mucic acid, and L-galactonic acid, but not D-galactose, galactitol, or L-galacturonic acid. I concluded that the following configuration is essential for the synthesis of the two enzymes:



Substitutions on carbon 1 (aldehyde, alcohol, or carboxyl) did not change its specificity but the remainder of the stereochemical configuration was essential

for induction (32). At that time little was known about the induction of enzymes (then referred to as "adaptation") but evidently compounds containing the above configuration were able to bind to a repressor and to inactivate it, making transcription and elaboration of the enzymes possible by the derepressed system.

Among the highlights of my graduate career was the annual spring seminar at the home of Professor C. B. van Niel in Carmel, where students in microbiology from the Berkeley campus met with van Niel's students of the Hopkins Marine Station at Pacific Grove and presented informal talks about their research. Professor van Niel, the first and most prominent student of Kluyver, was a great inspiration to the graduate students as Barker had so aptly described earlier (4). There I also met for the first time the late Michael Doudoroff, who later became one of my best friends. These seminars were important not only because of their scientific content, but also because they taught us how to approach and analyze scientific problems critically.

By 1943 I completed my research for the PhD and I began applying to various universities for a faculty position (postdoctoral training was not common in those days). During my graduate student years I had already realized that my early plan to return to the Netherlands, even after the end of the war, and enter the family winery business was no longer attractive and challenging to me. I saw much greater opportunities for a scientific career in the United States. During the period of job applications, however, Professor W. V. Cruess, then chairman, offered me a position in the Department of Food Technology. This practice of hiring someone in the same department where he had obtained his PhD degree, without extensive advertising of the position, was not uncommon in those days. I gladly accepted the offer and have remained with the department until the present.

A FACULTY POSITION AT BERKELEY, 1943–1953

My early research years were rather heavily devoted to the development of improvements in the dehydration of fruits and vegetables, activities necessitated by the war and sponsored by the Departments of the Army and Navy. Under the leadership of Emil Mrak we developed a steam-blanching process whereby apricots, peaches, and pears could be efficiently dried in dehydraters (31) rather than by the traditional slow and sometimes unsanitary sun-drying process. Advances were also made in the dehydration of vegetables, particularly in the effective inactivation of oxidative enzymes that could cause early discoloration and off-flavor production in storage at elevated temperatures (33). We also developed a process for sterilizing dried prunes, figs, and dates of high moisture content with ethylene or propylene oxide so that the fruit could be consumed directly from plastic packages. Although these epoxides

are excellent germicidal compounds and decompose rapidly into their respective glycols, the Food and Drug Administration decided after a number of years that potentially harmful products could be formed by epoxides reacting with fruit components. While we were testing the killing action of ethylene oxide on a strain of *Saccharomyces cerevisiae* we discovered that when most cells had been killed the residual population contained a high proportion of cells that upon plating produced smaller colonies than those produced by the parental strain. The variant differed from the parental strain only in its inability to respire glucose or ethanol and its lack of cytochrome oxidase (62). We thus discovered nonrespiratory mutants of *S. cerevisiae,* and can only blame ourselves for not following up on this observation as Ephrussi and co-workers did later on (9a).

In 1948 I married Marinka Boratynski after failure of an earlier marriage from before my immigration to the United States. In the fall of 1951 I took my first six-month sabbatical leave and returned with my wife and young stepdaughter Sasha to my alma mater in Delft. The reason for this choice was that Mia Lodder and Nel Kreger–van Rij were preparing the first comprehensive treatise on the taxonomy of all yeasts known at that time (18a). The extensive collection of yeast strains at the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS) stored at the Delft Laboratory for Microbiology and the inspiring leadership of Professor A. J. Kluyver made this enormous task possible. For me, it was a valuable and rewarding experience to review the manuscript in preparation and to update my knowledge of the advances and status of yeast taxonomy. I also started a small project on the reasons for the delayed fermentation of sucrose by osmophilic, haploid species of the genus *Saccharomyces (Zygosaccharomyces),* which I later published with D. Pappagianis (29), then a student in the Department of Food Technology at Berkeley. Besides providing scientific value, my leave gave me the opportunity to reestablish family ties that had almost been totally disrupted during the Second World War, which unfortunately had claimed a number of casualties among members of my family as a result of their activities in the resistance movement.

While in the Netherlands I received a letter from the dean of the College of Agriculture in Berkeley with the announcement that the Department of Food Technology was going to be transferred to the Davis campus of the University of California. Although space would be immensely improved in the new building, I had considerable misgivings about the move to a small campus with very little cultural activity and few strong science departments. In addition we had just built a house in Berkeley in an attractive location and we would have to go through this process once more in the flatlands of the Sacramento Valley.

Nevertheless, upon our return to Berkeley in 1952 we began planning for

the move to Davis. Through my wife Marinka's initiative we obtained the service of architect Rowan Maiden, a protégé of Frank Lloyd Wright, who designed a most interesting house which was completed in July of 1953. Since the official departmental move was made on July 1, 1952, I commuted to Davis by train three times weekly to fulfill my teaching obligations while continuing my research in Berkeley in my old departmental laboratory.

Several years before the move to Davis, Emil Mrak had become chairman of the department, succeeding William V. Cruess; as a result the time available to Mrak for laboratory work became progressively less, although his interest in yeast ecology and taxonomy remained strong. In 1948 and 1949 Mrak and I had published two extensive reviews on yeast dealing with broad biological aspects and with sexual reproduction and ascosporulation (26, 43, 43a). Together, we had been teaching a one-semester upper-division course with laboratory at Berkeley that was totally devoted to the taxonomy, ecology, and physiology of yeasts—one of the few or perhaps the only course of this kind in the United States. We transferred the course to Davis, made it a graduate course, and continued to be its instructors.

CONTINUATION OF MY CAREER AT DAVIS

At the time of my transfer to Davis I obtained tenure and decided to concentrate my research efforts in the broad area of yeasts and to disassociate myself from the applied research pertaining to dried fruits. Although the latter with its field work was enjoyable and productive, it virtually eliminated the summer for basic research and writing.

I was fortunate in my final years in Berkeley to attract three outstanding graduate students for the PhD program in microbiology: Bor Shiun Luh, a native of China, Moshe Shifrine, a native of Israel, and Arnold Demain from Michigan. Luh and I began a survey of various yeast species for the production of pectic enzymes. Because many species of filamentous fungi were known to produce these enzymes (including *Penicillium chrysogenum,* which I had used in my own doctoral research) we were interested to determine if there might be yeasts endowed with this ability and, if so, to characterize such enzymes. Screening was based on the ability of the various yeast species to clarify a pectin medium containing 0.2% glucose. *Saccharomyces fragilis* (later reclassified as *Kluyveromyces fragilis*) was one of the very few yeasts with this ability but the pectin molecule was only very slightly degraded. Polygalacturonic acid was hydrolyzed more extensively but not to completion. The reason for the very limited pectin hydrolysis was found to be the inability of *K. fragilis* to synthesize pectin esterase. Not only did the yeast polygalacturonase require de-esterified pectin as substrate, but we found that the end products consisted of tri-, di-, and monogalacturonic acids rather than

D-galacturonic acid as in the case of most fungi. Luh and I also developed a method to isolate pure di- and trigalacturonic acid from a polygalacturonic acid hydrolysate using the yeast enzyme, based on the differential solubilities of the acids as strontium and lead salts (38). These oligogalacturonides had not been isolated prior to that time and we beat a group of Swiss investigators in this effort by only a short time.

Further study of yeast polygalacturonase was done by Arnold Demain. We showed that the enzyme produced by *K. fragilis* was constitutive and was secreted in a synthetic glucose culture medium as a single species of pectic enzyme in nearly pure form (36). Surprisingly, the yeast was found to be unable to utilize D-galacturonic acid as a sole carbon source. We concluded that its enzymatic ability on polygalacturonic acid may have been a vestigial evolutionary property from a related pectolytic microorganism. Demain also developed the concept of affinity adsorption for concentrating this enzyme; he used insoluble pectic acid in suspension for its adsorption and eluted the enzyme in the cold with 1 M acetate buffer. By stopping the polygalacturonic acid hydrolysis at the stage where tetragalacturonic acid was the largest oligogalacturonide, we found it possible to isolate the tetramer as its cupric salt and the pentamer by paper chromatography. The availability of oligogalacturonides in substrate quantities enabled us to do kinetic studies and to determine the pattern of action of yeast polygalacturonase on the polymer as well as its oligouronides (7). Demain and I found that the hydrolysis of polygalacturonic acid takes place by the following series of reactions.

(*a*) Pectic acid → penta- + tetra- + tri- + digalacturonic acids (random hydrolysis);
(*b*) Pentagalacturonic acid → tetra- + galacturonic acids as well as tri- + digalacturonic acids;
(*c*) Tetragalacturonic acid → tri- + galacturonic acids;
(*d*) Trigalacturonic acid → di- + galacturonic acids.

Reactions *a* and *b* comprise the initial rapid linear phase of the hydrolysis. The optimum pH of *a* is 4.4. A slower linear phase, beginning at 25% hydrolysis, is characterized by reaction *c*, which is optimal at a pH of approximately 3.5. After about 50% hydrolysis, *d* is the main reaction, and it occurs at a very low rate. Finally, after 70% hydrolysis the reaction ceases, leaving the dimer and monomer as end products. Reaction *d*, like *c*, is much slower at the higher pH; hence at pH 4.5 the reaction appears to cease at 50% hydrolysis and the apparent end products are tri-, di-, and monogalacturonic acids.

It remained to be determined which glycosidic bonds in these oligogalacturonides were preferentially cleaved. This problem was solved by another graduate student, Denish Patel from India, who modified the reducing end of

the oligomers by oxidation to a carboxyl group or by reduction to an alcohol, and used these modified compounds as substrates for yeast polygalacturonase. By comparing the products of hydrolysis with those of the unmodified substrates we found that tetra- and trigalacturonic acids undergo terminal hydrolysis solely at the terminal, reducing galacturonic acid unit (30). Pentagalacturonic acid is the smallest substrate in which both the first and second glycosidic bonds from the reducing end are hydrolyzed.

Since *Kluyveromyces fragilis* also has the ability to ferment the linear fructose polymer inulin, Harry Snyder, another graduate student, determined the characteristics of the extracellular, inducible enzyme responsible for the breakdown of inulin (54). We found that the yeast enzyme was an exoenzyme, successively hydrolyzing single fructose units from a chain connected by β-(2→1) linkages until the chain was fully degraded, releasing a single glucose molecule from its end. By following the rate of glucose release with a glucose oxidase reagent, we demonstrated that the enzyme operated by the so-called single-chain mechanism. Yeast inulinase also hydrolyzed the outer chains of branched bacterial levan in which the fructose molecules are linked by β-(2→6) bonds. As a result of this last property yeast inulinase differs from the inulinase in Jerusalem artichoke tubers (in which the main storage carbohydrate is inulin), which can hydrolyze only the β-(1→2) bonds of inulin.

About 1957, researchers had discovered a new mechanism of pectin and pectic acid breakdown by bacteria in which the galacturonic acid residues are cleaved by enzymes known as *trans*-eliminases or lyases. In my laboratory we investigated such enzymes from two sources, the bacterium *Clostridium multifermentans* and the fungus *Aspergillus fonsecaeus*. The bacterial enzymes originally discovered in this area were random-splitting or endoenzymes. Graduate student James Macmillan and I described and characterized an exolyase from *C. multifermentans* that was specific for polygalacturonic acid; the exolyase sequentially split units of α, β-unsaturated digalacturonic acid from the reducing end of this substrate (19). Since the reaction goes to completion, this enzyme proved extremely useful; it enabled us to prepare pure unsaturated digalacturonic acid, a compound not available at that time, in large quantities.

Ron Edstrom, another graduate student, and I purified an extracellular, random-splitting endolyase from *Aspergillus fonsecaeus* that was specific for pectin rather than pectic acid. Fully methylated pectin (polymethyl polygalacturonate methyl glycoside) was the best substrate. When the rate of cleavage became very low, the products consisted of a mixture of unsaturated methylated oligogalacturonides with a degree of polymerization from 2 to 5. We then prepared substrate quantities of methylated derivatives of oligogalacturonides, as they were available in our laboratory as products of yeast

polygalacturonase. The methyl ester of hexagalacturonic acid produced the methyl esters of normal and unsaturated trigalacturonic acid. The pentamer ester was split into unsaturated trimethyl trigalacturonate plus the diester of digalacturonic acid, while the tetramer ester yielded unsaturated trimethyl trigalacturonate plus methyl galacturonate (9). The enzyme did not split the methyl esters of di- and trigalacturonic acid. The fungal lyase therefore had a clear tendency to split the third bond from the reducing end in the various oligomers, whereas it cleaved the polymer in a more random manner.

The studies on pectic enzymes described here and two excursions into fruit polygalacturonases were gratifying because well-defined substrates and highly purified enzymes were used, rather than those used in so many earlier studies in which experimental conditions were less precisely established. These biochemical studies were accompanied by a series of explorations of the natural habitats of yeasts, which led to the discovery of a number of new yeast species.

EARLY ECOLOGICAL FORAYS, 1948–1959

Although Mrak and I had looked into the yeasts responsible for the spoilage of various food products and those isolated as contaminants of commercial fermentation processes, I developed a greater interest in yeasts found in nature. Much less was known about yeasts associated with various plants and animals. Numerous yeasts had been isolated and described from various clinical materials, but the taxonomy and the elucidation of their natural habitats were in most cases confusing and not well established.

Mrak and I conducted an early survey of yeasts associated with freshly caught shrimp in the Gulf of Mexico. Among the marine yeasts isolated several were thought to represent new species, but only one, *Candida diddensii* (originally described as *Trichosporon diddensii*), is still a recognized species.

One of my early graduate students, Moshe Shifrine, looked into the yeasts associated with bark beetles of the genera *Ips* and *Dendroctonus,* which often cause massive destruction of *Pinus* spp. in California. Although such an association was known in Europe and in other parts of the United States, it was interesting to extend this information to the West Coast mountains and to make use of the newer yeast taxonomy of the 1952 treatise by Lodder and Kreger–van Rij (18a). Several species already known from this habitat in other geographic regions were identified but a number of new species were discovered as well. These included *Pichia haplophila, Torulopsis nitratophila,* and *Candida silvicola* (52). The last species was the most common, but Wickerham, who showed it to be a haploid, heterothallic yeast that produced

hat-shaped spores upon mixing of appropriate mating types, later placed it in the genus *Hansenula* as *H. holstii*.

The bark beetle yeast survey was Shifrine's MS thesis; he continued with a doctoral dissertation on the biology of a budding yeast regularly found in the stomach contents and feces of domestic rabbits (53). Although it had been observed microscopically in fecal material as early as 1845, when we began our study no one had yet been able to obtain this large budding yeast in culture. Shifrine found that the yeast could be enriched at 37°C in a liquid medium of yeast autolysate-proteose peptone-glucose adjusted to pH 3.0 when inoculated with rabbit pellets or preferably with the soft night feces that have a higher viable yeast concentration. Although the yeast multiplied vigorously in this medium, streaking on an agar medium of the same composition produced no colonies. Thinking that the medium must have dried out too rapidly at 37°C, Shifrine then incubated the plates in a small desiccator jar with some water in the bottom. Under these conditions colonies finally developed that could be restreaked and incubated under the same conditions, so that finally pure cultures on slants became a reality. Although at about the same time Parle in New Zealand also obtained pure cultures in a liquid medium by serial transfer, he too was unable to obtain colony development in an incubator on agar surfaces or in pour plates. The puzzle of why this yeast would grow only on agar plates in a small desiccator was finally solved a few years later by Richle & Scholer in Germany (48a). They discovered that the yeast had an unusual requirement for atmospheric carbon dioxide. The moisture in our desiccator was not the crucial factor; the yeast by its fermentative ability produced enough CO_2 in this closed environment (as it also does in liquid media) to permit growth and colony development.

The yeast under discussion has had a number of different names, most commonly *Saccharomycopsis guttulata,* given by Schiönning. In 1971 it was placed in a new genus by van der Walt and became *Cyniclomyces guttulatus*. The yeast is highly specific for rabbits and is maintained in the intestinal system by coprophagy, the consumption of the soft night feces by adults as well as weanlings. Shifrine and I found that ascosporulation occurred only at about 18–20°C, whereas vegetative growth in liquid cultures took place only between 30° and 40°C. We also found that the cultures of *C. guttulatus* were very short-lived at room temperature or above. Survival of cells was better at 5°C; a sporulated culture survived at that temperature for at least six months.

The effect of CO_2 on growth of *C. guttulatus* was studied in more detail by another graduate student, Ed Buecher (5). He used a gas mixing apparatus and a sparger in liquid medium to introduce various mixtures of CO_2, O_2, and N_2. It was found that at oxygen concentrations between 2 and 20% the growth rate was maximal when sufficient CO_2 was present, but without O_2 the cells grew

poorly, granulated, and died early. The growth rate in 2% O_2 increased proportionately with the CO_2 concentration until the latter reached at least 10–15%. With an optimal gas mixture the growth rates at 37°C were about the same at pH 2.0, 4.0, and 5.6 but the cells were longer-lived at pH 5.6. In the rabbit, where most of the growth occurs in the stomach at low pH, the cells soon move to the intestinal tract where growth stops when the pH reaches 7.0 or higher. Our group's involvement in the elucidation of the life cycle, physiology, and ecology of *C. guttulatus,* which had remained a mystery for over a hundred years, was truly exciting and rewarding.

My interest in yeast ecology then turned to species of *Drosophila* that require a source of yeast to complete their life cycles. Theodosius Dobzhansky, who had for many years actively followed chromosome changes in wild species of *Drosophila* in the Sierra Nevada of California, approached Emil Mrak and me with a proposal to study the yeasts that these flies might have in their crops. Since the feeding habits of these flies were not known, such a project not only would reveal what kinds of yeasts were present in these mountain areas (4000–10,000 ft), but also might give clues to the feeding substrates of these flies if the survey were combined with a sampling of local substrates that constitute yeast habitats.

We performed the first survey of this type with a graduate student from Egypt, El Tabey Shehata, principally in the southern California Sierra. In this study we collected the flies by attracting them to banana that had been fermented with baker's yeast and screened carefully with cheesecloth to prevent access of the flies to the bait. The flies were taken to the Berkeley laboratory under refrigeration to slow down or prevent rapid digestion of the yeasts in the alimentary canal. The crops were then removed and streaked on agar medium at pH 3.7 to reduce bacterial growth. Fungi were rarely present. This first survey was highly successful in terms of new species found in the flies but no definitive correlation was found between the yeasts from flies and those from several suspected feeding substrates (51). The new species were named *Saccharomyces drosophilarum, S. dobzhanskii,* and *S. phaseolosporus;* all three were later transferred to the genus *Kluyveromyces.*

The *Drosophila* work was continued in the Yosemite area of the Sierra Nevada with the help of Moshe Shifrine and a new graduate student of mine, Martin Miller. Also involved was Elisa Knapp, a visiting scientist from Brazil. The approach was generally the same as in the previous study except that the flies were dissected in the field within two hours of capture. The principal substrate samples in the environment were slime exudates of red firs and oaks that were considered possible breeding and/or feeding sites for the two main species of *Drosophila* in that area, *D. pseudoobscura* and *D. persimilis* (6). The most common yeast species from exudates and related sources were *Pichia pastoris* (then known as *Saccharomyces pastori), Pichia*

fluxuum (a new species initially described as *Debaryomyces fluxorum),* an asporogenous yeast phenotypically resembling *Hansenula mrakii,* and *Pichia silvestris* (later considered synonymous with *P. membranaefaciens*). Two additional new species were *Pichia quercuum* and *Pichia carsonii.* It may be of interest to bacteriologists that *P. carsonii* was considered at one time synonymous with *Pichia vini. P. vini* would have had priority if its authors had provided a Latin description, but as this was not the case this specific epithet became invalid under the Botanical Code. The 240 yeast isolates from *Drosophila* in the same area presented a different picture; there was almost no overlap with isolates from the environmental sources (40). Some of the species were the same as those found in southern California: *Kluyveromyces veronae* (syn, *K. thermotolerans*), *K. drosophilarum,* and *K. dobzhanskii.* Several new species were also isolated and described, including *Kluyveromyces wickerhamii, Saccharomyces kluyveri,* and *Trichosporon aculeatum.* Our results clearly indicated that adult *Drosophila* did not feed on the yeasts found in tree exudates or on yeasts associated with pine bark beetles (the subject of Shifrine's earlier study) although there is some evidence that adults occasionally use tree fluxes for oviposition. The presence of *Torulopsis stellata* and apiculate yeasts of the genera *Hanseniaspora* and *Kloeckera* in the crops of adults suggests that they periodically feed on rotting fruit of wild shrubs and trees in which such yeasts are commonly present. The main question, however, remains unanswered; this concerns the identity of the principal food sources that contain most of the yeasts found in crops of adult *Drosophila* in the California mountains. Nevertheless, wild species of *Drosophila* are excellent scouts to tell us something about the yeast flora present in a particular area. It is known, however, that *Drosophila* is differentially attracted to different yeast species (8). Thus the yeasts found in the alimentary tract may not be representative of the total yeast flora in a particular area.

EXPLORATIONS OF ENZYMES THAT AFFECT THE INTEGRITY OF THE YEAST CELL ENVELOPE

About 1960 my research interests in the biochemical area shifted from pectolytic enzymes to those that are capable of hydrolyzing the cell walls of yeasts. The rationale for exploring this area was to ascertain if determining the composition of the cell envelope of different yeasts by their susceptibility to specific hydrolytic enzymes might contribute to a better understanding of relationships among species and genera. My first review on yeast cell walls was published in 1963 (34) and was followed by a more detailed one in 1971 (35). These provided a basis from which to start, but also pointed out how incomplete our knowledge of this area still was at that time.

My first graduate student to explore this area was Hirosato Tanaka from

Japan. Tanaka began by preparing purified baker's yeast cell walls and used them as substrate in agar media to isolate bacteria that could hydrolyze the walls as indicated by clear zones around the colonies. From among a number of different microorganisms that showed activity we selected a strain of *Bacillus circulans* because of its strong extracellular lytic activity on walls of *Saccharomyces cerevisiae* (59). Only cell walls induced lytic activity. In a liquid mineral medium under shaking conditions the cell walls lysed and in the supernated fluid we demonstrated β-(1→3)-glucanase (substrate laminarin) and β-(1→6)-glucanase (substrate pustulan). The water-soluble mannan wall component was not attacked by *B. circulans* and could be quantitatively recovered from the supernatant culture fluid in a less degraded form than is possible by hot alkali extraction of walls. We then separated the two glucanase activities from each other by DEAE-cellulose chromatography. We applied these enzymes separately and together to wall suspensions of other yeast species (60) and observed profound differences in sensitivity. For example, *S. cerevisiae* showed complete lysis by β-(1→3)-glucanase and weak and incomplete lysis (clearing) by β-(1→6)-glucanase; *Hansenula anomala* showed complete lysis by each of the glucanases, while *Schizosaccharomyces* walls were poorly attacked by each or a combination of the two. These and other responses indicated the usefulness of such enzymatic analyses (including reverse experiments with cell wall inducers from other species) in making taxonomic interpretations and in drawing qualitative conclusions on cell wall composition.

However, the interpretation of the lytic activities turned out to be more complex than had been thought originally. Graham Fleet, a student from Australia, repeated the isolation of the two β-glucanases described above, but included several additional purification steps (10). He then determined the pattern of action of the two enzymes on laminarin and pustulan. To our surprise, however, the enzymes, either alone or in combination, no longer lysed baker's yeast cell walls. Since the crude mixture had done so, we concluded that during purification one or more lytic components of the enzyme complex had been removed. Frank Rombouts, a postdoctoral visitor from the Netherlands, re-analyzed the crude culture fluid that had lysed the cell wall substrate. He applied the principle of affinity adsorption: He treated the culture fluid with a slurry of alkali-insoluble yeast glucan, centrifuged the glucan, and allowed self-digestion of the glucan at pH 6.0. This digest, which exhibited lytic activity, was then chromatographed and resolved into one lytic β-(1→6)-glucanase and two lytic β-(1→3)-glucanases, which were further purified and characterized (49, 50). We postulated that the lytic process is initiated by a synergistic action of the three lytic glucanases and that the two nonlytic glucanases described above participate in the further hydrolysis of the soluble products to glucose, laminaribiose, and gentiobiose.

Tanaka had shown (60) that the crude mixture of β-glucanases induced by walls of *S. cerevisiae* produced only a partial lysis of cell walls of species of *Schizosaccharomyces*. Species of this genus are known to contain a major additional component in their walls, (1→3)-α-glucan. Melvin Meyer, a later graduate student, and I investigated the ability of *B. circulans* to produce an enzyme capable of hydrolyzing this polysaccharide by growing the bacterium on *Schizosaccharomyces* cell walls or on a highly purified (1→3)-α-glucan isolated from the mycelium of the fungus *Aspergillus niger*. We demonstrated endo-(1→3)-α-glucanase in the culture fluid, and Meyer purified the enzyme using affinity adsorption in the cold on colloidal (1→3)-α-glucan followed by column chromatography after autohydrolysis of the adsorption substrate (22). Because (1→3)-α-glucan is insoluble at pH 7.5, the optimum pH for this glucanase, we introduced the minimal number of carboxymethyl groups in the assay substrate to solubilize it. The advantage of having available (1→3)-α-glucanase is that it can be added to lytic enzymes used for the production of protoplasts that lack this enzyme (e.g. snail digesive fluid). Enzymes supplemented in this way can then be used for the production of protoplasts from *Schizosaccharomyces* or other yeasts that contain (1→3)-α-glucan in their cell walls.

Our interest in cell wall lysis was not limited to exogenous microbial enzymes for the removal of cell walls from yeasts. We also became interested in endogenous yeast glucanases that are involved in the lysis of ascus walls of yeasts whose ascospores are rapidly released from asci (the former cell walls) upon reaching maturity, as well as those involved in sexual conjugation (fusion of two separate cells).

Ahmed Abd-El-Al, an Egyptian graduate student of mine, was the first to study this problem in depth (1). In extracts and supernatant culture fluids of several yeasts he identified and purified several exo-β-glucanases that removed glucosyl units sequentially from laminarin as well as from pustulan. The maximum velocity of hydrolysis and the affinity of the enzymes for these substrates were greatest in yeasts whose asci lysed rapidly, so these nonspecific exo-β-glucanases were considered responsible for the rapid lysis of asci in *Kluyveromyces fragilis* and *Hansenula anomala*. However, their activity on isolated cell walls was found to be minimal. In a subsequent study of *Hanseniaspora valbyensis*, another yeast with rapidly lysing asci, we found only an endo-β-(1→3)-glucanase that was specific for laminarin and that caused random hydrolysis of this substrate (2).

Graham Fleet also worked on endogenous glucanases for his PhD research. He demonstrated an exo-β-glucanase in cell extracts and culture fluid of *Schizosaccharomyces versatilis*, but this enzyme had no lytic activity on autoclaved cell walls. He then discovered that cell walls of this yeast contained a tightly associated endo-β-(1→3)-glucanase that could be released

only by autohydrolysis of the walls (11). This enzyme caused extensive lysis of cell walls and presumably becomes activated during the fusion of haploid cells prior to sporulation and during ascus lysis following sporulation in the zygote. Similar endo-β-(1→3)-glucanases were found in wall preparations of a number of other yeast species. Their tight association with the cell wall explains why in our earlier studies (1) we detected only exo-β-glucanase in cell extracts and culture media.

A further study of *Kluyveromyces phaseolosporus* with Thomas Villa, a postdoctoral visitor from Spain, showed that this yeast not only produced the ubiquitous nonspecific exo-β-glucanase found in many other yeasts but, in addition, three other glucanases (61). Two were endo-β-(1→3)-glucanases and the third was an atypical exo-β-glucanase. A combination of the last three enzymes caused spheroplast formation in vegetative cells of this yeast. From our own work and that of a number of other laboratories it has now become clear that every yeast, when examined with sophisticated separation techniques, appears to contain a complex of various β-glucanases that is located primarily in the periplasmic space or in the cell wall itself. At appropriate times during asexual or sexual reproduction these enzymes become activated and contribute to wall softening during budding and conjugation or wall lysis during sporulation. Although the exact mechanism of activation is not fully understood, mating pheromones appear to be directly involved in wall extension and cell fusion during conjugation.

LIFE IN DAVIS IN THE 1960s AND NEW ADVENTURES IN YEAST ECOLOGY

Since my arrival in Davis in 1953 the campus and the community had begun to develop in a major way and most of my earlier misgivings about moving here proved to be unfounded. I still participated in a number of musical activities in the Bay Area but our own music department was slowly developing and began organizing informal chamber music performances. Emil Mrak had become chancellor of the Davis campus and was very supportive of the arts. I was appointed to the Committee for Arts and Lectures and ultimately became its chairman. It was most enjoyable to be involved in developing an arts program on the campus, meeting members of performing groups and entertaining them in our homes after performances. Special events in that period were visits with my friend Jerome Rosen of our music department to the Bay Area home of Darius Milhaud, who was commissioned to compose a symphony for the dedication of a new assembly hall on campus. The Symphonie Rurale was later performed there by the San Francisco Symphony Orchestra.

Although biochemical studies of yeasts continued to go well in my laboratory, ecologic and taxonomic aspects were not neglected. In the early 1960s Minoru Yoneyama came to Davis from Hiroshima University as a visiting scholar. We decided to study the yeast flora associated with bark beetles of the genus *Scolytus,* which attack primarily species of fir *(Abies)* and Douglas fir *(Pseudotsuga).* Species of these two tree genera are hosts to two species of *Scolytus* beetles, but in both beetles and in their larval galleries we found one principal haploid heterothallic yeast with a wide geographic distribution, which we named *Endomycopsis scolyti* (46). The sexual cycle of this yeast proved to be very difficult to demonstrate because mating was very rare when groups of strains were mixed on the sporulation medium that we improvised from barley sprouts and fir frass. Because of Yoneyama's extreme patience and perseverence we finally recognized two strains that represented haploid mating types, but mating was exceptionally rare. We then decided to isolate a diploid strain of the yeast in the hope that the poor sporulation was due to a block in the conjugation process, considered prezygotic breakdown. Auxotrophic mutants were prepared of the two mating types and after mixing on sporulation medium the mixture was streaked on minimal medium and a small number of prototrophic diploid colonies were isolated. To our delight these sporulated abundantly, usually with two to four hat-shaped spores per ascus. The new species was later reclassified as *Pichia scolyti.*

At about the same time another visitor came to my laboratory, Lidia do Carmo-Sousa from Portugal. In one of her projects she demonstrated in cooperation with Yoneyama (47) that the yeast community in a particular slime exudate of an *Ulmus* species on the Davis campus was approximately constant over a 12-month sampling period, with *Pichia pastoris* the dominant species and *Trichosporon penicillatum* in smaller numbers. *Prototheca moriformis,* a species of colorless *Chlorella,* was common in summer but disappeared during the winter months. In another project, do Carmo-Sousa and I studied some yeast isolates I had obtained in Oregon from bark beetle frass of the coastal hemlock *Tsuga heterophylla,* a tree species we had not studied for associated yeasts as yet. Although the sampling was small, four new yeast species were recognized besides *Hansenula capsulata,* a common species in beetle frass of *Pinus* spp. The new species were named *Cryptococcus skinneri, Candida oregonensis, Bullera tsugae,* and *Sporobolomyces singularis* (37). The last species was interesting because it lacked carotenoid pigments, in contrast to most species of the genus. In addition, the assimilatory pattern of *S. singularis* was unusual. Among the common sugars tested it assimilated only glucose, lactose, and cellobiose, but not galactose. To determine the fate of the galactose moiety when the yeast was grown on lactose, a chromatographic analysis was made of the culture fluid during growth. We found that galactose did not accumulate in the medium at low pH but instead a

new trisaccharide appeared, which seemed to be formed by transglycosylation of the galactosyl unit from the enzyme-galactosyl complex to a lactose acceptor molecule rather than to water. We believe this was the first observation of quantitative transglycosylation by a living yeast.

Transglycosylation in this yeast was studied in much greater detail a few years later when Frank Spencer from the Prairie Regional Laboratory in Canada spent a sabbatical year in my laboratory. In these studies, in cooperation with Phil Gorin, also from the Saskatoon laboratory, we demonstrated that at a pH of about 3.8 *S. singularis* grown on lactose transferred the galactosyl unit to lactose and attached it by a β-(1→4)-bond. Further transglycosylation resulted in formation of a tetrasaccharide in which the terminal galactosyl unit was also attached by the same linkage (12). To extend this remarkable synthetic ability *S. singularis* was also grown on lactose or on cellobiose with a large number of various monosaccharides as acceptors (13), and a whole array of new synthetic disaccharides was identified. The only requirement of the acceptor sugar or its derivative was the presence of two vicinal hydroxyl groups in the acceptor molecule. Chemical synthesis of such unusual disaccharides would be extremely difficult and laborious.

Frank Spencer, Martin Miller, and I identified and described two new yeast species that occur in mountainous regions of California, *Pichia trehalophila* from exudates of cottonwood trees and *Pichia salictaria* from insect frass in willows (41). The regular presence of interesting yeast species in slime exudates of trees induced Miller (who had then become a faculty member in Food Science) and me to apply to the National Science Foundation for a grant under the US-Japan Cooperative Science Program to do a broad study of the yeasts in tree exudates on all of the major Japanese islands and on the West Coast of North America. This program required the participation of an equal number of Japanese scientists who would be supported by the Japanese Society for the Promotion of Science. Our Japanese counterparts were Minoru Yoneyama, who had previously worked in my laboratory, and Masumi Soneda. We were fortunate to receive funding for two years and we began the first phase of the project in 1967 in Japan. It was a most interesting experience; we crisscrossed the Japanese islands for about two months by train and locally by jeep, mainly in forested mountain areas, staying in forest lodges, Japanese inns, and occasionally university dormitories. Our Japanese colleagues were extremely well organized in arranging for transportation and facilities. We carried out the yeast isolations in improvised field laboratories or occasionally in university laboratories. This trip gave Miller and me an insight into Japanese country life that few scientists with Japanese collaborators have had the privilege to enjoy.

In July of the next year, 1968, Miller and I took a university vehicle from

Davis to Anchorage, Alaska where we met our two Japanese colleagues and began collecting samples of tree exudates along Alaskan roads and the Alcan Highway, with various side trips through the Yukon Territory, British Columbia, and the states of Washington, Oregon, and California. During this trip the samples were streaked and analyzed in the field, usually in forest campsites and occasionally in a motel. Our Japanese colleagues were as impressed by the magnificent countryside we traveled through as Miller and I had been in Japan.

We collected about 400 yeast strains in Japan and somewhat more in North America. The monumental task of identifying so many yeast strains took several years but was facilitated considerably by a hand-operated multipoint inoculating device that was designed and built in the machine shop of the Department of Food Science. The results were finally presented at an international yeast symposium held jointly with the Fourth International Fermentation Symposium in Kyoto (42).

Many interesting results came of the two surveys. Because of the widely separated geographic areas and the very different tree floras in Japan and the Pacific Northwest some profound differences in yeast flora were noted. For example, *Dipodascus aggregatus* and *Nadsonia elongata* were extremely common in Japan but were not found in the Pacific Northwest. Conversely *Pichia pastoris* and two species of *Torulopsis* were common in the Pacific Northwest but were not found in Japan. We postulated that the differences in yeast flora may have been due to the presence or absence of certain insect vectors that utilize tree exudates in the two areas. Some isolates were difficult to classify while others represented new species.

In Japan we isolated a number of strains of a fermenting, carotenoid-producing species. This was most unusual since all carotenoid-containing yeasts up to that time were strictly respiratory. In addition, the principal carotenoid pigment in this yeast was identified as astaxanthin (3), which does not occur in other "red" yeasts but is present in the flesh of salmonids and certain crustaceans. I felt greatly honored when my three colleagues from the collecting team decided to create a new genus for the fermenting red yeasts and to name it *Phaffia* (25). *Phaffia rhodozyma* has some industrial potential as a dietary pigment source for pen-reared salmonids and crustaceans. The flesh of salmon reared in ponds is white owing to a lack of a natural source of astaxanthin. The astaxanthin from whole cells of *P. rhodozyma* is not available to fish, but when fish are fed with broken yeast their flesh becomes orange-red. Because the cell walls of this yeast are tough and difficult to break we tried using *Bacillus circulans* as a cell wall–degrading organism, as related earlier in this chapter; this resulted in complete extractability of the pigment (16).

ASSOCIATION WITH THE DEPARTMENT OF BACTERIOLOGY AND A BOOK ON YEAST

Toward the end of the 1960s a number of events took place that affected my career and professional activities at Davis. Emil Mrak and I were approached by Kenneth Thimann, then at Harvard University, to write a book on the biology of yeasts. Because of Emil's duties as chancellor of the Davis campus, we invited our colleague Martin Miller to be a co-author. We prepared a manuscript entitled *The Life of Yeasts* (39), written to give the nonspecialist a background on the broad aspects of yeast biology. The first edition was published by Harvard University Press in 1966; it was followed by a second, extensively revised edition in 1978. A Japanese translation was later prepared by my friend Susumu Nagai from Nara. The book was useful in our teaching program and also provided a background on aspects of yeasts, especially ecology, not generally known to biochemists, geneticists, and other specialists. In 1969 I was awarded the Faculty Research Lectureship on the Davis campus, an honor that came as a total surprise to me. I chose the title for my lecture, "Changing Aspects in Yeast Systematics," on the basis of my developing interest in molecular taxonomy of yeasts.

At about the same time I was asked to join the faculty of the Department of Bacteriology on a part-time basis and our graduate course on yeasts was transferred from the Department of Food Science to the Department of Bacteriology; in return a course on industrial microbiology was transferred to the Department of Food Science. In 1970 I was asked to serve as chairman of the bacteriology department, an assignment I accepted with mixed feelings because I did not think that administrative ability was one of my strong points and my research program was in another department. When the five years of my appointment were over my teaching appointment remained with the Department of Bacteriology, but I could spend more of my time on NIH-sponsored research, which then started to produce important results. In addition I had become editor of the *Yeast Newsletter,* which Emil Mrak had started in 1950. This publication provided a valuable service to the yeast community. The *Yeast Newsletter* also later became the official organ of the International Commission on Yeasts and Yeast-like Organisms under the International Union of Microbiological Societies, a commission that sponsored meetings on general and specialized aspects of yeasts. I also became an associate editor of the *International Journal of Systematic Bacteriology*. I enjoyed this activity since it broadened my contact with the bacterial world as my association with the Department of Bacteriology had before.

FROM TRADITIONAL TO MOLECULAR TAXONOMY

During the identification of the numerous yeast isolates from various natural habitats it became increasingly clear that in spite of significant improvements in the older taxonomic systems (18, 18a) species delimitations were often arbitrary and were sometimes based on only one or a few genes coding for hydrolytic enzymes that enabled a yeast to utilize certain di- and trisaccharides. Towards the end of the 1960s Sally Meyer decided to carry out her doctoral thesis work in my laboratory and on the basis of other very rewarding results in molecular bacterial systematics she encouraged me to apply to the National Institutes of Health for a research grant on molecular systematics of yeasts. I was fortunate to be funded and we were able to purchase the necessary equipment for studies on DNA base composition and DNA-DNA hybridization. Many technical difficulties had to be overcome because procedures that worked well with bacteria had to be radically modified for yeasts. However, through her efforts and those of later students and competent technicians the methodology for yeasts gradually became workable and yielded a number of significant contributions to the systematics of yeasts.

We began by determining the mean base composition of the nuclear DNA of various yeasts [guanine plus cytosine (GC) content] by the so-called melting technique or thermal denaturation procedure. Since the results obtained by this method can be markedly affected by impurities in the DNA or by the presence of minor DNA species we shifted to the buoyant density method using cesium chloride when an analytical ultracentrifuge became available in the department. Because this method is considerably less influenced by the above problems, the data became more accurate and reproducible. Our results and those of other laboratories (mainly in Japan) showed that the DNA base composition of well-documented species varied little and thus this information offered a valuable exclusionary function: A difference of more than about 1.0–1.5 mol% GC determined by buoyant density virtually precludes the sharing of similar base sequences between two yeasts and indicates that they are not recent descendents from a common ancestor. Yeasts with similar base composition could have similar or different base sequences and thus require comparison of their genomes by DNA-DNA hybridization or other methods. Sally Meyer and I also noted that with very few exceptions yeasts with GC contents greater than 50 mol% had basidiomycetous affinity whereas those with GC contents below 50% were ascomycetes or asporogenous forms thereof. A similar relationship did not apply to filamentous fungi. Yeasts with GC contents close to the 50% level could not be assigned with certainty. This information is especially helpful as

it offers one of several clues for relating asporogenous yeasts to one of the above classes (23).

As with determination of base composition, many initial problems had to be overcome in experiments on genome comparison involving determination of DNA complementarity. For example, it was necessary to isolate DNA with sufficiently high yield, to label reference DNA in vivo with a radioactive tracer, and to perfect the reannealing technique on nitrocellulose filters. Sally Meyer and I used tritium-labeled DNA on nitrocellulose filters. In spite of relatively low counts and leaching problems with the filter paper method, the new approach to taxonomy was beginning to give results that were contrary to previous taxonomic views. For example, certain varieties proved to be unrelated to their corresponding parental species, some postulated imperfect forms of sporogenous yeasts proved to be unrelated, and some accepted species were shown to be synonymous with others from which they were separated only by the assimilation of certain disaccharides (24).

Further work in this direction was done by Chet Price, whose doctoral dissertation dealt with species of *Saccharomyces (Torulaspora* group), *Pichia, Schwanniomyces,* and *Debaryomyces* (48). By that time we had begun labeling the reference DNA with ^{32}P in vivo, doing the reassociation in a liquid system and analyzing for duplex DNA by its adsorption on hydroxylapatite. This system allowed up to 85% reannealing of the reference DNA and gave highly reproducible information on DNA relatedness among the species tested. Again, many species that had been differentiated on relatively trivial grounds showed high DNA relatedness (80–100%) and were thus reduced to synonymy. Most of the results in that study revealed either very high or low DNA relatedness among the species tested. In only one case was an intermediate value obtained: *Debaryomyces subglobosus,* then considered a synonym of *D. hansenii,* showed only 40% DNA complementarity with the type strain of *D. hansenii,* suggesting that these two taxa had diverged only recently from each other. At that time we concluded that the technique of DNA hybridization showed mainly close kinships or distant relationships among yeast species. Further support for this view was obtained by Leda Mendonça-Hagler, a postdoctoral visitor from Brazil, whose DNA hybridization experiments demonstrated that four species of psychrophobic yeasts were very closely related and could be reduced to one sporogenous species, *Saccharomyces telluris,* and its imperfect form, *Torulopsis pintolopesei* (including the variety *slooffii*) (21). At about the same time another foreign visitor had joined my group, Alessandro Martini from the University of Perugia in Italy. He worked on the systematics of species in the genus *Kluyveromyces* and developed an optical method for DNA-DNA hybridization in yeasts (20), a procedure that does not require labeling of reference DNA with a radioactive tracer. This method was later perfected by Cletus Kurtzman in Peoria and

provided data in good agreement with those obtained by the use of radioactive reference DNA. Martini's results, supplemented later by those of Heather Presley and myself, eliminated a number of *Kluyveromyces* species as they were found to be synonyms of other earlier described species. Our findings demonstrated that the two spore morphologies in the genus (kidney shaped and spheroidal) were not significant taxonomically, and showed that strains of some species of different geographic origin showed significant genome divergence (65–100% DNA complementarity). The latter finding suggested that evolutionary divergence might be caused, at least in part, by allopatry, strain separation under the influence of the chemical makeup of different host plants or other environmental influences.

André Lachance, a graduate student from Canada, explored another molecular approach to the taxonomy of species of *Kluyveromyces*. Because of our previous discovery of the apparently universal presence of nonspecific exo-β-glucanases in various yeast species, Lachance made a comparative study of the molecular weights of this enzyme in different species and then determined the immunological relatedness of the enzymes by the microcomplement fixation technique (17). In general, there was an excellent correlation between our previous DNA relatedness data and the immunological enzyme comparison. He confirmed our earlier conclusion that in *Kluyveromyces* ascospore morphology was not a sound criterion for species differentiation.

ECOLOGICAL EXPLORATIONS IN CACTUS NECROSES

About 1970 Bill Heed from the University of Arizona in Tucson contacted me with the request to look at some yeast strains that he had isolated from rotting cactus tissue in the Sonoran Desert, the breeding site of certain desert-adapted species of *Drosophila*. These strains did not appear very interesting at first as they keyed by Lodder's taxonomic system (18) to *Pichia membranaefaciens*, a very common yeast found in deciduous tree exudates as well as a common film yeast in wine and fermented olives. However, a much larger number of strains isolated by Heed and his students between 1971 and 1974 from cactus rots and associated *Drosophila* species in southern Arizona and the state of Sonora and northern Baja California in Mexico revealed two new asporogenous species of yeasts that appeared to be specific in cactus necroses. These were named *Torulopsis sonorensis* and *Cryptococcus cereanus* (55). The last species was interesting because it showed multilateral budding and other characteristics typical of yeasts with ascomycetous affinity, whereas all other *Cryptococcus* species known at that time had basidiomycetous affinity. On the basis of its GC content (approximately 49 mol%) it was at the borderline between the two classes of fungi. The basis for assigning it to *Cryptococcus* was its strong growth on inositol as the sole carbon source (18).

Its taxonomic position was resolved in 1978 by Rodrigues de Miranda, who demonstrated ascosporulation in the type strain and placed it in a new genus, *Sporopachydermia* (48b).

Bill Heed spent a sabbatical year in 1971 at Davis and participated in the identification of the more than 300 yeast isolates from cactus. The most common yeast encountered (about 190 strains) again was initially identified as *Pichia membranaefaciens* on the basis of the key in Lodder's taxonomic treatise (18). Although the strains fitted that species well, we decided to determine GC contents of representative strains and found to our surprise that they fell into two levels that were 8 and 12 mol% lower than that of *P. membranaefaciens* (about 44 mol%). Strains with base composition of about 36 mol% were described as a new species, *Pichia cactophila* (57), that turned out to be the most cosmopolitan species among all cactophilic yeasts. Fortunately, we discovered a phenotypic trait to differentiate *P. cactophila* from *P. membranaefaciens;* this was the ability of the former to grow well when the sole carbon source was D-glucosamine, a compound rarely used for species differentiation at that time. However, the strains with a GC content of about 32 mol% were negative on D-glucosamine, like *P. membranaefaciens*. Thus far we have not found a simple phenotypic character except habitat to separate these new isolates, which were named *Pichia heedii* (44), from *P. membranaefaciens*. *P. heedii* is much more restricted in its habitat than *P. cactophila* and is found in only two species of columnar cactus, *Carnegiea gigantea* (saguaro) and *Pachycereus schottii* (senita).

Tom Starmer, who obtained his PhD degree with Bill Heed, joined my group in 1976 as a postdoctoral visitor, and our stimulating and productive collaborative effort on yeast ecology and evolution has continued to the present. Starmer, now on the faculty at Syracuse University in New York, and I continued to study the yeast-cactus-*Drosophila* ecosystem under a National Science Foundation cooperative research grant.

Collecting areas were greatly expanded to include Baja California, southern Mexico, northern Venezuela, 12 islands in the Caribbean Sea, Texas, Hawaii, and eastern Australia. Each survey yielded new species in addition to cosmopolitan cactus-specific yeasts already known from earlier studies (58). Most of the new species fitted in the genus *Pichia*. Although at first some were keyed to known species using Lodder's taxonomy (18), determinations of their DNA base compositions and DNA-DNA hybridizations showed that they were distinct and cactus-specific. This approach also showed that several of these species were evolutionarily related. For example, *Pichia pseudocactophila* (14), although it has the same GC content and the same physiological properties as *Pichia cactophila,* showed only about 35% DNA relatedness with the latter. In addition, Don Holzschu, my last PhD student, demonstrated

that the two species differed consistently in the electrophoretic mobility of four major metabolic enzymes. His technique has been helpful in distinguishing other closely related cactus-specific species (15), and has also been found useful for other yeasts by Japanese investigators.

The evolutionary divergence between the heterothallic, four-spored *P. pseudocactophila* and the homothallic, two-spored *P. cactophila* may have been influenced by host-plant chemistry. The former has been found in only two related species of columnar cacti (cardon and saguaro), while the latter is a host-plant generalist. Other effects of host plant chemistry have been noted. For example, the two varieties of *Pichia amethionina* (a naturally occurring methionine auxotroph found only in cacti) are found in alternate subtribes of cacti. *P. amethionina* var. *amethionina* occurs in species of the Stenocereinae that are rich in triterpene glycosides, whereas *P. amethionina* var. *pachycereana* occurs in species of the Pachycereinae that lack these compounds. The former variety is resistant to triterpene glycosides and the latter is sensitive; thus there is potential for gene pool separation and evolutionary divergence (56).

Because of evolutionary convergence among a number of new cactus-specific species with very similar physiological and morphological properties, we have been looking for new phenotypic properties for their separation. Growth on acetone, 2-propanol, ethyl acetate, or *N*-acetyl glucosamine as the sole carbon compound appears to be a useful character. For example, a recently described species, *Pichia deserticola* (45), resembles *P. cactophila* but can be distinguished by its strong growth on ethyl acetate and its lack of glucosamine utilization. The cactus-*Drosophila*-yeast ecosystem has given us a wealth of information on the evolution and speciation of yeasts that no other ecosystem has been able to supply.

CONCLUDING REMARKS

As may be evident from the preceding pages, my main research interest in microbiology ultimately developed into concern for ecological and resultant taxonomic questions. My early training in chemistry was undoubtedly influential in my interest in understanding metabolic pathways of unusual species recovered during my many ecological forays. Molecular approaches to the systematics of yeast also contributed greatly to the success of yeast studies in my laboratory.

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Unfortunately, space limitations do not permit me to acknowledge all of my many students and co-workers who have contributed to the story given here. I

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