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Bacteria of the genus Klebsiella have been found to be

associated with redwood water storage reservoirs, redwood

chips, and liquid expressed from within living redwood. A

1,000 gallon experimental redwood reservoir was employed to

determine how to control the presence of Klebsiella and

Enterobacter in drinking water stored in these tanks. Over

a twenty month period of operation, these coliforms were

not detected in tank water. Control of Klebsiella and

Enterobacter was dependent on three factors: maintenance of

a free chlorine residual of 0.2-0.4 ppm in the tank water,

maximum retention times of four to seven days, and the use

of a separate overhead inlet water pipe. Klebsiella

multiplied to 10^6 per ml in dechlorinated water samples from

the experimental tank, utilizing nutrients leached from the

redwood. The predominant metabolizable carbon sources in

aqueous extracts of redwood are cyclitol compounds, of which

myo-inositol is the parent compound. Pinitol and sequoyitol,
two other cyclitols, are also encountered in redwood. 100%,
97%, and 68% of the combined Klebsiella isolates from
clinical and environmental origins fermented inositol,
sequoyitol, and pinitol, respectively. These compounds can
also be used as a sole source of carbon and energy by
Klebsiella. Similar results were obtained with Enterobacter
isolates, but most other enteric bacteria tested could not
metabolize cyclitols. Strains of Klebsiella multiplied to
levels exceeding 10^5/ml in aqueous extracts of non-sterile
redwood within 6 days. Most other enteric bacteria did not
grow in these extracts. Cyclitol metabolism correlated well
with the ability to multiply in redwood extract in the
presence of cyclitol-negative indigenous bacteria. Other
experiments were conducted with three small redwood tanks
in order to assess the effects of chlorine residuals,
retention times, and cyclitol concentrations in redwood
tank water on coliforms and on Klebsiella. As chlorine
residuals were increased, coliform and total bacterial
counts decreased. Longer retention times resulted in the
accumulation of cyclitol compounds and a decrease in
chlorine residuals. A decrease in cyclitol concentration
was found to correlate with a drop in coliform and
Klebsiella densities. No cyclitol degradation by
indigenous coliforms was observed in water taken from the

tanks, which partially explained why water flow is required to completely remove cyclitols from a redwood tank. In another study, gas chromatographic analysis was employed to demonstrate in situ cyclitol utilization in redwood extracts by isolates of Klebsiella, Enterobacter, and other gram-negative bacteria. In aqueous redwood extracts, all of the Klebsiella tested reached densities exceeding 5.0×10^6 cells/ml within 4 days and all utilized pinitol and sequoyitol, as did Enterobacter. Other enteric bacteria did not utilize cyclitols in this extract. A defined minimal medium, containing the carbohydrates and cyclitols in redwood, was used to determine which carbon sources are preferentially utilized by Klebsielleae and other bacteria. It was found that D-glucose and L-arabinose were consumed by Klebsiella before the cyclitols were utilized. Pinitol utilization proceeded more slowly than that of sequoyitol and myo-inositol. Cyclitol utilization in the defined medium was also observed for strains of Yersinia, Erwinia, and Salmonella. E. coli isolates did not utilize cyclitols. The ability to use cyclitols as a carbon source can explain the presence of Klebsiella and Enterobacter in redwood water storage reservoirs and in redwood lumber. This ability may also be related to their presence and growth in other botanical material containing cyclitol compounds. Other experiments demonstrated the utilization of myo-inositol

hexaphosphate (phytic acid) by Klebsielleae, and the transfer of antibiotic resistance between strains of Klebsiella in aqueous extracts of redwood sawdust with cell densities of Klebsiella likely to occur in nature.

NUTRITIONAL FACTORS ASSOCIATED WITH THE PRESENCE AND GROWTH
OF <u>KLEBSIELLA</u> IN REDWOOD ENVIRONMENTS

by

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Chapter 1

Introduction

Coliform bacteria of the genus Klebsiella are unique among the Enterobacteriaceae, because they occupy such a broad range of habitats. Klebsiella is an opportunistic pathogen (16,20,30,50,67,68), especially implicated in nosocomial infections (16,20,30,50,67), and it also causes diseases (including mastitis) in domestic animals (13,26,37, 46,71). In addition, Klebsiella is found in significant numbers in many botanical environments, usually outnumbering other enteric bacteria (2,12,14,23,24,28,32,38,41,43,48,51, 52,53).

Klebsiella isolated from environmental sources have been shown to be indistinguishable from pathogenic Klebsiella isolates, based on biochemical, serological, and DNA homology studies (44,58). In addition, environmental isolates have been shown to be potentially as pathogenic as clinical strains, based on virulence tests in mice (10,44). In one report, 17% of environmental Klebsiella were found to be fecal coliform (FC) positive (9), and in another study 16% of the environmental isolates tested possessed multiple antibiotic resistance (65).

There is no doubt about the significance of <u>Klebsiella</u>
in nosocomial infections. These organisms can cause respi-
ratory infections, genito-urinary infections, and surgical
wound infections (60), usually in debilitated or compromised
patients (60,63). <u>Klebsiella</u> has also been reported as a
cause of severe septicemia in cancer patients (68). It is
believed that <u>Klebsiella</u> initially colonizes the gastro-
intestinal tract, which can later lead to more disseminated
disease (47,60). In one study carried out with acutely
burned children in a hospital, the incidence of <u>Klebsiella</u>
in the intestinal tract of 58 of these patients was 77% (63).
In contrast, in a group of 104 patients in generally good
health, the incidence of <u>Klebsiella</u> was only 26%. <u>Klebsiella</u>
was first detected in the majority of the acute patients
after their stay in the hospital. Another investigation with
40 healthy infants admitted consecutively to a nursery showed
that within 4 days of admission, 90% of the infants became
gastro-intestinal tract carriers of a kanamycin-resistant
strain of <u>Klebsiella</u> (1). It has also been shown that
patients can become intestinal carriers of the same
serotype of <u>Klebsiella</u> that subsequently infects them;
antibiotic therapy selects for colonization by resistant
organisms (60).

The fact that a high percentage of <u>Klebsiella</u> isolated
in clinical situations are multiply antibiotic-resistant may

complicate the treatment of disease caused by these organisms (1,3,27,50). A large number of multiply antibiotic-resistant <u>Klebsiella</u> are capable of conjugal transfer of this resistance to other non-resistant strains via R-plasmids (3,27,31,57,69). The incidence of R-plasmid bearing strains of <u>Klebsiella</u> has risen greatly over the past several years, making this organism a greater health risk (27,31).

In addition to the diseases in humans caused by <u>Klebsiella</u>, there are several animal diseases where it has been implicated. <u>Klebsiella</u> is the primary agent of bovine coliform mastitis (13), and has caused infections in horses (46), monkeys (26), guinea pigs (37), and muskrats (71). It has also been found associated with small green pet turtles (45).

As mentioned earlier, <u>Klebsiella</u> (and another member of the <u>Klebsielleae</u> tribe, <u>Enterobacter</u>) are present in a wide range of botanical habitats. <u>Klebsielleae</u> have been found in samples of soil, pine needles, and bark in a forest environment, where they comprised 90% of the coliforms present (24). <u>Klebsiella</u> alone accounted for 71% of the coliforms. Some 35% of these <u>Klebsiella</u> were fecal coliform positive. In this same study, <u>Klebsiella</u> and <u>Enterobacter</u> were isolated from fresh produce. <u>Klebsiella</u> has also been reported in sugarcane wastes (51) and in pulp mill effluents (32,38,41), where the majority of coliforms isolated were <u>Klebsielleae</u> (no <u>Escherichia coli</u> were detected) and high cell densities

of Klebsiella were observed. Textile mill effluents have been a source of Klebsiella as well (23). Some of the pulp mill isolates possess the ability to fix nitrogen (41,48).

An association of Klebsiella with living trees has also been discovered. Klebsiella and Enterobacter have been isolated from living white fir trees (2), and these organisms have also been found within living redwood and hemlock (12). Other enteric bacteria such as Escherichia coli were not detected. Klebsiella isolated from fresh and used sawdust bedding has been implicated as a possible reservoir for mastitis-causing organisms (49).

The presence of Klebsiella on fresh vegetables consumed raw by humans is well documented. In one investigation, 50% of the vegetables examined and 7 of 7 seed samples yielded Klebsiella (14). Viable counts for this organism generally were about 10^3/gram of vegetable peel or seed. Other studies reported similar results with tomatoes, radishes, celery and carrots (24).

When botanical material, including vegetables, was inoculated with human as well as environmental strains of Klebsiella, it was observed that Klebsiella are capable of rapid multiplication on this material regardless of origin; Klebsiella grew to levels exceeding 10^6/ml in sterile pulp waste and 10^3/gram on surface peels of potatoes and on lettuce leaves (40). This is further proof of the biochemical homogeneity of Klebsiella, and in situ regrowth might be a

partial explanation as to why fecal coliform positive
Klebsiella can be isolated from habitats where recent
fecal contamination is not evident (14,24).

In light of the association of Klebsiella with fresh
vegetables, and the evidence that known pathogenic strains
as well as potentially pathogenic environmental strains can
grow on botanical material, the presence of Klebsiella on
produce consumed in hospitals is of concern, especially since
intestinal colonization of patients has been demonstrated.
It has been shown that Klebsiella can be present in densities
of 10^2-10^6/gram on vegetable salads prepared for patients (70).
Klebsiella has also been found on salads served at schools (62).
Flower vases present in surgical wards and burn units in
hospitals have yielded high densities of Klebsiella (66).
Hospital equipment has been proven to be a source of
Klebsiella as well (60,66). Clearly, several vectors are
involved in contamination of debilitated patients with
Klebsiella.

Although Klebsiella has been recovered from chlorinated
water supplies, its presence in unpolluted waters is erratic
(15,39,56). However, the discovery of Klebsiella in finished
drinking water emanating from redwood water storage reservoirs
led to the investigation of another example of the associa-
tion of these bacteria with botanical material. In 1964, a
descriptive report appeared on the presence of coliform

bacteria in redwood tanks, but no mention of Klebsiella was made (36). Recently the connection between redwood (Sequoia sempervirens) and Klebsiella was established, when it was discovered that 20 of 33 water systems in Oregon employing redwood storage tanks were contaminated with Klebsiella and Enterobacter; no obvious source of contamination could be found (59). Klebsiella densities were found to be higher in newer reservoirs. It was suggested that redwood and other botanical material have water-soluble nutrients that select for coliforms of the Klebsielleae tribe, since no other enteric bacteria were found in the reservoirs.

An examination of the literature concerning redwood reveals that water soluble extractives make up between 15 and 30 percent of the dry weight of redwood heartwood, from which water storage reservoirs are constructed (4). In heartwood, the extractives are located chiefly in the cell cavities of the wood ray parenchyma and the longitudinal parenchyma. 63% of the extractives are tannin and poly-phenolic compounds; polysaccharides and free sugars (in heartwood, D-glucose and L-arabinose) comprise only 2%, coloring matter 0.5%, and other material (undefined) 4.5%. The remainder of the extractives (38%) are cyclitol (alicyclic polyalcohol) compounds, of which myo-inositol is the parent compound. Myo-inositol and two other cyclitols, pinitol, and sequoyitol, are present in redwood (Figure 1 of Chapter 3).

Pinitol makes up 61% of the cyclitols in redwood, sequoyitol 31%, and myo-inositol 7% (5). Among the Enterobacteriaceae, the tribe Klebsielleae is one of the few groups that is capable of fermenting inositol (25,42). Inositol fermentation by Klebsiella is the basis for a selective medium recently developed for the isolation of Klebsiella (11). There is no corresponding data available for the utilization of pinitol and sequoyitol.

Cyclitol compounds, including inositol, pinitol, and sequoyitol, have a wide distribution, and have been detected in many botanical habitats (54). Of all cyclitols, myo-inositol has the widest occurrence in plant material, and the distribution of other cyclitols is somewhat irregular (6). Pinitol is the most widely distributed of the cyclitol methyl ethers, having been found in 6 families of gymnosperms and in 13 families of angiosperms, including legumes (54). Sequoyitol has been detected in a large number of gymnosperms; its presence always appears to accompany that of pinitol (55). Other related cyclitol methyl ethers, such as bornesitol, ononitol, and quebrachitol also may be found in vegetable material (55). Quebrachitol has been detected in 11 families of angiosperms (54).

A considerable amount of the myo-inositol in nature occurs in the form of inositol polyphosphates. Prominent among these phosphates is inositol hexaphosphate (phytic

acid), which usually is found in the form of a mixed
calcium-magnesium salt (17,55). Phytic acid is encountered
in a wide variety of vegetable seeds, such as pea and
lentil, and also is present in hemp and sunflower seeds (55).
In seeds, up to 90% of the organically bound phosphorus may
be in the form of phytic acid (17). It has also been
detected in the roots of carrots, parsnips, and potatoes,
and is a component of soil organic material (17,55). Dur-
ing seed germination, phytic acid undergoes a stepwise
dephosphorylation to yield free myo-inositol, which is
incorporated into cell wall constituents (55). Enzymes
for the degradation of phytic acid have been detected in
microorganisms (18,19,29,33,34,35,61).

As stated earlier, myo-inositol is the parent com-
pound of the cyclitols (8). Myo-inositol is synthesized
in plant material from D-glucose via ATP kinase, cyclase,
and phosphatase reactions. From myo-inositol the rest of
the cyclitols are synthesized; in the case of pinitol and
sequoyitol this involves a methylation of myo-inositol with
S-adenosylmethionine to form sequoyitol. An epimerase
reaction converts sequoyitol to pinitol (22).

There is little information concerning the degrada-
tion of cyclitols by bacteria. It is believed that a myo-
inositol dehydrogenase and NAD, in what at the time was
named Aerobacter aerogenes, catalyzes the first step in a

catabolic series of reactions that results in the eventual formation of dihydroxyacetone phosphate, acetyl CoA, and CO_2 (7,64). Reports have stated that the myo-inositol degrading enzymes are inducible, and that transport into bacteria is mediated by an active transport system (7,21,64).

The present investigation involved several aspects of the association of Klebsielleae with botanical material, and especially with redwood. Studies were conducted with an experimental 1,000 gallon (3788 liter) redwood storage reservoir constructed for research purposes in order to determine which design and maintenance factors were necessary to assure Klebsielleae-free water in similar tanks.

The second stage of the project examined cyclitol utilization among the Enterobacteriaceae and among Klebsielleae in particular, and its correlation with the growth characteristics of these organisms in aqueous saw-dust extracts. This was done in order to assess the relationship between cyclitol utilization and the ability of Klebsiella and Enterobacter to colonize a wide variety of botanical habitats.

Another study involved the effects of chlorine residuals, retention times, and cyclitol concentrations on Klebsielleae in redwood water storage reservoirs. Antibiotic-resistant Klebsiella strains were employed to assess the effects of some of these parameters. Three small 90 liter redwood tanks were used in these experiments.

Gas chromatographic techniques were then used to monitor the degradation of cyclitols in aqueous extracts of redwood by _Klebsielleae_ and other gram-negative bacteria. In addition, a defined basal salts medium supplemented with cyclitols and carbohydrates was employed in an attempt to show which of these two groups of compounds are pre-ferentially utilized by enteric bacteria.

Other experiments were conducted concerning the utilization of phytic acid by gram-negative bacteria. In addition, antibiotic resistance transfer experiments between strains of _Klebsiella_ were performed in aqueous extracts of redwood sawdust in order to demonstrate that conjugal transfer of antibiotic resistance may occur at cell densities of _Klebsiella_ likely to occur in nature.

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Chapter 2

Control of Coliform Bacteria in Finished Drinking Water
Stored in Redwood Tanks

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Abstract

An experimental 1000 gal (3788 l) redwood water storage reservoir was employed to study the control of coliform contamination in finished drinking water caused by Klebsiella pneumoniae and Enterobacter species. Over a twenty month period of operation, coliforms were never detected in tank water. Control of coliforms is dependent on three factors: maintenance of a free chlorine residual of 0.2-0.4 ppm in the tank water, maximum retention times of four to seven days, and the use of a separate overhead inlet water pipe. Klebsiella can multiply to 10^6 per ml in dechlorinated water samples from the experimental tank, utilizing nutrients leached from the redwood.

Introduction

Redwood tanks are used to store finished drinking
water in many locations in the western United States,
Canada, South America, and the Pacific Islands. In the
western United States, they are often used in state and
federal recreation areas and serve small communities as
well as larger cities. Those in state and federal recrea-
tion areas in Oregon alone serve over 1 million annual day
visitors. Reservoir capacities are commonly available to
store 5000 to 500,000 gal (18,940 to 189,400 1); and the
tanks are relatively inexpensive, easily assembled, and
durable (Fig. 1). However, in two studies, coliform con-
tamination of drinking water has been traced to redwood
tanks, and some doubt has arisen as to their usefulness
for the storage of finished drinking water.

The presence of coliform bacteria in water stored in
redwood reservoirs was first reported by Jones and Green-
burg in 1964 (1). In that study, it was found that potable
water entered the tanks, but water with high coliform counts
left them. The problem was not caused by uncovered tanks,
or any obvious source of contamination. The authors pro-
posed that the contamination was associated with the redwood
used to construct the tank, and suggested that chlorination
be employed to alleviate the problem.

A recent study by Seidler et al. has reported the presence of coliforms in excessive amounts in redwood water storage reservoirs in Oregon (2). In that survey, total coliform counts in water emanating from the redwood tanks were ten to 40 times higher than that allowed under federal membrane filtration guidelines. These tanks were part of both public (state parks) and private water systems; coliforms were present in nine of ten private drinking water systems and eleven of 23 water systems in state and federal parks. As in the previous investigation, no source of contamination other than the tank itself could be found. Coliforms isolated from tank water were identified as Klebsiella pneumoniae and Enterobacter species.

Klebsiella pneumoniae has an extensive distribution in the environment, in addition to being an opportunistic pathogen for humans as well as animals (3,4,5). It has been isolated from vegetables, from within living white fir trees, and from industrial effluents containing botanical material, such as those from pulp and textile mills (6,7,8,9,10,11). Klebsiella of environmental origins, including redwood tanks, appear to be biochemically in-distinguishable from clinical isolates (12), and based on mouse virulence tests are potentially as pathogenic as clinical isolates (13,14).

This investigation was conducted in order to find a way to control coliform contamination (i.e., <u>Klebsiella</u> and <u>Enterobacter</u>) in redwood water tanks. Seidler et al. were able to correlate three main deficiencies with contamination in redwood reservoirs: 1) lack of an automated chlorination apparatus or insufficient chlorine to maintain a free residual in the outlet water, 2) a common inlet/outlet plumbing system, and 3) long retention times for water stored in the tanks. It was speculated that low chlorine residuals allow coliforms to multiply on nutrients leached from the redwood; the other two factors would lead to stagnation of water and accumulation of these nutrients. Based on these findings, design modifications were made in a 1000 gal (3788 1) experimental tank which was constructed for research purposes (Fig. 2). An automated chlorine metering system was employed, and an inlet water pipe separate from the outlet was installed at the top of the tank. Regular monitoring (usually daily) of physical parameters of the water (chlorine residuals, optical density, and pH) was performed. Samples of water were frequently examined for bacteriological quality, and material from the interior surfaces of the staves of the tank was scraped at intervals and examined for coliforms and other bacteria. Tank water samples collected at various times during the study were inoculated with a strain of <u>Klebsiella</u> <u>pneumoniae</u> to determine their capabilities to support growth of this organism.

Materials and Methods

<u>1000 gal (3788 l) experimental redwood tank</u>. A 1000 gal (3788 l) redwood tank was constructed for use in this investigation by National Tank and Pipe Co., Portland, Ore. It measured 71 in. (1.80 m) in height with an interior radius of 33 in. (0.84 m). The 42 staves of the tank each measured 2 1/16 in. (6.51 cm) by 5 3/8 in. (13.65 cm). A hinged plywood lid covered the top. Incoming water, from municipal city lines, entered the tank at the top and was sprayed evenly to all sides. A float system controlled the level of the water and drainage was through a valve at the bottom of the tank (Fig. 2).

Various retention times for tank water were achieved with a motor timer which controlled a solenoid valve. The timer monitored both the length of time between drainings (Timer #1) and the length of time the drain was open (Timer #2).

Chlorine concentrations in the incoming water were regulated by a metering pump on an 80 gal (303 l) holding tank. The stock chlorine solution was prepared by adding 1 gal. (3.79 l) of commercial bleach to 14 gal. (53.03 l) of tap water (1:15 dilution). A dial on the pump was adjusted to provide appropriate chlorine concentrations for water entering the tank.

Retention times. It was found that water drained
from the tank at the rate of 3.1 gal (11.74 l) per minute.
The timers were adjusted to provide retention times based
on this drain rate.

Preliminary adjustments and decontamination of tank
interior. The manufacturer's recommendations were fol-
lowed. In order to leach tannins from the wood, which
affect odor, color, and taste of the water, 5 lb. (2.27 kg)
of soda ash was added to a full tank and held for 24 hr.
After drainage, enough stock chlorine solution was added
to bring the chlorine concentration in the full tank to
200 ppm. Granular dry chlorine was used in preparing the
stock solution. The tank was put into service in October
1975; however, in November 1975 it was necessary to per-
form a similar soda ash treatment of 7 days duration. This
second treatment greatly improved the taste, odor, and
color of the water.

Preliminary studies with the tank at 20 percent
capacity were then carried out between the first month
of operation and the third month to determine the amount
of chlorine necessary to achieve an adequate free chlorine
residual (0.2 to 0.4 ppm) in the drain (outlet) water. The
effect of lengthened retention time on residual chlorine
was also examined. During this period, bacteriological
examinations of the water were performed regularly. No

coliforms were detected during this period; total bacterial counts averaged 350 per ml and never exceeded 2000 per ml.

During the third month, the experimental tank was filled to capacity and set on a 1 day retention time. With this schedule, approximately one-sixth of the tank water was drained every 4 hrs. Capacity was maintained at all times by a float system. Retention times were later increased to two, four, eight and finally twelve days. During the sixteenth month, the chlorine amendments were terminated in order to observe the effects of lowered chlorine residuals on the bacteriological quality of tank water; water entering the tank then carried a free residual of 0.5 ppm, due to the chlorination of the municipal water.

Daily observations of physical parameters of tank water. Free chlorine residuals and total chlorine residuals in the drain water and inlet water were determined by the N,N-diethyl-para-phenylenediamine (DPD) method. Optical density readings and pH were also determined for water samples.

Bacteriological examination of tank water and staves. Water leaving the tank via the drain at the bottom was periodically examined for total coliforms and total bacterial counts, using standard procedures (15). Total coliforms were enumerated by the membrane filtration technique on mENDO agar LES. Sodium thiosulfate was added

to all samples to remove any free chlorine in the water.
Varying amounts of drain water (usually 100 ml or 1000 ml)
were tested. On eight occasions, the water level was
lowered and the interior surfaces of the tank were scraped
with sterile glass microscope slides. Surface material was
dispersed and tested for total coliforms by the spread
plate technique on mENDO agar LES and ENDO agar, and with
phenol red lactose broth. In addition, some non-coliform
bacteria were isolated for identification.

Identification of isolates from staves. Media pre-
paration and procedures used for the identification of
coliform organisms were those recommended by Edwards and
Ewing (16). Final identifications were made using the
API 20E System. Isolates obtained from plate count agar
were identified using 9-digit profiles of the API 20E
System, or by procedures outlined in the eighth edition
of Bergey's Manual of Determinative Bacteriology (17,18).

Nutrient potential of redwood tank water. Samples
of water from the 1000 gal (3788 l) experimental tank were
collected at various times during the investigation and
stored at -17°C. The samples were thawed and filter
sterilized using membrane filters. They were inoculated
with low cell densities (less than 100 cells/ml) of an 18
hr. culture of Klebsiella pneumoniae and incubated with

shaking at 35°C. Plate counts were performed at 48 hr.
and 96 hr. All glassware was acid washed to remove any
extraneous nutrient sources. Sodium thiosulfate was used
to remove chlorine when necessary.

Results

Total bacterial counts in the outlet water ranged from 2000 per ml to <10 per ml during the twenty month period (Table 1). Generally, the counts were in the 10-50/ml range when an adequate free residual of 0.2-0.4 ppm of chlorine was maintained. After the chlorine amendments were terminated in the sixteenth month, total counts increased to about 2000/ml, but by the nineteenth month they had returned to the previous level.

At no point in this investigation were coliforms detected in water emanating from the experimental tank. Coliforms were not observed with the membrane filter procedure, even when volumes of 1000 ml were filtered. Furthermore, none of the isolated colonies picked from plates used for total bacterial counts were coliforms.

Coliforms were detected in only one of the eight experiments in which the interior surfaces of the tank were scraped and examined (Table 1). Five of the eighteen staves sampled on this occasion yielded coliforms. All coliforms isolated were identified as <u>Klebsiella</u> <u>pneumoniae</u>; all were fecal coliform (FC) negative. Subsequent stave scraping experiments failed to detect any coliforms.

Non-coliform isolates from the stave material were also identified. Of thirteen colonies picked, five were

non-fluorescent _Pseudomonas_ spp. and three were _Moraxella_.
One _Pseudomonas_ _fluorescens_ and one _Xanthamonas_ were
isolated, and three gram-positive bacteria were identified:
one _Bacillus_, one _Micrococcus_, and one _Staphylococcus_
epidermidis.

As the experimental tank aged, the chlorine demand
decreased (Table 1). For example, from the sixth month
to the fifteenth month, the amount of chlorine needed to
maintain a free residual of 0.4 ppm decreased from 5.1 ppm
to 1.5 ppm. As of the twentieth month, it appeared that
the chlorine demand had stabilized.

It was also determined that retention time has a
definite effect on chlorine residuals in new redwood tanks
(Table 1). In the fourth month (1 day retention), 2.1 ppm
at the inlet maintained a residual of 0.9 ppm, but 5.1 ppm
was needed two months later (4 day retention) to maintain
a lower residual of 0.4 ppm. Similarly, in the seventh
month (8 day retention), 8.2 ppm in the inlet water was
required to give 0.3 ppm in the drain water. Longer reten-
tion times, therefore, require greater amounts of chlorine
in the inlet water.

An increase in the level of water in the tank also
increases the chlorine demand. Evidence for this can be
seen in Table 1 when the capacity of the tank was increased
from 20 percent to 90 percent during the third month. This

observation is probably related to the increased leaching of chlorine-inactivating compounds from newly immersed wood surfaces.

Of fifteen redwood tank water samples tested for the capability to support growth of <u>Klebsiella</u> <u>pneumoniae</u>, five were positive (Table 2). In these five samples, the strain of <u>Klebsiella</u> used multiplied to numbers exceeding 10^6 per ml. Glassware was acid washed, so the only source of nutrients was the redwood of the tank. No correlation was found between those samples that supported growth and those that did not.

Discussion

With careful monitoring and simple adjustments of
design, redwood water reservoirs can be operated without
coliform contamination. However, there are three main
factors that must be considered.

First, an adequate free chlorine residual of 0.2-
0.4 ppm should be maintained throughout the system, to
prevent bacteria from multiplying in the tank water. This
can most conveniently be accomplished with an automated
chlorination system to maintain the necessary chlorine
level in inlet water. The amount of chlorine added to the
inlet water will vary, depending on tank age, percentage
of tank capacity being utilized, and retention time. The
combination of a new reservoir and lengthy retention time
(7 days or more) will exert a great chlorine demand and
therefore require great chlorine amendments to control
coliform contamination. In the experimental reservoir,
it was necessary to use 20 ppm or more to maintain a
chlorine residual during the second and third months of
operation with 8 to 12 day retention times. Routine
monitoring in all newer systems is therefore necessary to
insure that the chlorine residual is sufficient in the
outlet water, since tanks of large capacities may take
7 to 10 days to equilibrate after chlorine amendments have
been altered. Bacteriological monitoring of coliform and

total counts should be performed as prescribed in federal
and state regulations. Small water purveyors or private
systems serving only a few connections may be exempt from
routine monitoring requirements. Nevertheless, routine
chlorine analyses should be made for at least the first
two years of operation.

As previously implied, the use of lengthy retention times
of 7 days or more should be avoided. If necessary, the
reservoir should be operated at minimal capacity to achieve
more rapid water turnover. Ordinarily, inlet chlorine
residuals of 2 to 4 ppm will fulfill requirements of most
new reservoirs with retention periods of 1 to 4 days. It
should also be noted that long retention times will increase
stagnation of water in the tank and the leaching of growth-
promoting nutrients for coliform bacteria. As a redwood
reservoir ages, the use of longer retention times and
greater percentages of tank capacities will be possible,
since the amount of added chlorine needed to maintain a
residual in the outlet water will decrease. Presumably,
most of the compounds that may be capable of reacting with
and inactivating free chlorine in the water will be
leached from the redwood staves as they age. However, the
times necessary for the chlorine demand to decrease may
vary with reservoir capacity.

As stated earlier, both free chlorine residuals and
total chlorine residuals were measured throughout this

investigation. Only free chlorine residuals have been reported, however, since combined chlorine residuals were never detected. If there are compounds present in redwood which are capable of combining with and inactivating free chlorine in tank water, then the combined chlorine may be present at the wood/water interface, and therefore escape detection. Another possibility is that the method used for determining chlorine residuals in this study (DPD) failed to detect these combined chlorine compounds. Regardless of the chemical form of the combined chlorine, based on our observations (2), it is doubtful that it is effective in the control of bacteria in the water.

Finally, all redwood water systems should utilize a plumbing arrangement at the top of the tank for inlet water, that sprays the water evenly to all sides. This design modification partially alleviates stagnation in the tank. When water enters at the top and exits at the bottom, a greater amount of mixing takes place than when water enters and exits at the bottom. In addition, spraying water evenly into the tank promotes aging of the wood and leaching of nutrients. This alteration of design would be even more significant with larger tanks with capacities of 25,000 gal (94,700 l) or more.

The physical characteristics of water from the experimental tank, such as odor and color, improved markedly after

the second soda ash treatment of seven days duration. The manufacturer's recommendation of one 24 hr treatment proved to be inadequate; a treatment of five to seven days should give better results. Optical density readings taken during the second treatment showed that considerable material was leached from the wood even after the third day of treatment. After this treatment, the odor and color of the tank water were quite acceptable. In fact, optical density readings demonstrated no significant difference in clarity between tank water and municipal city water. The pH of tank water was consistently in the 7.0-7.2 range.

Coliforms were never detected in water emanating from the experimental tank, but the detection of <u>Klebsiella</u> <u>pneumoniae</u> in one stave scraping experiment demonstrates their presence and emphasizes the importance of good maintenance, especially with newer reservoirs. As a redwood tank ages, however, it appears that the likelihood of coliform contamination decreases. The termination of added chlorine amendments to the municipal water supply after sixteen months with no development of coliforms in the water or on the staves, supports this view. This is probably due to the decreased concentration of growth supporting nutrients emanating from the wood.

Non-coliform bacteria isolated and identified during this study do not seem to be appreciably different from those found in other water storage systems. None of the

bacteria could be considered dangerous to the health of those drinking water from redwood systems (19). Total counts in tank water can be kept within reasonable limits, generally with a chlorine residual of 0.2-0.4 ppm. Occasionally, chlorine residuals may be increased temporarily to lower total counts, especially in newer reservoirs.

Laboratory experiments have demonstrated that Klebsiella pneumoniae can multiply to high densities in at least some samples of redwood tank water. No correlation can be found between those samples where Klebsiella multiplied and those where it did not. Perhaps, a trace amount of an essential mineral or other nutrient was present in five samples and not in the others. In any case, it is certain that the nutrients utilized in the five samples supporting growth were derived from redwood. There fore, if coliforms are present in redwood reservoirs and measures are not taken to control them, rapid multiplication will take place and a contamination problem can ensue.

The likely source of the Klebsiella isolated in this investigation and previous ones of redwood water systems has now been determined. Experiments conducted with samples of redwood have demonstrated the presence of Klebsiella (and Enterobacter) in liquid aseptically pressed from freshly cut wood (20). Presumably, Klebsiella are present in the wood as the tanks are constructed. Later, as

redwood tanks are filled with water, <u>Klebsiella</u> are leached from the wood along with the nutrients that support their growth. Lack of a chlorine residual in the water allows their multiplication to detectable and/or excessive levels.

In summary, redwood tanks can be a satisfactory means to store water for drinking purposes, providing a few guidelines are followed and a regular monitoring schedule of chlorine residuals and bacteriological sampling is maintained. This is especially important for new redwood tanks, where the likelihood of coliform contamination is increased. When properly managed, however, redwood water systems can be expected to provide potable drinking water throughout the life of the tank.

Acknowledgments

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Table 1. Monitoring of physical and microbiological parameters in the 1000 gal (3788 1) experimental redwood tank

Month of operation	Retention time (days)	Number of days sampled	Free chlorine at inlet (ppm)	Free chlorine at outlet (ppm)	pH	OD_{600}	Average total count/ml	Stave scraping
1	2[a]	7	1.7	0.3	7.0	.009		
1	4[a]	9	4.9	0.1	7.1	.018	225	NCD[b]
1	8[a]	6	11.5	0.2	7.4	.020	1040	
2	8[a]	13	23.3	0.5	7.0	.009	123	
2	12[a]	11	18.2	0.1	7.0	.012	15	NCD
3	12[a]	6	30.0	0.1	7.0	.008		
3	1	20	8.6	3.7	7.1	.001	3	
4	1	26	2.1	0.9	7.1	.001	>1000	NCD
5	1	5	1.9	0.2	7.0	.001	>1000	
5	2	4	3.0	0.4	7.1	.003	>2000	
5	4	21	3.9	0.7	7.1	.001	>2000	NCD
6	4	14	5.1	0.4	7.0	.001	10	
7	8	19	8.2	0.3	6.8	.001	95	
8	8	28	8.6	0.5	7.0	.000	<10	NCD
9	8	24	5.8	0.2	7.0	.001	25	
10	8	27	5.8	0.5	7.1	.000	<10	NCD
11	8	28	4.2	0.6	7.3	.000	<10	
12	8	19	3.3	0.4	7.2	.000	40	NCD
12	12	7	3.9	0.3	7.2	.000		
13	12	28	3.0	0.4	7.2	.000	20	
14	12	23	2.7	0.5	7.3	.000	<10	Klebsiella pneumoniae FC[c]-
15	12	23	1.5	0.4	7.2	.000	21	NCD
16	12	19	0.5	0.1	7.1	.000	2060	
17	12	7	0.5	0.2	7.2	.000	1090	
18	12	7	0.5	< 0.1	7.2	.000	860	
19	12	6	0.5	< 0.1	7.0	.000	<10	
20	12	3	0.5	< 0.1	7.0	.000	48	NCD

[a] Tank operating at 20 percent capacity
[b] NCD - no coliforms detected
[c] FC-; fecal coliform negative

NOTE: Data for months 1-6 contained in this table are from the thesis of Jan E. Morrow, M.S. 1975, Oregon State University.

Table 2. Growth of <u>Klebsiella</u> in redwood tank water.

Date sample collected	Inoculum	Viable count/ml 48 hr	96 hr
10/24/75	4.5×10^1	3.0×10^2	3.0×10^2
11/13/75	2.3×10^0	$< 1.0 \times 10^1$	1.0×10^1
12/18/75	6.0×10^1	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
2/7/76	5.5×10^1	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
2/26/76	9.0×10^1	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
4/5/76	7.0×10^1	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
6/30/76	2.3×10^0	3.7×10^5	1.6×10^6
7/29/76	2.3×10^0	1.5×10^6	1.5×10^6
9/15/76	7.0×10^1	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
10/10/76	8.0×10^1	1.3×10^7	9.4×10^6
11/19/76	4.0×10^1	5.0×10^0	$< 1.0 \times 10^1$
1/11/77	2.3×10^0	2.5×10^6	1.8×10^6
2/10/77	8.0×10^1	4.2×10^6	5.0×10^6
3/10/77	9.5×10^1	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
5/18/77	6.0×10^1	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
Municipal water	7.5×10^1	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
Double distilled water	9.5×10^1	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$

Figure 1.   Redwood water storage reservoir; 50,000 gal capacity (189,400 l).
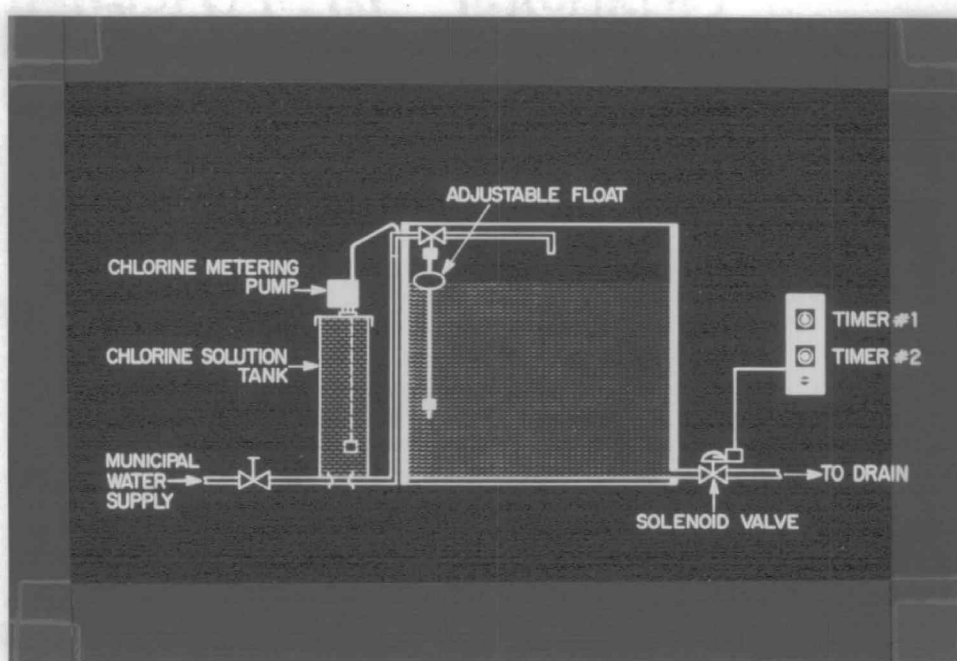
Figure 2. Diagram showing design modifications included
in the 1,000 gal (3,788 l) experimental tank.

Chapter 3

Cyclitol Utilization Associated with the Presence of

Klebsielleae in Botanical Environments

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Abstract

Bacteria of the tribe Klebsielleae are capable of
metabolizing a class of compounds present in aqueous
extracts of redwood known as cyclitols, of which myo-
inositol is the parent compound. Pinitol and sequoyitol,
two other cyclitols, are also encountered in redwood. 100%,
97%, and 68% of the combined Klebsiella isolates from
clinical and environmental origins fermented inositol,
sequoyitol and pinitol, respectively. These compounds can
also be used as a sole source of carbon and energy by
Klebsiella. Similar results were obtained with Enterobacter
isolates, but most other enteric bacteria tested could not
metabolize cyclitols. Strains of Klebsiella multiplied to
levels exceeding 10^5/ml in aqueous extracts of non-sterile
redwood within 6 days. Most other enteric bacteria did not
grow in these extracts. Cyclitol metabolism was found to
correlate well with the ability to multiply in redwood
extract in the presence of cyclitol-negative indigenous
bacteria. The capacity to use cyclitols, which are present
in a variety of plant material, might afford Klebsielleae
of both environmental and clinical origins an advantage in
competing for nutrients and colonizing botanical environ-
ments.

Introduction

The association of coliform bacteria of the tribe
Klebsielleae with botanical environments has been well
demonstrated. Klebsiella and Enterobacter have been
isolated from living trees (1), wood chips (10) and sawdust
(23), sugarcane wastes (24), fresh vegetables (8,10,12,
29,31), industrial effluents containing botanical material
(9,14,15,17,22), and more recently from colonized staves
and potable drinking water stored in redwood reservoirs (28,
30), and from within redwood lumber (6). Klebsiella
isolated from these environments have been found to be
indistinguishable from pathogenic Klebsiella isolates from
human and animal sources, based on biochemical, serological,
and mouse virulence tests (4,21,27).

In most instances, other coliform and enteric bacteria
(including Escherichia coli) are not found in comparable
densities with Klebsielleae in these botanical environments
(6,10,14,28,31). The nutritional bases for this unique
colonization are unknown, especially since carbohydrate
utilization characteristics throughout the enteric group are
similar, particularly between Klebsielleae and E. coli (11).
However, an important biochemical distinction between the
Klebsielleae tribe and E. coli is the fermentation of myo-
inositol by the former; E. coli and nearly all other enteric
bacteria lack this ability (11). This phenomenon is the

basis for a selective medium recently developed for
Klebsiella (5). Inositol, a "sugar alcohol", is a member
of a class of compounds known as cyclitols, which have an
extensive distribution in plant material (26).

The recent reports that document the association of
Klebsiella with redwood suggest that there are water-
soluble nutrients present in redwood that selectively
support the growth of Klebsielleae (28,30). A study by
Anderson (2) illustrated that cyclitol compounds comprise
38% of the water-soluble extractives of redwood while free
sugars make up only 0.5%. Of the cyclitols in redwood,
pinitol constitutes 61%, sequoyitol 31%, and myo-inositol
7% (3). The remainder of the extractives of redwood are
either inhibitory or not metabolizable by most bacteria.

In this investigation, relationships between cyclitol
utilization in phenol red broth and in defined media and
the ability to colonize aqueous extracts of redwood sawdust
with and without the presence of indigenous flora were
evaluated for Klebsiella of environmental and clinical
origins. Several other genera of gram-negative bacteria
were similarly examined.

Materials and Methods

Bacterial cultures. Klebsiella isolates used in this study
were obtained from sources reported earlier (4,8,27,28). Three
of the Klebsiella tested were ATCC cultures, as were the
Enterobacter aerogenes and Serratia liquefaciens cultures.
The Enterobacter agglomerans and the Erwinia carotovora
isolates were obtained from the International Collection of
Phytopathogenic Bacteria in Davis, California, and the
Yersinia isolates were obtained from the Center for Disease
Control in Atlanta, Georgia. The remainder of the cultures
were obtained from culture collections at Oregon State
University.

Cyclitol utilization in phenol red broth base. Myo-
inositol was obtained from Sigma. The pinitol was a gift
from the laboratory of Dr. Arthur Anderson, University of
California Forest Products Laboratory, Richmond, Calif.,
and the sequoyitol was a gift from Dr. Laurens Anderson,
Department of Biochemistry, University of Wisconsin.
Cyclitols were supplied in phenol red broth (Difco) at a
0.5% concentration. Fewer cultures were tested with
sequoyitol, since limited amounts were available.

Cyclitol utilization in defined medium. The defined
medium used contained the following ingredients in g/l:
Tris (hydroxymethyl aminomethane) buffer to pH7, 10 g; NH_4Cl,
2g; KCl, 2g; KH_2PO_4, 1g; and Na_2SO_4, 0.1g (this was combined

to form solution A). Solution B contained in g/100ml: $CaCl_2 \cdot 2H_2O$, 0.05g; and $MgCl_2$, 0.02g. 900 ml of sterile solution A, 10 ml of sterile solution B, and 100 ml of the test carbon source to achieve 0.2% of the latter were combined to prepare one liter of the basal salts medium. Cultures examined for utilization of cyclitols were first propagated in the medium in the presence of a growth limiting amount of glucose or inositol (0.02%). Cultures were shaken at 35°C (Yersinia and Erwinia at 28°C) and growth was monitored with turbidity measurements at 600 nm using a Bausch and Lomb Spectronic 20 colorimeter.

Isolation of antibiotic resistant mutants. Spontaneous mutants resistant to 1000 µg/ml of streptomycin sulfate (Sigma) were isolated by growing cultures to high cell densities in penassay broth (Difco) on an incubator shaker, then adding enough streptomycin to give a 1000 µg/ml concentration. The cultures were incubated another few hours, centrifuged, and resuspended in 1ml of sterile 0.01M Tris buffer. This suspension was spread on plate count agar (Difco) containing 1000 µg/ml of streptomycin. Colonies appearing within 48h were picked and restreaked on the antibiotic medium. All antibiotic mutants and their non-resistant parent cultures were inoculated on a glucose basal salts medium to determine if any of the mutants were auxotrophic and/or dependent on streptomycin for growth.

The identity of all antibiotic resistant mutants used was verified with the API 20E system (Analytab Products, Plainview, N.Y.), or by the methods of Edwards and Ewing (11).

Aqueous extracts of sawdust. Aqueous extracts of sawdust were prepared by adding 0.25g of non-sterile or sterile redwood sawdust to 600 ml sterile double distilled water. This concentration was used since it was neither growth inhibitory due to high polyphenolic concentrations, nor provided sufficient extraneous contaminants to indiscriminately allow the growth of cyclitol-negative species. The suspension was allowed to stand at room temperature for 2 days while nutrients were leached from the sawdust. At 2 days, a few drops of sterile 0.1% sodium hydroxide was added to neutralize the extract.

Cultures were grown overnight on an incubator shaker in penassay broth with 1000 μg/ml streptomycin, diluted to 10^{-4} in Tris buffer, and inoculated into the extract to give initial densities of 100-500 cells/ml. The extract was incubated statically at room temperature. Plate counts were made at intervals on plate count agar plus streptomycin, containing 0.05% cycloheximide, in order to inhibit indigenous bacteria and fungal flora. The indigenous flora (uninoculated sawdust) were enumerated on plate count agar, which was incubated at room temperature.

Identification of indigenous bacterial flora.

Indigenous bacterial flora of redwood sawdust were identified using 9 digit profiles of the API 20E system.

Results

Figure 1 illustrates the structures of myo-inositol, pinitol, and sequoyitol, and presented in Table 1 are the results obtained from the cyclitol fermentation experiments in phenol red broth. With few exceptions, positive reactions (acid production) in phenol red inositol broth were restricted to the Klebsielleae tribe. All of the Klebsiella isolates tested were positive for acid production, and all but one of the Enterobacter and Serratia strains (an E. cloacae isolate) also were able to utilize this compound. Two of five Salmonella tested were positive, as were isolates of Citrobacter, Erwinia and Yersinia. With the exception of Salmonella, the non-Klebsielleae cultures gave weaker or delayed positive reactions with inositol as compared to those of Klebsiella and Enterobacter.

A similar cyclitol utilization pattern was observed with pinitol and sequoyitol (Table I). The only isolates other than Klebsielleae able to utilize these compounds were the inositol-positive Salmonella (pinitol and sequoyitol) and one isolate of Yersinia (sequoyitol only). Within the Klebsielleae tribe, Klebsiella had a higher percentage positive with sequoyitol (97%) and with pinitol (68%) than either Enterobacter or Serratia. Klebsiella strains from clinical and environmental sources in general had a similar percentage of positive reactions on all three

cyclitols. In no instance was a positive reaction with pinitol or sequoyitol observed without a corresponding positive reaction with inositol.

Figure 2 shows the growth curves with 4 isolates of Klebsiella of different origins when inositol or pinitol were supplied as sole sources of carbon and energy in a defined basal salts medium. All 4 isolates were able to utilize inositol and pinitol as a carbon source, although growth rates varied with generation times of 1-2 hrs. at 35C. Control flasks of the same medium without a carbon source had negligible turbidity.

Table 2 contains the results of a similar growth experiment with Klebsiella and other genera of gram-negative bacteria, including Pseudomonas aeruginosa. It can be seen that utilization of inositol as a carbon source was, with few exceptions (Yersinia, Erwinia, Salmonella) restricted to the Klebsielleae tribe. The results of these experiments in a defined medium agree with the fermentation of inositol in phenol red broth.

Table 3 shows the results of growth experiments in 0.04% w/v non-sterile aqueous extracts of redwood sawdust (i.e., containing the indigenous flora). Four Klebsiella isolates multiplied to levels exceeding 10^5/ml, and three isolates to levels greater than 10^4/ml, within 6 days after inoculation. For most of the Klebsiella tested, viable

counts approached maximum levels by the sixth day, and remained at that level throughout the sampling period of 31 days. The indigenous flora achieved levels of 10^6-10^7 cells/ml. No clear correlation between the origin of Klebsiella isolates and growth and persistence in redwood extract was observed, although one water isolate and two of three mastitis isolates tested had higher cell densities than most of the other strains tested.

Other members of the Klebsielleae tribe (Enterobacter and Serratia) grew to lower cell densities than Klebsiella. Of the remaining gram-negative bacteria tested, only the Yersinia and Erwinia isolates were capable of growth and persistence in the extract. The Citrobacter isolate was recovered throughout the 31 day sampling period, but did not increase in numbers. The remainder of the cultures tested were not recoverable at the end of the experiment. Control flasks containing double distilled water inoculated concurrently with the redwood extract demonstrated no more than a 10-fold increase in cell numbers.

In order to assess the influence of indigenous flora on colonization potential in redwood environments, similar experiments were conducted in sterile aqueous redwood extracts. These results are presented in Table 4. Of the

13 Klebsiella isolates tested, 8 grew to levels greater

than 10^6/ml, and only three did not exceed 10^5/ml. Two

isolates that did not exceed 10^3/ml in non-sterile extract

(UT1500 and MH31) multiplied to levels greater than 10^6/ml

within 6 days in the sterile extract. The Enterobacter and

Serratia isolates also attained somewhat higher levels of

growth in sterile as compared to non-sterile extracts, as

did E. coli and Citrobacter freundii.

Table 5 presents the correlation between cyclitol

utilization in phenol red broth base and the ability to

grow in non-sterile aqueous redwood extract. Cyclitol

utilization seems to be a prerequisite for successful

colonization of non-sterile redwood environments, but

cyclitol utilization itself is not always sufficient for

growth in this medium. For example, Klebsiella MH31 and

S. enteritidis failed to grow in the non-sterile sawdust

extracts but fermented all 3 cyclitols. The Klebsiella

grew well in sterile redwood extract, but the Salmonella

isolate did not.

Enumeration and identification of the indigenous

flora of redwood sawdust revealed that two organisms, both

non-fluorescent Pseudomonas spp., were the predominant

members of the bacterial flora of redwood. These organisms

increased in numbers from approximately 10^2/ml to 10^6/ml

within 6 days in uninoculated non-sterile redwood extract.

Neither of the organisms isolated utilized cyclitols when tested in OF medium (20) with cyclitols as the carbon source.

Discussion

Cyclitol compounds, including inositol, sequoyitol, and pinitol, are found in many types of botanical material, including species of redwood, fir, pine, and hemlock, as well as in several families of angiosperms (26). Based on data obtained in this investigation, it appears that bacteria of the tribe Klebsielleae, which also have been found to be associated with plant material, are one of the few groups among the Enterobacteriaceae with the capacity to utilize cyclitols as a carbon source. Some isolates of Yersinia, Erwinia, and Salmonella are also capable of metabolizing cyclitols, but fewer members of these genera possess this trait as compared to Klebsiella and Enterobacter. Salmonella did not multiply in the aqueous sawdust extracts, however. Within the genus Klebsiella, the data indicate that this cyclitol utilization capacity is present in all groups of isolates, whether they are of environmental or clinical origin.

The ability to metabolize cyclitol compounds and use them as a carbon source likely gives Klebsiella and Enterobacter an advantage over other bacteria of the enteric group in colonizing certain botanical environments, which involves competition for nutrients with the cyclitol-negative indigenous flora of these habitats. Evidence for this can be found in the experiments conducted with extracts of redwood.

In the absence of indigenous organisms in sterile redwood extracts, several cyclitol-negative and cyclitol-positive isolates both attained significant levels of growth. Cyclitol-negative enteric bacteria were probably able to use the carbohydrates present in redwood extract as a carbon source, since experiments with phenol red broth demonstrated that almost all of the isolates tested gave a positive reaction with most, if not all of the carbohydrates in redwood.

In contrast, in non-sterile redwood extract only cyclitol-positive isolates (of both environmental and clinical origins) were capable of competing with the indigenous organisms; most cyclitol-negative strains were not recoverable at the end of the experiment. Presumably the carbohydrates that cyclitol-negative organisms could utilize were consumed by indigenous organisms, but cyclitol compounds remained. Therefore, it appears that the presence of cyclitols as a carbon source in an environment with a significant density of indigenous flora may select for cyclitol-positive organisms, and Klebsielleae in particular.

Of the three other genera tested in this study that could use a cyclitol as a carbon source (Erwinia, Yersinia, and Salmonella), Erwinia and Yersinia are environmentally associated (7,13,18), and in most cases grew well in the

experiments in redwood extracts. The cyclitol-positive _Salmonella_ did not grow in sterile extracts of redwood, indicating that some compound required by this organism is not present in the extract, or some inhibitory compound may be present that prevents its multiplication.

It would appear that cyclitol metabolism is a significant part of the nutritional bases involved with the presence of _Klebsielleae_ in drinking water emanating from redwood water storage reservoirs. Although redwood is one of the few tree species where cyclitol content is well characterized, cyclitols have also been detected in species of fir, pine, and hemlock (26). The recent isolation of _Klebsiella_ from within redwood, hemlock, and fir (6) (pine was not tested) supports this association of cyclitol compounds with the isolation of _Klebsielleae_ (and not other enteric bacteria) from botanical habitats.

Other botanical material such as pulp and textile mill effluents (9,14,15), fresh vegetables (8,10,31), and plant roots (19,25), have been significant sources of _Klebsiella_ in the environment. If cyclitol compounds are present in these effluents and in other plant material, they may be part of the nutritional bases that promote the profileration of _Klebsiella_ of environmental as well as clinical origins (16).

Acknowledgments

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Table 1. Fermentation of cyclitols in phenol red broth by gram-negative bacteria.

	Inositol		Pinitol		Sequoyitol	
	No. Positive / No. Tested	% Positive	No. Positive / No. Tested	% Positive	No. Positive / No. Tested	% Positive
Aeromonas	0/3	0	0/3	0	not tested	
Escherichia coli	0/26	0	0/26	0	0/4	0
Shigella	0/3	0	0/3	0	0/2	0
Salmonella	2/5	40	2/5	40	2/4	50
Citrobacter	1/2	50	0/2	0	0/1	0.
Klebsiella (ATCC)	3/3	100	3/3	100	3/3	100
Klebsiella (environmental)	60/60	100	52/60	87	17/18	94
Klebsiella (clinical)	43/43	100	39/43	91	7/7	100
Klebsiella (mastitis)	30/30	100	23/30	77	5/5	100
Klebsiella (ATCC-oxytoca)	2/2	100	2/2	100	2/2	100
Enterobacter aerogenes	10/10	100	9/10	90	1/1	100
Enterobacter agglomerans	21/21	100	11/21	52	0/2	0
Enterobacter hafniae	1/1	100	0/1	0	not tested	
Enterobacter cloacae	7/8	88	1/8	13	0/1	0
Serratia marcescens	2/2	100	0/2	0	1/2	50
Serratia liquefaciens	2/2	100	2/2	100	1/1	100
Proteus vulgaris	0/2	0	0/2	0	0/1	0
Erwinia carotovora	1/2	50	0/2	0	0/2	0
Erwinia (other)	1/5	20	0/5	0	not tested	
Yersinia enterocolitica	7/7	100	0/7	0	1/4	25
Yersinia pseudotuberculosis	0/2	0	0/2	0	not tested	

Table 2. Growth of gram-negative bacteria in 0.2% inositol.

Culture	$O.D._{600}$		
	at 0 h	at 13 h	at 20 h
Klebsiella ATCC 13182	.010	.320	.396
Enterobacter aerogenes ATCC 13048	.012	.368	.568
Enterobacter agglomerans ICPB EH103	.009	.039	.112
Enterobacter cloacae OSU	.004		.008
Serratia liquefaciens ATCC 14460	.009	.200	.288
Serratia mercescens OSU	.012	.050	.105
Citrobacter freundii OSU	.009	.005	.001
Erwinia carotovora ICPB EC153	.010	.040	.109
Escherichia coli DS5-1 OSU	.009	.011	.009
Proteus vulgaris OSU	.009		.009
Pseudomonas aeruginosa OSU	.015	.015	.012
Salmonella enteritidis ser. paratyphi B OSU	.005		.120
Shigella dysenteriae OSU	.005		.005
Yersinia enterocolitica CDC 867	.009	.021	.032

Cultures were pregrown in limiting glucose and a few drops were inoculated into basal salts plus 0.2% inositol. Cultures were incubated on a shaker at 35°C (28°C for Erwinia and Yersinia).

Table 3. Growth of gram-negative bacteria in aqueous extracts of redwood sawdust in the presence of indigenous flora.

Culture	Origin	Viable cell count, days after inoculation (per ml)[a]		
		2	6	31
Klebsiella				
PC$_2$	redwood tank water	1.5×10^4	7.0×10^5	5.6×10^5
ATCC 13882		7.4×10^2	3.2×10^5	8.2×10^3
PS401LR	bovine mastitis	9.4×10^4	3.0×10^5	1.9×10^5
PSB49-1	bovine mastitis	4.3×10^4	1.4×10^5	2.8×10^5
SL-1	human medical	4.7×10^3	3.4×10^4	1.0×10^4
JH$_4$2	drinking water	1.8×10^3	3.0×10^4	4.3×10^4
ATCC 13182		3.4×10^3	1.6×10^3	8.0×10^3
U010994	human medical	5.6×10^2	3.6×10^3	2.9×10^3
UT1500	human medical	2.1×10^3	3.3×10^3	1.8×10^3
ATCC 13883		5.5×10^2	9.2×10^2	1.1×10^3
MH31	human medical	6.0×10^2	5.3×10^2	5.2×10^2
U011065	human medical	1.8×10^2	1.7×10^2	4.0×10^1
MSU1684	bovine mastitis	1.1×10^2	3.0×10^1	3.0×10^1
Enterobacter agglomerans ICPB EH103		3.2×10^3	3.1×10^5	1.4×10^2
Erwinia carotovora ICPB EC153		1.0×10^5	1.5×10^4	6.6×10^2
Serratia liquefaciens ATCC 14460		7.0×10^3	7.3×10^3	1.1×10^2
Yersinia enterocolitica CDC 867		1.1×10^4	5.3×10^3	6.0×10^3
Enterobacter aerogenes ATCC 13048		3.7×10^2	1.1×10^3	5.3×10^2
Serratia marcescens OSU		3.8×10^2	4.1×10^2	3.0×10^2
Citrobacter freundii OSU		5.5×10^2	2.5×10^2	5.5×10^2
Enterobacter cloacae OSU		3.7×10^2	2.1×10^2	$< 1.0 \times 10^1$
Escherichia coli DS5-1 OSU		5.0×10^2	1.8×10^2	$< 1.0 \times 10^1$
Proteus vulgaris OSU		2.0×10^2	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
Salmonella enteritidis ser. paratyphi B OSU		$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
Salmonella typhi OSU		$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
Shigella dysenteriae OSU		$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
Indigenous flora (uninoculated)		1.9×10^5	6.0×10^6	9.5×10^6

[a] Initial viable counts ranged from 1.0×10^2 to 5.0×10^2/ml. Counts were recorded on days 0,2,6,14, and 31.

Table 4. Growth of gram-negative bacteria in sterile aqueous extracts of redwood sawdust.

Culture	Origin	Viable cell count, days after inoculation (per ml)[a]		
		2	6	31
Klebsiella				
UT1500	human medical	3.0×10^4	1.5×10^6	1.2×10^6
PC$_2$	redwood tank water	4.3×10^4	1.3×10^6	1.8×10^6
MH31	human medical	1.8×10^4	1.2×10^6	1.4×10^6
PS401LR	bovine mastitis	2.1×10^4	1.1×10^6	9.5×10^5
PSB49-1	bovine mastitis	6.7×10^3	1.1×10^6	1.8×10^6
SL-1	human medical	4.7×10^3	1.1×10^6	1.3×10^6
ATCC 13882		1.0×10^3	1.1×10^6	9.5×10^5
ATCC 13182		8.8×10^2	1.1×10^6	1.3×10^6
JH$_4$2	drinking water	1.3×10^4	5.6×10^5	8.0×10^5
UO10994	human medical	5.8×10^2	8.7×10^4	4.3×10^5
UO11065	human medical	9.5×10^2	6.8×10^4	3.2×10^4
ATCC 13883		1.3×10^3	2.1×10^4	$<1.0 \times 10^1$
MSUI684	bovine mastitis	9.0×10^1	2.0×10^1	$<1.0 \times 10^1$
Erwinia carotovora				
ICPB EC153		2.3×10^4	2.5×10^6	1.6×10^5
Serratia liquefaciens				
ATCC 14460		1.7×10^3	1.2×10^6	5.6×10^6
Enterobacter agglomerans				
ICPB EH103		1.2×10^2	3.7×10^5	6.5×10^5
Enterobacter aerogenes				
ATCC 13048		1.2×10^3	1.5×10^5	1.5×10^5
Citrobacter freundii OSU		6.6×10^2	1.1×10^4	3.0×10^5
Escherichia coli		7.6×10^2	3.3×10^3	5.2×10^4
DS5-1 OSU				
Serratia marcescens OSU		3.1×10^2	8.9×10^2	2.1×10^5
Enterobacter cloacae OSU		6.1×10^2	8.0×10^2	1.4×10^2
Yersinia enterocolitica		3.1×10^2	2.4×10^2	4.9×10^6
CDC 867				
Proteus vulgaris OSU		1.2×10^3	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Salmonella enteritidis ser.		3.0×10^1	1.0×10^1	$<1.0 \times 10^1$
paratyphi B OSU				
Shigella dysenteriae OSU		1.1×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$

[a] Initial viable counts ranged from 1.0×10^2 to 5.0×10^2/ml. Counts were recorded on days 0,2,6,14, and 31.

Table 5. Correlation between growth level in non-sterile aqueous redwood extract and cyclitol fermentation in phenol red broth.

Culture	Maximum viable count (per ml)	Response with: Inositol	Pinitol	Sequoyitol
Colonizing bacteria ($>10^3$/ml)				
Klebsiella				
PC$_2$	7.0×10^5	+G[a]	+G	+G
PS401LR	3.6×10^5	+G	+	+
ATCC 13882	3.2×10^5	+G	+G	+
PSB49-1	2.9×10^5	+G	+	+G
JH$_4$2	4.6×10^4	+G	-	±
SL-1	3.4×10^4	+G	+G	+G
ATCC 13182	1.6×10^4	+G	+G	+G
UO10994	3.8×10^3	+G	+	+G
UT1500	3.3×10^3	+G	-	-
ATCC 13883	1.1×10^3	+G	+	+G
Enterobacter agglomerans ICPB EH103	3.1×10^5	±	±	-
Erwinia carotovora ICPB EC153	5.4×10^4	+	-	-
Yersinia enterocolitica CDC 867	9.1×10^3	+	-	+
Serratia liquefaciens ATCC 14460	7.3×10^3	+	-	+
Enterobacter aerogenes ATCC 13048	1.3×10^3	+G	-	+G
Non-colonizing bacteria ($<10^3$/ml)				
Klebsiella				
MH31	6.0×10^2	+G	+G	+G
UO11065	2.6×10^2	+G	±	+
MSU1684	1.1×10^2	+G	-	±
Salmonella enteritidis ser. paratyphi B OSU	6.6×10^2	+G	+G	+G
Citrobacter freundii OSU	5.9×10^2	-	-	-
Escherichia coli DS5-1 OSU	5.0×10^2	-	-	-
Serratia marcescens OSU	4.1×10^2	+	-	-
Enterobacter cloacae OSU	3.7×10^2	-	-	-
Shigella dysenteriae OSU	3.2×10^2	-	-	-
Proteus vulgaris OSU	3.1×10^2	-	-	-
Salmonella typhi OSU	1.6×10^2	-	-	-

[a]G : gas production.

MYO-INOSITOL
7%

SEQUOYITOL
31%
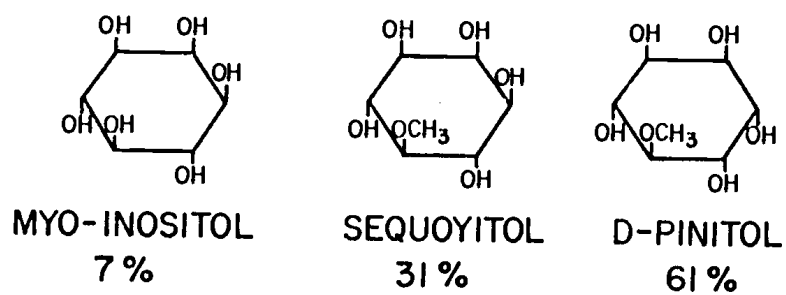
D-PINITOL
61%

Figure 1. Cyclitol compounds present in redwood and their
relative percentages.
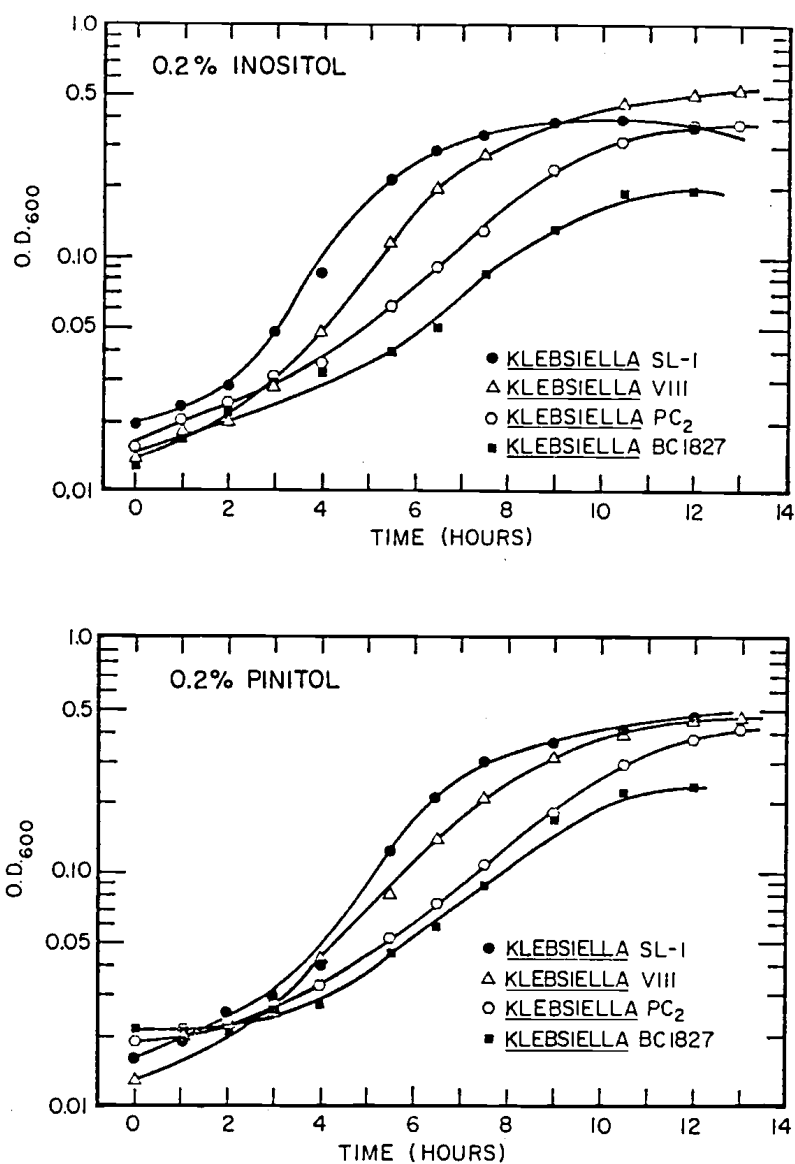
Figure 2. Growth curves with Klebsiella grown in a basal salts medium supplemented with 0.2% inositol or pinitol. Origin of strains tested: SL-1, human medical; Vlll, vegetable; PC_2, redwood tank water; BC 1827, pulp mill effluent.

Chapter 4

Effects of Chlorine Residuals, Retention Times, and
Cyclitol Concentrations on Coliforms in Redwood
Water Storage Reservoirs

Abstract

Experiments were conducted in three small 90 liter
redwood tanks in order to assess the effects of chlorine
residuals, retention times, and cyclitol concentrations in
redwood tank water on coliforms and on Klebsiella. As
chlorine residuals increased in tank 1 (a result of a 5 day
retention schedule) coliform and total bacterial counts
decreased to 5.3/ml and 9.9×10^4/ml, respectively. No
cyclitol compounds were detected in tank water after 22
weeks of service. Tank 2 was maintained in a stagnant con-
dition, and cyclitol concentrations, coliform counts, and
optical density readings of tank water increased. pH
values decreased during this period. In tank 3, a decrease
in cyclitol concentration correlated with a drop in coliform
and Klebsiella densities. From stave slime material,
isolates of Klebsiella, Enterobacter agglomerans, and Entero-
bacter cloacae were identified, as well as several species
of aerobic gram-negative bacteria. No cyclitol degradation
by indigenous coliforms was observed in samples of water
taken from tanks 2 and 3. The fact that some samples of
water will not support the growth of Klebsielleae partially

explains why cyclitols are gradually consumed in redwood reservoirs by these organisms, and that water flow is required to completely remove them from the tanks.

Introduction

Among the factors affecting the presence and growth of coliform bacteria (including <u>Klebsiella</u>) in redwood water storage reservoirs are free chlorine residuals in the water and retention times for water storage (6,7) and presumably cyclitol concentrations. Low chlorine residuals allow multiplication of coliform bacteria in the water, long retention times result in stagnation of tank water and leaching of water soluble nutrients, and cyclitol concentrations reflect the nutrient potential for cyclitol-positive coliforms.

This study was conducted with three small 90 liter redwood tanks in order to assess the effects of these three parameters. In one of the tanks, an antibiotic resistant strain of <u>Klebsiella</u> was inoculated, and growth and survival characteristics were monitored as conditions were varied. Indigenous coliforms were monitored in the other two tanks. Samples of water from the tanks were taken at intervals and analyzed for cyclitol content using gas chromatographic techniques. Scraping of slime from the staves of the tanks was performed, and coliforms as well as other indigenous bacteria were identified from this material. Regular sampling of tank water was performed in order to enumerate total coliforms, <u>Klebsiella</u>, and to determine total bacterial counts. An additional experiment

was conducted where water was removed from two of the tanks and incubated with shaking in an attempt to demonstrate cyclitol utilization in tank water by indigenous coliforms.

Materials and Methods

Redwood tanks. Three small redwood reservoirs (capac-
ity 90 liters) were constructed by National Tank and Pipe
Co., Portland, Ore., for use in this investigation (Figure 1).
Each tank had two holes drilled, one at the top and one at
the bottom. Tap water from Corvallis municipal lines
entered at the bottom and overflowed out the top hole. Water
was dripped into the tanks from carboy containers situated
above water level. Valves on the carboys were adjusted to
regulate flow rates and retention times.

Observations of physical parameters of tank water.
Free chlorine residuals and total chlorine residuals in
tank water were determined by the N,N-diethyl-para-phenylene
diamine (DPD) method. Optical density readings were taken
with a Bausch and Lomb Spectronic 20 colorimeter, pH read-
ings were also determined for some water samples.

Bacteriological examination of tank water. Water in
all three tanks was frequently examined for total coliforms,
Klebsiella, and total bacterial counts. Coliform counts
and total bacterial counts were determined using standard
procedures (1); Klebsiella were enumerated on a MacConkey-
Inositol-Carbenicillin (MCIC) medium (3). Both spread plate
and membrane filtration techniques were employed for enu-
meration of coliforms and Klebsiella.

Bacteriological examination of staves. The water level
was lowered in the tanks and the interior surfaces were
scraped with sterile glass microscope slides. Surface
material was dispersed in 0.01M tris (hydroxymethyl amino-
methane) buffer and tested for total coliforms by the spread
plate technique on mENDO agar LES (Difco), and for Klebsiella
on MCIC medium. Total bacterial counts were enumerated on
plate count agar (Difco).

Identification of isolates from stave material. Media
preparation and procedures used for the identification of
coliform organisms were those recommended by Edwards and
Ewing (5). Final identifications were made using the API
20E System (Analytab Products, Plainview, N.Y.). Isolates
obtained from plate count agar were identified using 9-digit
profiles of the API 20E System, or by procedures outlined
in the eighth edition of Bergey's Manual of Determinative
Bacteriology (4).

Gas chromatographic analysis of cyclitol concentrations
in tank water. In all cases 0.5 ml samples of tank water
were analyzed. Quebrachitol, (a gift from Dr. Laurens
Anderson, Department of Biochemistry, University of Wiscon-
sin) a cyclitol not present in redwood, was used as an inter-
nal quantitation standard. 0.05 ml of a 1 mg/ml aqueous
solution of quebrachitol was added to all samples with a
digital microliter pipet, immediately before dry heat

evaporation.   Dry samples were silylated with 0.2 ml of Tri

Sil Z (Pierce Chemical Co., Rockford, Ill.) for 3 hours

at room temperature and 2 µl was injected into the gas

chromatograph.

A Hewlett-Packard Model 5710A gas chromatograph with

a flame ionization detector was used in these experiments.

This instrument was equipped with a 6 foot 3% OV-17 station-

ary phase on a Gas Chrom Q support (100-120 mesh) for

analysis of redwood tank water samples.   The oven tempera-

ture used was 160°C, with a carrier gas (helium) flow of

20 ml/min.   Injection port temperature was 260°C; detector

temperature was 300°C.   Peaks were recorded with a Hewlett-

Packard Model 7123 A strip chart recorder, using a chart

drive speed of 15 in/hr.   The FID control was set with a

range of 10 and attenuation of 2 for all analyses.

Quantitative determination of cyclitol concentrations

was determined by first preparing a tracing on uniform

thickness vellum tracing paper.   The peaks were cut out and

weighed on a Mettler H20 analytical balance and the mass

compared to that of the internal standard.

Results and Discussion

The results of experiments with tank 1 are contained in Table 1. This set of data best illustrates the effects of retention times and chlorine residuals on coliform counts and on total counts. In the period between week 2 and week 17, this tank was on a 6 day or 8 day retention schedule. With these retention times, the amount of free chlorine in the water was below detection. During this period, total coliforms present averaged about 10^3/ml, with not a great deal of variation. Similarly, total counts were consistently between 10^5 and 10^6 cells/ml. Klebsiella, as monitored by counts on MCIC/medium, were consistently detectable.

During week 17, the retention schedule on tank 1 was changed to 5 days. With this turnover rate, a low chlorine residual was detected by week 18. During week 19, the turnover schedule was increased to 3 days. Chlorine residuals in the water increased to 0.1-0.2 ppm. Concurrent with this increase in chlorine residual, both the coliform count and total counts decreased. By week 22, coliform counts were 5.3/ml (Figure 2), and total counts were down to 9.9×10^4/ml. These levels, however, are still far in excess of federal drinking water standards (1), and additional chlorine would be needed to lower them to acceptable levels.

The effects of continuous water flow through a redwood tank can be seen in the cyclitol concentration and optical

density data for tank 1. Although early data for cyclitols was not obtained with tank 1, by week 17 the cyclitol concentration was at a relatively low level, and by week 22 was undetectable (Figure 3). The optical density readings reflect the leaching out of water soluble extractives (mostly phenolic compounds) that give redwood its characteristic color (2). A decrease in these extractives was observed between week 7 and week 18, when the optical density dropped from 0.010 to 0.008. By week 22, the optical density of tank water was 0.000. Tap water was used as a blank.

Based on these results with tank 1, it appears that a 22 week period with the retention times used here is sufficient to leach nearly all of the cyclitol compounds (which support the growth of Klebsielleae) from a small redwood tank. In addition, other phenolic compounds that may be involved in inactivating free chlorine and thus contribute significantly to the chlorine demand of redwood reservoirs are also removed by this time. Larger redwood tanks, however, might require a longer period of time to reach this stage.

Table 2 contains the results of experiments with tank 2. From week 2 to week 9, this tank was set on a 6 day or 8 day retention schedule. During week 9, the inlet water was shut off and the water in the tank was stagnant through week 22. Chlorine residuals were below detection levels

throughout the period of investigation. During this time
of stagnation, coliform counts increased (Figure 2), al-
though no Klebsiella were detected. Total counts remained
at a level greater than 10^6 cells/ml. The cyclitol concen-
trations increased from 32 μg/ml (Figure 4) to almost 60μg/ml
by week 17, which correlates with the increase in coliforms.
Figure 5 illustrates the cyclitol profile at week 22.
Similarly, optical density readings increased from 0.013
at week 7 to 0.040 by week 18, reflecting the accumulation
of water soluble extractives that are leached from the red-
wood staves of the tank. The pH of tank water dropped from
6.55 during week 3 to 6.45 by week 17, which also reflects
the accumulation of tannin and phenolic compounds.

Tank 3 results are displayed in Table 3. In contrast
to tanks 1 and 2, tank 3 was stagnant from week 1 to week
16, then put on an 8 day retention schedule from week 17
to week 22. Throughout the period of investigation,
chlorine residuals were undetectable in this tank. The
effects of prolonged stagnation are reflected in the
cyclitol concentration and pH data. Total amounts of
cyclitol compounds increased to 80 μg/ml by week 17.
(Figure 6) and the pH of the water decreased from 6.25
during week 3 to 5.60 by week 17. However, after water
began to flow through the tank, the cyclitol concentration
dropped rapidly to undetectable levels by week 22 (Figure 7).

Presumably the cyclitols were completely leached from the tank by this time. Concurrent with the decrease in cyclitol concentration was a drop in coliform density from 3.2×10^4/ml (week 17) to 1.6×10^2/ml by week 22 (Figure 2). Klebsiella densities also decreased during this time, as did total bacterial counts.

Figure 8 represents Klebsiella densities in tanks 1 and 3 throughout the experimental period. Included in this figure are the viable counts for SL5, a strain of Klebsiella inoculated at a low density into tank 3 during week 1. SL5 was resistant to kanamycin, neomycin, and streptomycin, and was recovered on MacConkey agar (Difco) containing these antibiotics. The figure shows that SL5 at certain periods comprised up to 10% of the total Klebsiella present. When the cyclitol concentrations dropped after week 17, both the SL5 counts and total Klebsiella counts exhibited a 10 to 15 fold decrease. Klebsiella counts in tank 1 decreased significantly after week 13; this is presumably due to a decrease in cyclitol concentration along with an increase in chlorine residual.

On three occasions, slime material that had accumulated on the staves of the tanks was scraped and examined for total coliforms, Klebsiella, and other bacteria. In tank 1, coliforms identified were Enterobacter agglomerans and Klebsiella. Both indole-negative and indole-positive

(oxytoca group) Klebsiella were found. Non-coliform

bacteria identified were Pseudomonas fluorescens and

Moraxella spp. From tank 2, Enterobacter agglomerans and

Enterobacter cloacae were isolated, but no Klebsiella were

found. The reason why Klebsiella were never detected in

this tank is unknown, especially since the other two tanks

had indigenous Klebsiella. Other bacteria identified from

slime in tank 2 were Chromobacterium spp. and Pseudomonas

fluorescens. Tank 3 slime yielded Enterobacter agglomerans,

indole-positive and indole-negative Klebsiella, Pseudomonas

putida, and Pseudomonas paucimobilis.

During week 17, samples of water from tanks 2 and 3

were taken and incubated at 28°C with shaking for four

days, in an attempt to demonstrate cyclitol degradation

in tank water by indigenous coliforms. Samples for gas

chromatography, coliform counts, and total counts were

taken at inoculation and at 4 days. Although cyclitols

were detectable in both tank samples, no degradation of

these compounds was observed in the 4 day period. Neither

coliform counts or total counts increased significantly.

Since other experiments in redwood extracts have shown

cyclitol utilization by Klebsielleae, with similar but

somewhat higher cyclitol concentrations (Chapter 5), some

other compound(s) are probably limiting growth in these

samples. Experiments in Chapter 2 demonstrated that

<u>Klebsiella</u> can grow to densities exceeding 10^6 cells/ml in some samples of water from redwood tanks, but that in other samples no growth was observed. The fact that some samples of water will not support the growth of <u>Klebsielleae</u> helps explain why cyclitols are gradually consumed in redwood tanks by these organisms, and that water flow is required to completely remove them from the tanks.

Acknowledgments

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Table 1. Physical and bacteriological parameters in experimental tank 1

Week	Retention time (days)	Free chlorine residual (ppm)	Total coliforms (per ml)[a]	Klebsiella (per ml)[b]	Total bacteria (per ml)[c]	Total cyclitol concentration (μg/ml)	pH	OD_{600}
1	5	< 0.1	1.0×10^2	ND[d]	1.3×10^6			
2	5-6	0.1	1.6×10^2	ND	1.7×10^6			
3	6	< 0.1	2.6×10^2	ND	1.0×10^6		6.6	
4	8	< 0.1	3.5×10^2	ND	3.1×10^6			
5	8	< 0.1	2.1×10^3	ND	3.7×10^5			
6	8	< 0.1	4.4×10^3	1.4×10^1	5.6×10^5		6.6	
7	8	< 0.1	4.9×10^3	2.9×10^1	4.8×10^5			0.010
8	8	< 0.1	2.2×10^3	3.8×10^1	7.8×10^5			
9	8	< 0.1	6.8×10^3	1.7×10^1	7.6×10^5			
10	8	< 0.1	2.3×10^3	1.2×10^2	9.2×10^5			
11	8	< 0.1	3.0×10^3	1.7×10^2	1.2×10^6			
12	8	< 0.1	1.8×10^3	3.3×10^2	1.2×10^6			
13	8	< 0.1	9.6×10^2	1.0×10^3	1.0×10^6			
14	8	< 0.1	8.5×10^2	5.6	5.2×10^5			
15	8	< 0.1	4.5×10^2	4.2	2.6×10^6			
16	8	< 0.1	4.6×10^2	3.4	3.3×10^6			
17	5	< 0.1	1.9×10^2	2.4	4.7×10^5	4	7.1	
18	5	0.1-0.2	1.5×10^1	4.0	4.9×10^5			0.008
19	3	0.1	3.0×10^1		1.2×10^6			
20	3	0.1	7.7		1.2×10^6			
21	3	0.1-0.2	9.5	6.7	2.4×10^5			
22	3	0.1-0.2	5.3		9.9×10^4	ND		0.000

a green sheened colonies on mENDO agar LES within 24 hours
b enumerated on MCIC medium
c enumerated on plate count agar
d ND-not detected

Table 2. Physical and bacteriological parameters in experimental tank 2

Week	Retention time (days)	Free chlorine residual (ppm)	Total coliforms (per ml) [a]	Klebsiella (per ml) [b]	Total bacteria (per ml) [c]	Total cyclitol concentration (μg/ml)	pH	OD_{600}
1	5	< 0.1	ND [d]	ND	5.5×10^5			
2	5-6	0.1	ND	ND	1.1×10^6			
3	6	< 0.1	ND	ND	3.7×10^6		6.55	
4	8	< 0.1	ND	ND	3.7×10^6			
5	8	< 0.1	ND	ND	2.8×10^6			
6	8	< 0.1	ND	ND	2.2×10^6	32	6.50	
7	8	< 0.1	3.2	ND	1.3×10^6			0.013
8	8	< 0.1	2.3	ND	2.1×10^6			
9	stagnant	< 0.1	ND	ND	1.8×10^6			
10	stagnant	< 0.1	ND	ND	1.4×10^6			
11	stagnant	< 0.1	ND	ND	1.5×10^6			
12	stagnant	< 0.1	1.0	ND	1.5×10^6			
13	stagnant	< 0.1		ND	2.3×10^6			
14	stagnant	< 0.1	3.7×10^3	ND	4.3×10^6			
15	stagnant	< 0.1	2.0×10^4	ND	2.3×10^6			
16	stagnant	< 0.1	2.1×10^2	ND	2.9×10^6			
17	stagnant	< 0.1	2.6×10^2	ND	3.6×10^6	58	6.45	
18	stagnant							0.040
19	stagnant							
20	stagnant							
21	stagnant	< 0.1	1.4×10^2	ND	8.9×10^5			
22	stagnant	< 0.1	1.8×10^2	ND	1.1×10^6	55		0.041

a green sheened colonies on mENDO agar LES within 24 hours
b enumerated on MCIC medium
c enumerated on plate count agar
d ND-not detected

Table 3. Physical and bacteriological parameters in experimental tank 3

Week	Retention time (days)	Free chlorine residual (ppm)	Total[a] coliforms (per ml)	Klebsiella[b] (per ml)	Total[c] bacteria (per ml)	Total cyclitol concentration (µg/ml)	pH	OD_{600}
1	stagnant	< 0.1	ND[d]	ND	7.1×10^5			
2	stagnant	< 0.1	ND	ND	8.8×10^5	41		
3	stagnant	< 0.1	1.9×10^3	ND	1.6×10^6		6.25	
4	stagnant	< 0.1	1.7×10^3	1.0×10^1	5.5×10^5			
5	stagnant	< 0.1	4.8×10^4	ND	2.9×10^6			
6	stagnant	< 0.1	8.7×10^4	ND	5.1×10^6	75	5.90	
7	stagnant	< 0.1	5.9×10^4	4.0×10^1	4.0×10^6			0.019
8	stagnant	< 0.1	4.0×10^4	1.7×10^3	7.2×10^6			
9	stagnant	< 0.1	4.4×10^4	1.7×10^3	6.3×10^6			
10	stagnant	< 0.1	1.3×10^3	1.3×10^3	6.8×10^6			
11	stagnant	< 0.1	2.0×10^3	1.0×10^3	6.3×10^6			
12	stagnant	< 0.1	1.1×10^3	1.0×10^3	8.1×10^6			
13	stagnant	< 0.1	9.7×10^2	1.1×10^3	7.8×10^6			
14	stagnant	< 0.1	1.2×10^4	9.2×10^2	7.6×10^6			
15	stagnant	< 0.1	2.1×10^4	1.1×10^4	7.4×10^6			
16	stagnant	< 0.1	1.4×10^4	7.9×10^3	1.2×10^7			
17	8	< 0.1	3.2×10^4	1.4×10^4	8.3×10^6	80	5.60	
18	8	< 0.1	9.9×10^3	2.4×10^3	2.5×10^6			0.015
19	8	< 0.1	4.7×10^3	3.0×10^3	5.9×10^6			
20	8	< 0.1	1.9×10^3	2.4×10^3	3.2×10^6			
21	8	< 0.1	9.3×10^2	9.0×10^2	8.6×10^5			
22	8	< 0.1	1.6×10^2		3.0×10^5	ND		0.020

a green sheened colonies on mENDO agar LES within 24 hours
b enumerated on MCIC medium
c enumerated on plate count agar
d ND-not detected

Figure 1. 90 liter redwood reservoirs used in these
            experiments.
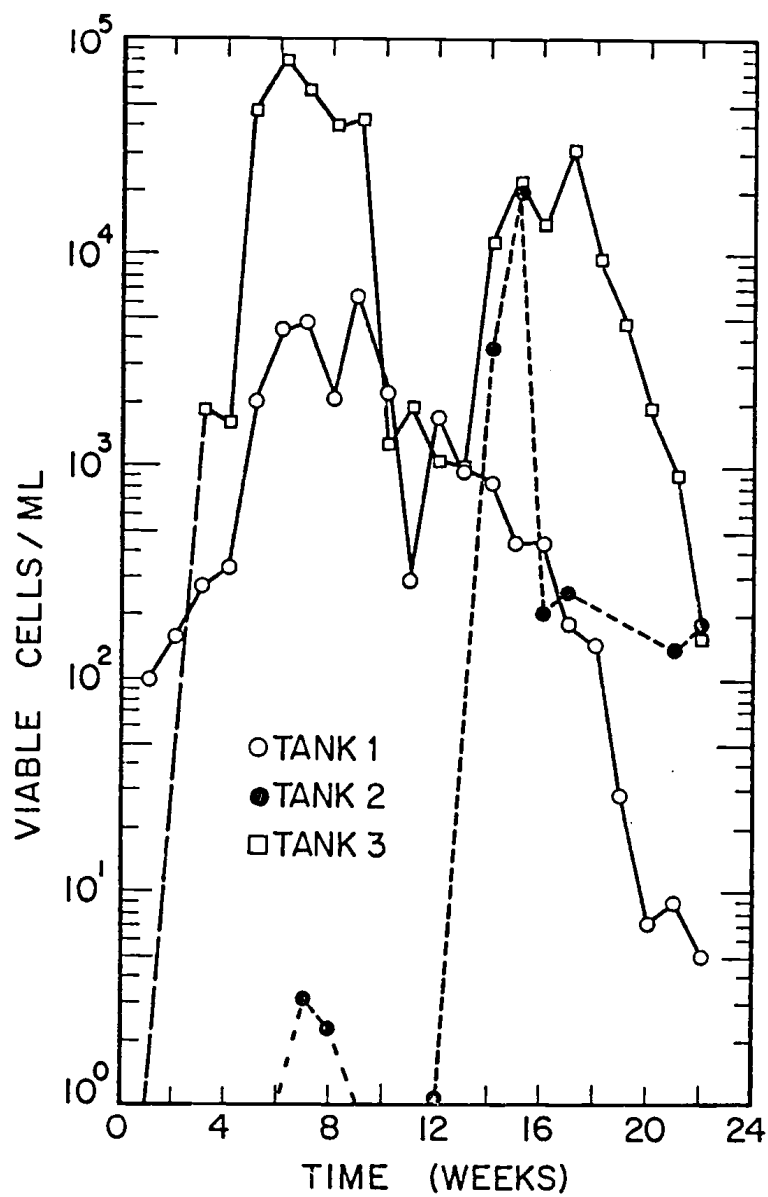
Figure 2. Coliform levels in water stored in the
experimental redwood tanks.
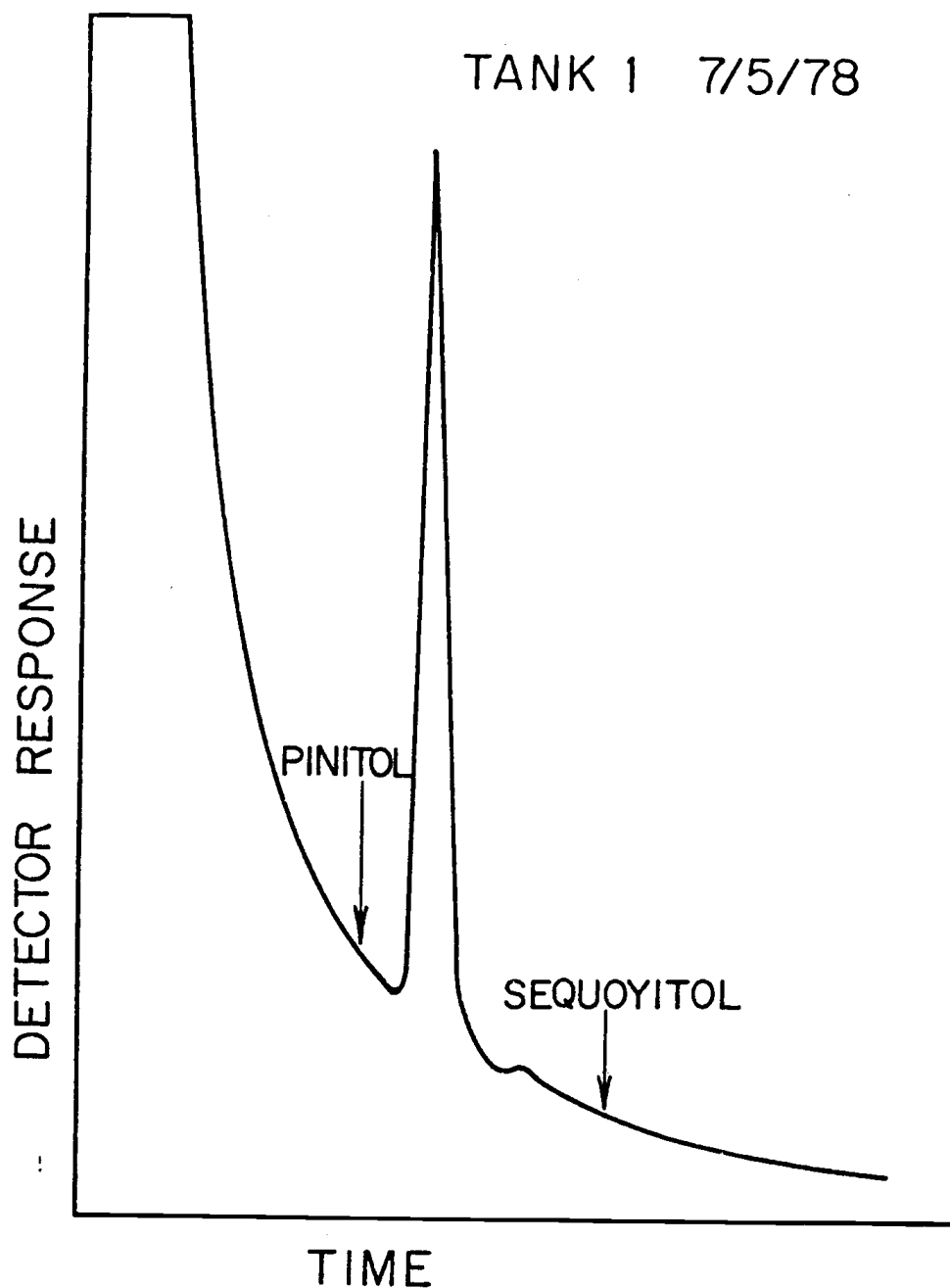
Figure 3. Gas chromatographic analysis of tank 1 water
collected on 7/5/78 (3 day retention schedule).
The large peak is that of quebrachitol, the
internal standard. Column temperature was 160°C.
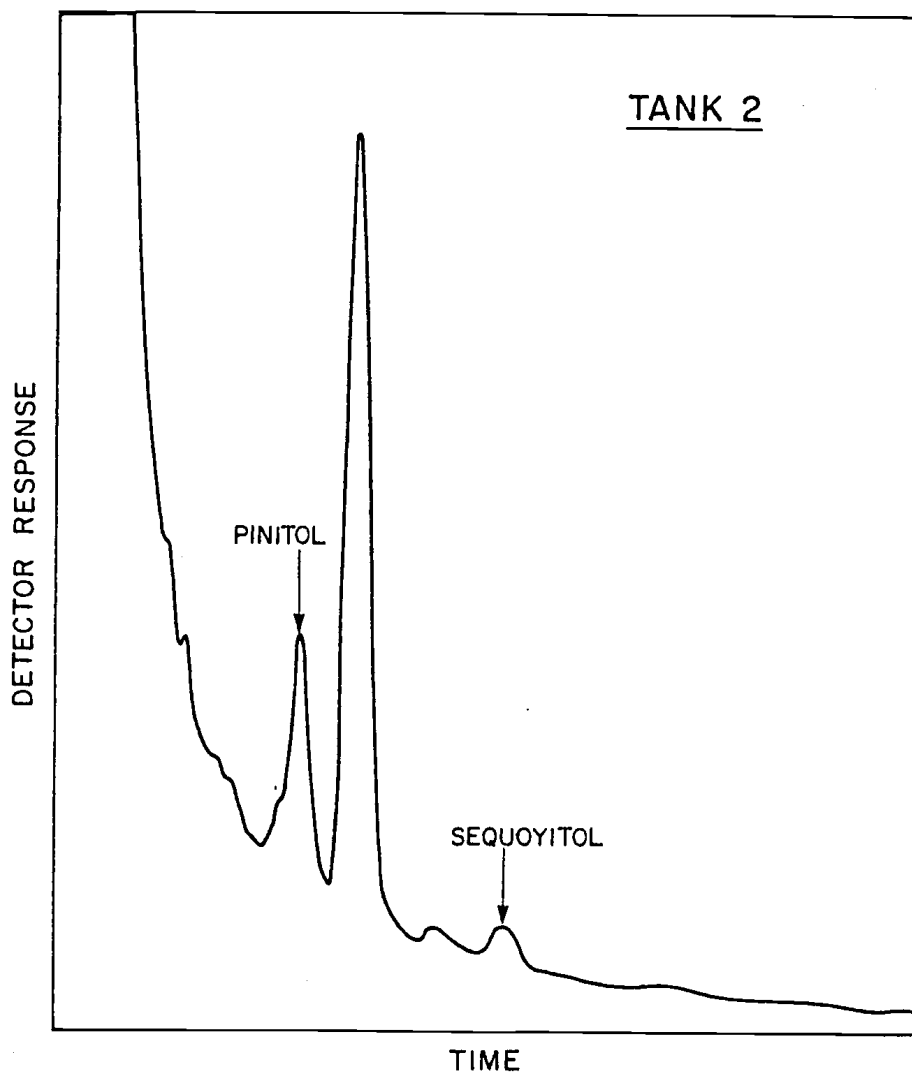
Figure 4. Analysis of tank 2 water collected on 3/14/78
(8 day retention). Column temperature was
160°C.
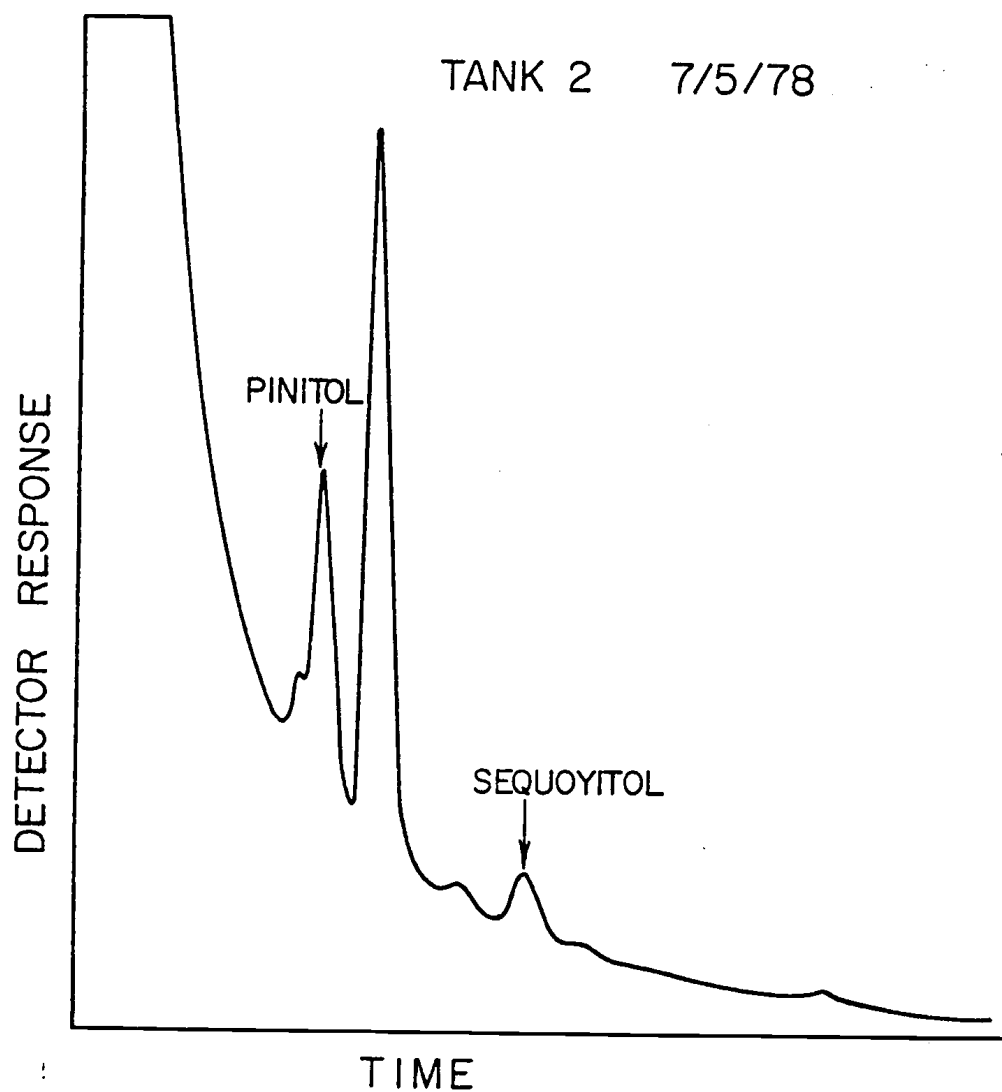
Figure 5. Tank 2 water collected on 7/5/78 (stagnant tank).
Column temperature was 160°C.
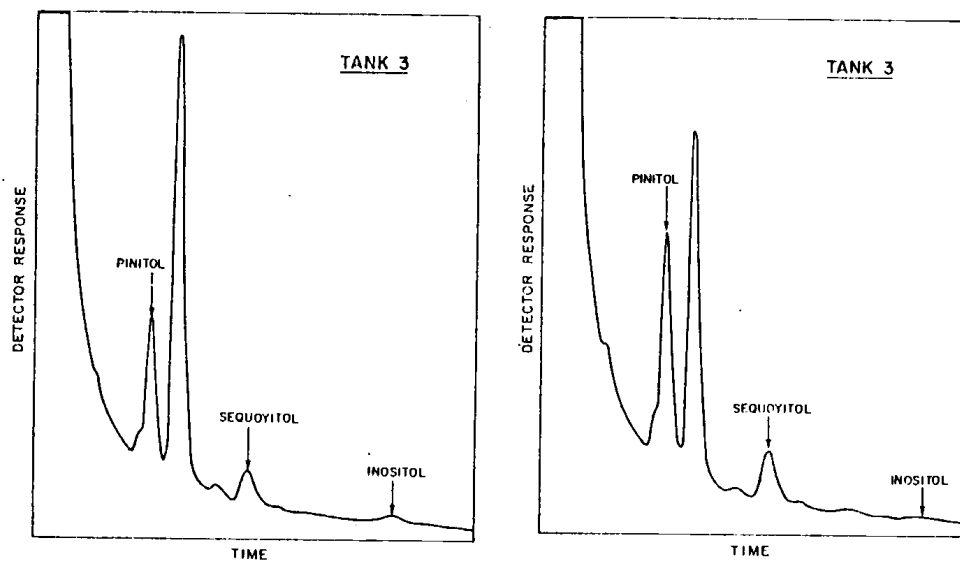
Figure 6.   Tank 3 water collected on 2/10/78 (stagnant tank) and on 3/14/78 (stagnant).   Column temperature was 160°C.
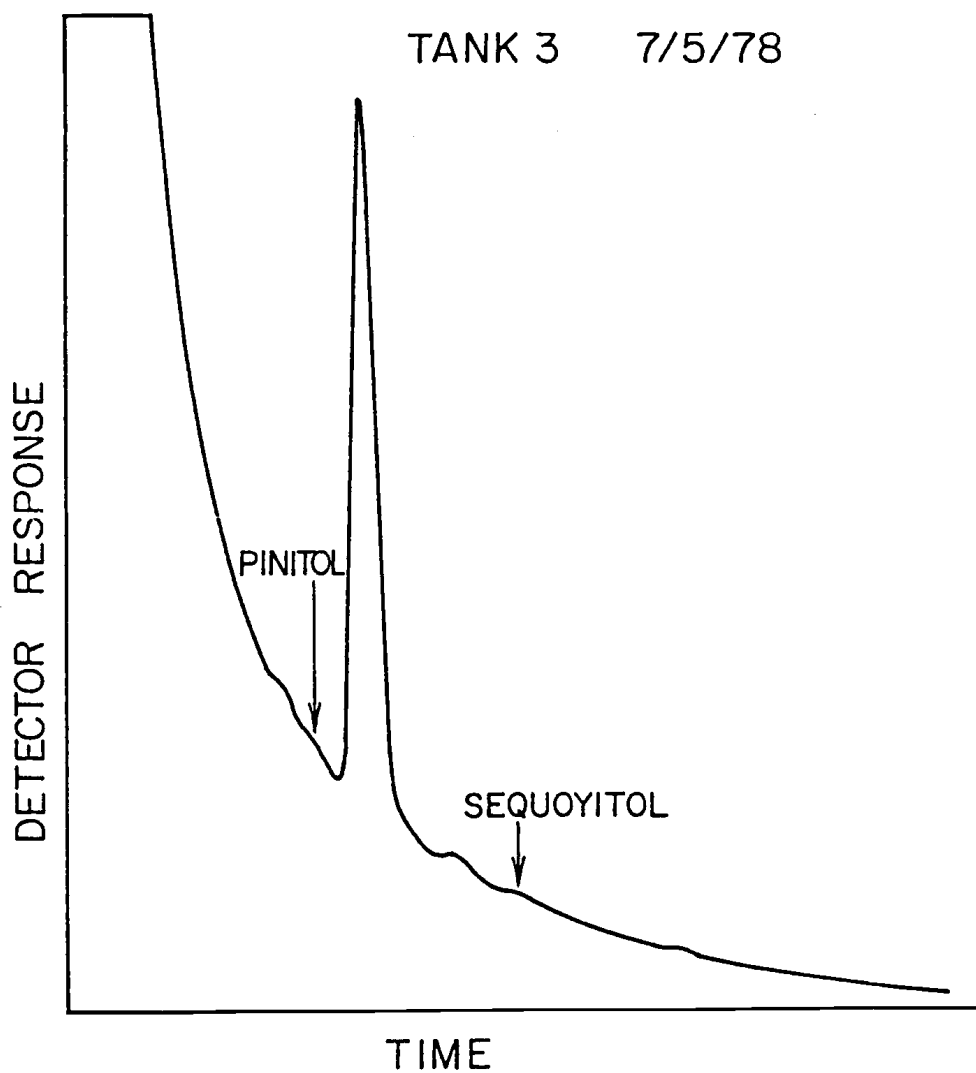
Figure 7.   Tank 3 water collected on 7/5/78 (8 day
            retention). Column temperature was 160°C.
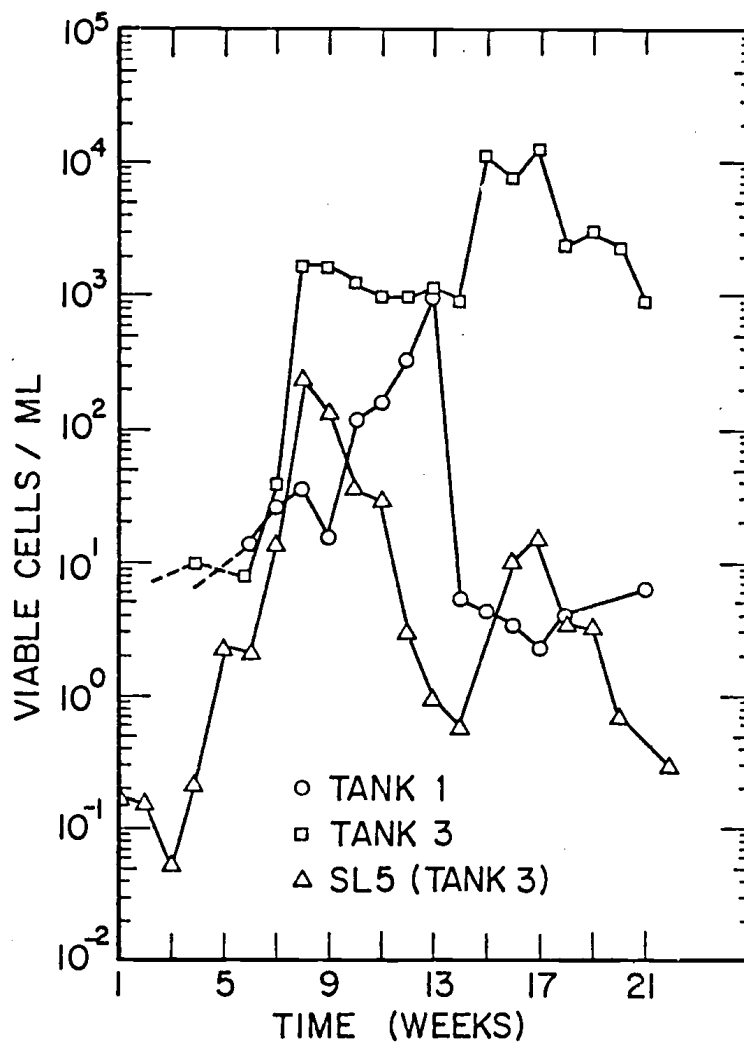
Figure 8. <u>Klebsiella</u> densities observed in water stored in redwood tanks 1 and 3. Included are densities for <u>Klebsiella</u> SL5, an antibiotic resistant culture.

Chapter 5

Gas Chromatographic Analysis of <u>In</u> <u>Situ</u> Cyclitol Utilization

by <u>Klebsielleae</u> Growing in Redwood Extracts

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Abstract

Gas chromatographic analysis was employed to demonstrate <u>in</u> <u>situ</u> cyclitol utilization in aqueous extracts of redwood by isolates of <u>Klebsiella</u>, <u>Enterobacter</u>, and several other genera of gram-negative bacteria. In aqueous redwood extracts, all of the <u>Klebsiella</u> isolates tested reached densities exceeding 5.0×10^6 cells/ml within 4 days and all utilized pinitol and sequoyitol, as did <u>Enterobacter</u>. Other enteric bacteria did not utilize cyclitols in this extract. A defined minimal medium, containing the carbohydrates and cyclitols in redwood (including myo-inositol), was used to determine which carbon sources are preferentially utilized by <u>Klebsielleae</u> and other bacteria. It was found that D-glucose and L-arabinose were consumed by <u>Klebsiella</u> before the three cyclitols were utilized. Pinitol utilization proceeded more slowly than that of sequoyitol and myo-inositol. Cyclitol utilization in the defined medium was also observed for strains of <u>Yersinia</u>, <u>Erwinia</u>, and <u>Salmonella</u>. <u>E</u>. <u>coli</u> isolates did not utilize cyclitol compounds. The ability to use cyclitols as a sole source of carbon can explain the presence of <u>Klebsielleae</u> in redwood water reservoirs and in redwood lumber. This ability may also be related to their presence and growth in a variety of

other botanical material containing cyclitol compounds.

Introduction

Recent reports have documented the association of bacteria of the _Klebsiella_ and _Enterobacter_ genera with redwood. These organisms have been found within redwood lumber (5) and in drinking water and on colonized staves in redwood reservoirs (19,21). _Klebsiella_ and _Enterobacter_ are capable of growth in aqueous redwood extracts in the presence of indigenous bacteria; most other enteric bacteria are not (22).

The predominant carbon sources in redwood that can be metabolized by _Klebsielleae_ (and not by most other enteric bacteria) are the cyclitol compounds pinitol, sequoyitol, and myo-inositol (2). Cyclitol metabolism in redwood extracts has been postulated to be an important nutritional basis for the association of _Klebsielleae_ with redwood (22). Cyclitols have also been detected in many other types of botanical material (16,17).

In this study, gas chromatographic techniques were used to demonstrate _in situ_ cyclitol utilization in aqueous redwood extracts by _Klebsiella_ of environmental and clinical origins. Several other gram-negative bacteria were similarly examined. Other experiments, monitored by gas chromatography, were conducted using a defined mineral medium supplemented with the three cyclitols and two carbohydrates found in redwood, in order to determine which

carbon sources are preferentially utilized by <u>Klebsiella</u>

and other bacteria.

Materials and Methods

Bacterial cultures. The source and origin of all but three of the cultures used in these experiments have been previously described (4,6,18,19,22). Escherichia coli ES5-1 was isolated from potable drinking water, Klebsiella E44.5-1 was isolated from redwood chips, and the Pseudomonas aeruginosa culture was obtained from the department culture collection at Oregon State University.

Aqueous redwood extracts. Aqueous extracts of redwood sawdust used in these experiments were prepared in the following manner: 1.67 g or 4 g of sterilized redwood (heartwood) sawdust was placed into 1000 ml of sterile double distilled water and allowed to stand at room temperature for 4 days. At this time, sterile 0.1% sodium hydroxide was used to neutralize the extract. The extract was sterilized by membrane (0.22 μm) filtration and pipetted into sterile flasks for the cyclitol utilization experiments. Preliminary studies showed that the concentration of 1.67 g sawdust/1000 ml water resulted in good growth of inoculated organisms, with a cyclitol concentration providing optimum peak sizes for analysis. This cyclitol concentration is similar to those observed in redwood tank water (authors' unpublished observations). The 4 g sawdust/1000 ml water extract was used only for the quantitation of cyclitols in crude redwood sawdust extracts.

Inoculation of aqueous redwood extracts. Cultures
inoculated into sterile aqueous redwood extract were grown
overnight in penassay broth (Difco), then diluted in
0.01M tris (hydroxymethyl aminomethane) buffer and added to
the extract to give initial densities of 10^3 to 10^4 viable
cells/ml. The extract was incubated with shaking at 28°C
for 4 days. At this time, viable cell counts were performed
on plate count agar (Difco) and 0.5 ml samples were taken
for gas chromatographic analysis.

Defined minimal salts medium. A defined minimal
salts medium was used to monitor carbohydrate and
cyclitol utilization by selected enteric bacteria (22).
L-arabinose (Sigma), D-glucose (J. T. Baker), pinitol,
sequoyitol, and myo-inositol (Sigma) were each present at
a concentration of 0.04%, to give a total carbon source
concentration of 0.2%. The pinitol was a gift from the
laboratory of Dr. Arthur Anderson, University of California
Forest Products Laboratory, Richmond, Calif., and the
sequoyitol was a gift from Dr. Laurens Anderson, Department
of Biochemistry, University of Wisconsin. Cultures
examined for utilization of carbohydrates and cyclitols
were first propagated in the minimal medium in a growth
limiting amount of D-glucose (0.02%); inoculation, incubation
and growth monitoring procedures were previously described
(22). Samples (0.5 ml) for gas chromatographic analysis

were taken at various times after inoculation, and sodium
azide was added with a digital microliter pipet to give
a 0.05% final concentration.

Gas chromatographic analysis of cyclitol and
carbohydrate concentrations. In all cases 0.5 ml samples
of redwood extract or defined medium were analyzed.
Quebrachitol (a gift from Dr. L. Anderson, University of
Wisconsin), a cyclitol not present in redwood, was used as
an internal standard. 0.05 ml of a 1 mg/ml aqueous
solution of quebrachitol was added to all samples with a
digital microliter pipet, immediately before dry heat
evaporation. In the case of the more concentrated sawdust
extract (4 g sawdust/1000 ml water), it was necessary to
remove excess phenolic compounds. This was achieved with
lead acetate precipitation followed by treatment with
sodium oxalate to remove excess lead (11). Dry samples
were silylated with 0.2 ml of Tri Sil Z (Pierce Chemical
Co., Rockford, Ill.) for 3 hours at room temperature and
2 μl was injected into the gas chromatograph.

A Hewlett-Packard Model 5710 A gas chromatograph
with a flame ionization detector was used in these
experiments (Figure 1). This instrument was equipped with
a 6 ft. 3% OV-17 stationary phase on a Gas Chrom Q
support (100-120 mesh) for analysis of samples for
carbohydrates and cyclitols. The oven temperature used for

analysis of redwood extract was 160°C; analysis of the mineral medium was accomplished with a temperature program of 145°C for the first 8 minutes, followed by heating at 4°C/min. to 160°C. This was necessary to enhance separation of the L-arabinose peaks. Carrier gas (helium) flow for all analyses was 20 ml/min. Injection port temperature was 260°C; detector temperature was 300°C. Peaks were recorded with a Hewlett-Packard Model 7123 A strip chart recorder. The FID control was set with a range of 10 and attenuation of 2 for all analyses.

Quantitative determination of cyclitol concentrations was accomplished by first preparing a tracing on uniform thickness vellum tracing paper. The peaks were cut out and weighed on a Mettler H20 analytical balance and the mass compared to that of the internal standard.

<u>Results</u>

The cyclitol profile of aqueous redwood extract
is illustrated in Figure 2. A 4 g/1000 ml concentration of
sawdust to water was used in order to detect myo-inositol.
This extract had pinitol, sequoyitol, and myo-inositol at
concentrations of 60 µg/ml, 20 µg/ml, and 8 µg/ml,
respectively. Pinitol comprised 68% of the total cyclitols,
sequoyitol 23%, and myo-inositol 9%. Carbohydrates
constitute only some 4% of the total cyclitol concentration
(2) and were not detected in these experiments.

A less concentrated redwood extract (1.67 g sawdust/
1000 ml water) was inoculated with gram-negative bacteria
in an attempt to demonstrate cyclitol utilization under
conditions similar to those encountered in a redwood water
storage reservoir. Myo-inositol was not detectable at
this concentration. The results of these experiments are
presented in Table 1. All of the <u>Klebsiella</u> strains,
regardless of origin, reached densities exceeding 5.0×10^6
cells/ml within 4 days from an inoculum of 10^3-10^4 cells/ml
and all metabolized pinitol and sequoyitol. Figure 3
illustrates the utilization of cyclitols by <u>Klebsiella</u> as
monitored with gas chromatography. All of the detectable
sequoyitol and between 54 and 90% of the detectable pinitol
were utilized by the <u>Klebsiella</u> tested. Of the other
members of the <u>Klebsielleae</u> tribe examined, the <u>Enterobacter</u>

agglomerans isolate metabolized virtually 100% of the

cyclitols present (Table 1). The Enterobacter aerogenes

strain grew to lower densities than the aforementioned

cultures (4.0×10^5 cells/ml) and cyclitol utilization was

slight. Both Serratia cultures had significantly lower cell

densities indicating very little if any growth, and no

cyclitol utilization was detected.

Of the remaining bacteria studied in redwood extract,

only the Escherichia and Citrobacter strains attained

densities exceeding 10^5 cells/ml. The Erwinia, Yersinia

and Salmonella isolates, which ferment cyclitols in phenol

red broth (22), decreased in viable cell count throughout

the 4 day period. None of the non-Klebsielleae cultures

utilized cyclitol compounds.

A defined minimal medium containing as carbon sources

the carbohydrates and cyclitols of redwood heartwood was

employed to determine which compounds are preferentially

used by certain enteric bacteria. A gas chromatographic

profile of the carbon sources in this medium is illustrated

in Figure 4. The 5 carbon sources present were each included

at the same concentration (0.04%), for a total carbon source

concentration of 0.2%. The calculated amount of carbon

source present, based on gas chromatography was close for

the three cyclitols and D-glucose. The concentration of

L-arabinose, however, was calculated to be significantly

less than presumably present, because of the small, multiple peaks in the chromatogram.

The results obtained with the Klebsielleae cultures in the defined medium are presented in Table 2. Among the Klebsiella isolates, the carbohydrates present in the medium were consumed before the cyclitols were utilized. For example, in the 3.5 hour to 5 hour samples, less than 10% of the detectable carbohydrates remained, yet 100% of the cyclitols were present. Figure 5 depicts the progressive consumption of the carbon sources by Klebsiella strain PC$_2$. By 4.5 hours, D-glucose and L-arabinose were almost completely undetectable in the medium, yet the cyclitols were present at the same concentration as the uninoculated control. Other Klebsiella isolates from various habitats demonstrated the same preferential use of carbohydrates.

Regarding cyclitol utilization among Klebsiella, it appeared that sequoyitol and inositol were more rapidly metabolized than pinitol. The data obtained with strains MH31 at 10.5 hours, SL-1 at 5.5 hours, and PC$_2$ at 10.5 hours support this observation. With strain PC$_2$ at 10.5 hours (Figure 5), over 50% of the pinitol remained, yet sequoyitol and inositol were almost completely consumed. Other strains of Klebsiella exhibited a similar cyclitol utilization pattern, but rates of degradation varied. However, all of the Klebsiella strains but PSB49-1, which

was not monitored past 4.5 hours, completely consumed the carbohydrates and cyclitols.

The two Enterobacter isolates examined completely degraded the cyclitols and carbohydrates present (Table 2). The Enterobacter aerogenes culture at 25.5 hours exhibited to some degree a preferential use of carbohydrates over cyclitols.

Among the non-Klebsielleae isolates tested, the Erwinia, Yersinia, and Salmonella cultures each demonstrated cyclitol utilization (Table 3). The Salmonella strain exhibited a preferential use of carbohydrates before cyclitols in the 4.5 hour and 10.5 hour samples, and in the 29.5 hour sample illustrated a more complete utilization of sequoyitol and inositol, similar to the Klebsiella cultures. The Yersinia isolate completely utilized all of the carbon sources present, but at a significantly slower rate than the other cultures. The Erwinia culture metabolized only the carbohydrates and inositol. Both Escherichia isolates utilized the carbohydrates but not the three cyclitols (Figure 5).

Discussion

In a previous study, 100, 97, and 68% of the
Klebsiella tested were capable of fermenting, in phenol
red broth, myo-inositol, sequoyitol, and pinitol, respec-
tively (22). In the present study, all of the Klebsiella
monitored by gas chromatography, regardless of origin,
demonstrated in situ utilization of cyclitols during growth
in aqueous redwood extracts and in a defined medium with
cyclitols as the source of carbon and energy. Certain
enteric bacteria such a E. coli and Citrobacter are capable
of some growth in sterile redwood extracts but are not
capable of utilizing cyclitols as energy sources in
defined media. Presumably their growth in sterile redwood
extracts is supported by the low level of carbohydrates
present (2). These organisms do not compete well with
indigenous bacteria in non-sterile extracts, however (22).
Other genera such as Erwinia, Salmonella, and Yersinia do
not readily grow in all redwood extracts even though they
can metabolize cyclitols (Table 3). Erwinia and Yersinia
previously demonstrated growth in a more dilute extract
(22). This indicates that these organisms are inhibited
by the tannins and polyphenolic compounds present in
redwood extracts or by other unknown factors. Klebsielleae
are evidently more resistant to these inhibitors and have

the nutritional advantage for competing with other
indigenous bacteria which do not utilize cyclitols (22).

Collectively the present results are consistent with
previous observations which illustrated high cell densities
of Klebsielleae associated with drinking water emanating
from redwood reservoirs and with liquid expressed from
various wood specimens (5,19). Other enteric bacteria
were rarely encountered in these specimens.

The results of experiments in the defined medium
agreed with an earlier finding that enzymes for cyclitol
metabolism among the Klebsielleae are inducible (3,20).
The carbohydrates are clearly the preferred substrate when
provided simultaneously with cyclitols in a defined minimal
medium.

Among the Klebsielleae, as well as the Salmonella
isolate examined, it appears that the metabolism of
sequoyitol and inositol proceeds more rapidly than that
of pinitol. This is an interesting observation in terms of
enzymology. In Klebsielleae the myo-inositol degradation
pathway is known (3,20), but no information is available
concerning the degradation of pinitol and sequoyitol.
Among Klebsielleae, the percentage of strains capable of
cyclitol degradation decreases in the order myo-inositol→
sequoyitol→pinitol (22). In plants, cyclitol synthesis
proceeds in the same direction. A methylation reaction is

involved between inositol and sequoyitol, and an

epimerization occurs between sequoyitol and pinitol (7).

This information suggests that in bacteria sequoyitol and

pinitol may be converted to myo-inositol first and then

follow the myo-inositol degradation pathway, which begins

with myo-inositol, dehydrogenase (3,20). The slower utiliza-

tion of pinitol relative to sequoyitol and myo-inositol may

reflect the induction of an enzyme converting pinitol to sequoyitol.

Cyclitol degradation by _Klebsielleae_ may be important

in other botanical environments in addition to redwood.

Myo-inositol has the widest distribution in plant material,

but pinitol is found in 6 families of gymnosperms

(including species of pine, fir, and hemlock) and in 13

families of angiosperms (16,17). Sequoyitol has been

detected in a large number of gymnosperms (17). The

isolation of _Klebsielleae_ from redwood, fir, and hemlock

(1,5), and from pulp and textile mill effluents (8,10,12,13),

fresh vegetables (6,9,23), and plant roots (14,15) may be

related to the cyclitol content of these habitats.

In addition to the three cyclitol compounds

studied here, there are several other cyclitols present in

botanical material (16,17). Whether _Klebsielleae_ can also

metabolize these compounds is at present unknown.

Acknowledgments

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Table 1. Utilization of cyclitols in aqueous redwood extract by gram-negative bacteria, after 4 days incubation.

Culture	Origin	Viable[a] cells/ml	Concentration of cyclitols (µg/ml) as detected Pinitol	Sequoyitol
Uninoculated control			$27\text{-}34^{b}$	$7\text{-}11^{b}$
Klebsiella				
E44.5-1	redwood	1.2×10^{7}	9	ND^{c}
MH24	vegetable	1.1×10^{7}	7	ND
BC1959	pulp mill effluent	7.2×10^{6}	13	ND
PSB-49-1	bovine mastitis	6.9×10^{6}	3	ND
PC_2	redwood tank water	5.8×10^{6}	14	ND
Enterobacter agglomerans ICPB EH103		1.2×10^{7}	2	ND
Escherichia coli DS5-1 OSU		1.8×10^{6}	32	8
Enterobacter aerogenes ATCC 13048		4.0×10^{5}	23	7
Citrobacter freundii OSU		1.2×10^{5}	32	9
Serratia liquefaciens ATCC 14460		1.8×10^{4}	30	8
Pseudomonas aeruginosa OSU		1.5×10^{4}	34	11
Serratia marcescens OSU		4.8×10^{3}	32	8
Erwinia carotovora ICPB EC 153		$< 1.0 \times 10^{2}$	NS^{d}	NS
Yersinia enterocolitica CDC 867		$< 1.0 \times 10^{2}$	NS	NS
Salmonella enteritidis ser. paratyphi B OSU		$< 1.0 \times 10^{2}$	NS	NS

a all cultures were inoculated at an initial density of $10^{3}\text{-}10^{4}$ viable cells/ml
b range obtained when several replicate samples from the same flask were processed
c ND: not detected
d NS: not sampled

Table 2. Utilization of carbohydrates and cyclitols in a defined minimal medium by <u>Klebsielleae</u>.

Culture	Origin	Hours after inoculation	Concentration of carbon source (μg/ml) as detected				
			L-Arabinose	D-Glucose	Pinitol	Sequoyitol	Myo-Inositol
Uninoculated control			$13\text{-}17^a$	$29\text{-}37^a$	$41\text{-}51^a$	$36\text{-}44^a$	$43\text{-}53^a$
Klebsiella							
MH31	human	5	3	ND^b	49	43	46
	medical	10.5	1	ND	23	11	13
		29.5	ND	ND	ND	ND	ND
MH24	vegetable	5.5	2	ND	44	36	53
		8.5	ND	ND	ND	ND	ND
SL-1	human	3.5	4	5	44	38	43
	medical	5.5	1	1	38	3	5
		7.5	ND	ND	ND	1	1
PC$_2$	redwood tank	4.5	2	3	47	44	53
	water	10.5	ND	ND	32	4	ND
		29.5	ND	ND	ND	ND	ND
PSB-49-1	bovine mastitis	4.5	1	ND	46	31	39
Enterobacter aerogenes ATCC 13048		25.5	ND	ND	ND	2	6
		29.5	ND	ND	ND	ND	ND
Enterobacter agglomerans ICPB EH103		25.5	ND	ND	ND	ND	ND

a values represent range obtained when several replicate samples were processed
b ND: not detected

Table 3. Utilization of carbohydrates and cyclitols in a defined minimal medium by non-Klebsielleae.

| Culture | Hours after inoculation | Concentration of carbon source (µg/ml) as detected | | | | |
		L-Arabinose	D-Glucose	Pinitol	Sequoyitol	Myo-Inositol
Uninoculated control		13-17[a]	29-37[a]	41-51[a]	36-44[a]	43-53[a]
Escherichia coli						
DS5-1 OSU	9.5	ND[b]	ND	51	43	53
	29.5	ND	ND	51	44	47
ES5-6 OSU	7.5	2	2	41	37	44
	29.5	ND	ND	47	44	53
Erwinia carotovora	5	4	ND	48	39	44
ICPB EC153	29.5	ND	ND	48	47	ND
Yersinia enterocolitica	36	ND	ND	ND	ND	ND
CDC 867						
Salmonella enteritidis	4.5	ND	ND	45	40	52
ser. paratyphi B OSU	10.5	ND	ND	44	37	45
	29.5	ND	ND	20	ND	ND

a values represent range obtained when several replicate samples were processed
b ND: not detected

Figure 1.   Hewlett-Packard Model 5710A gas chromatograph.
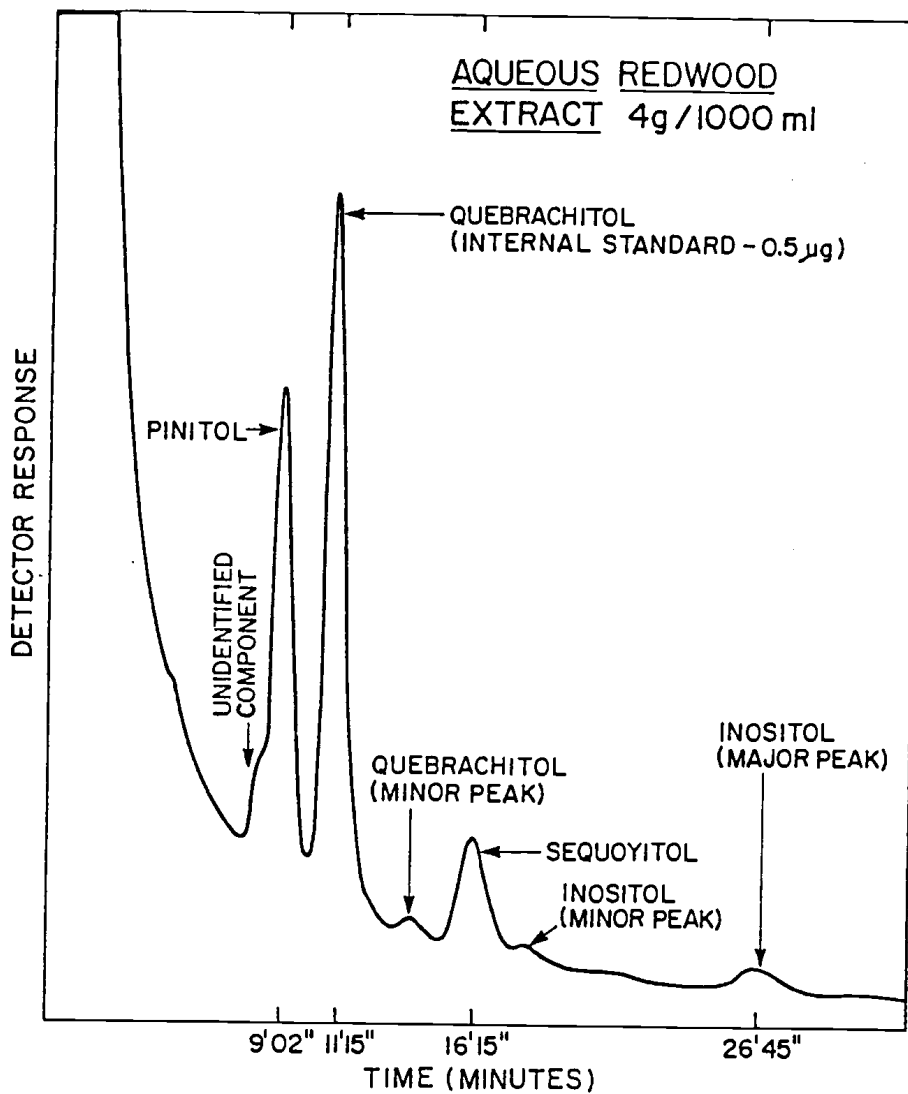
Figure 2. Gas chromatographic profile of a 4 g sawdust/
1000 ml water extract of redwood sawdust.
Column temperature was 160°C.
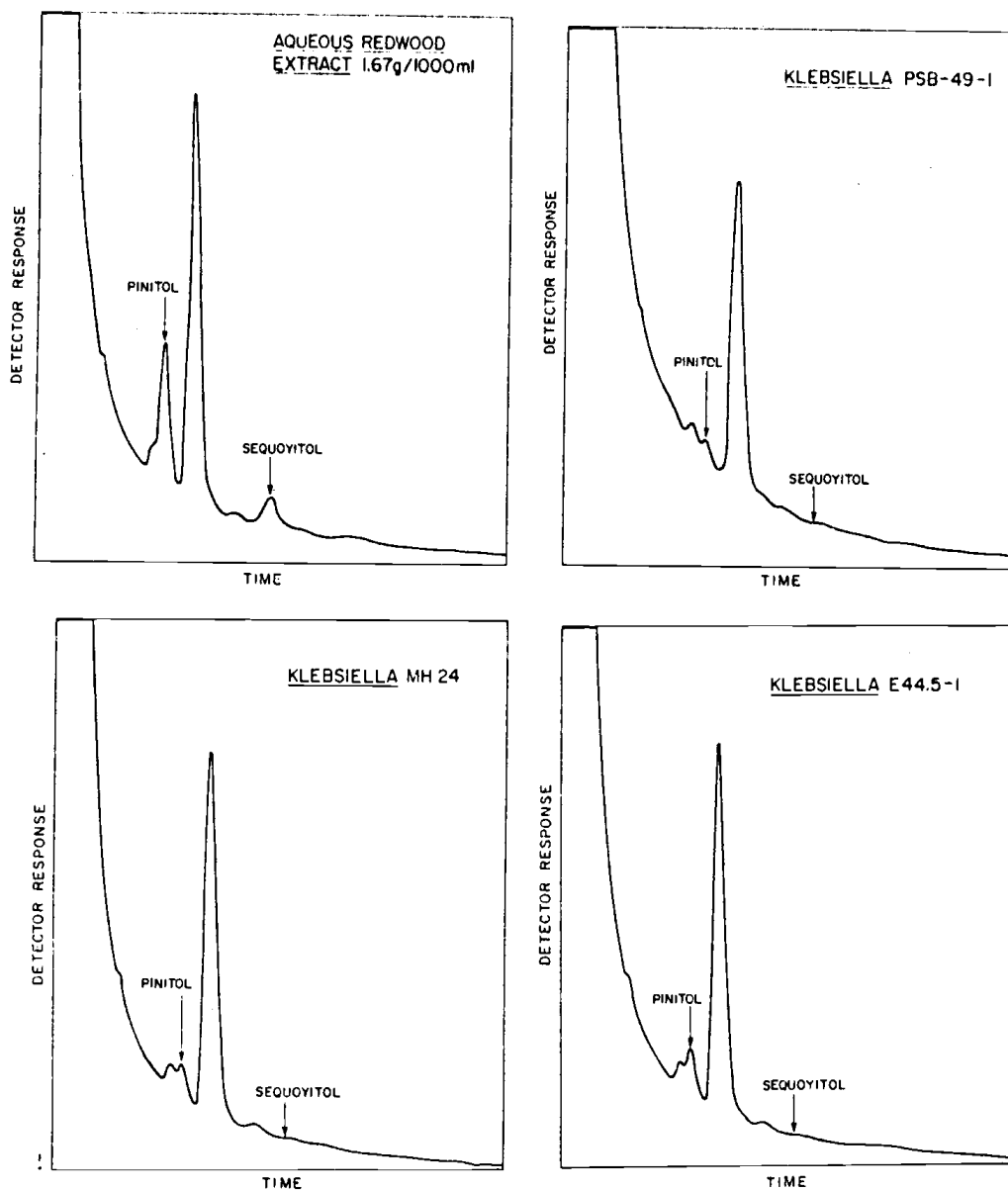
Figure 3. Gas chromatographic analysis of cyclitol
utilization by Klebsiella in a 1.67 g sawdust/
1000 ml water extract of redwood. Column
temperature was 160°C. Origin of isolates tested:
PSB49-1, mastitis; MH24, vegetable; E44.5-1,
redwood.
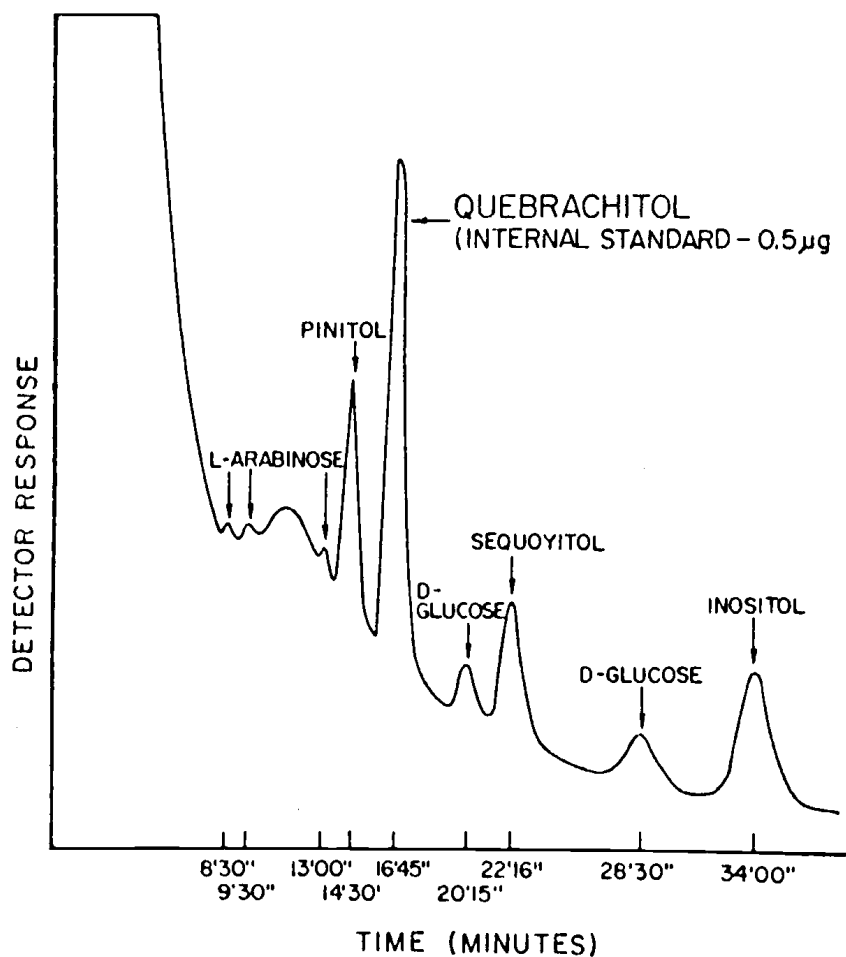
Figure 4.   Analysis of the sterile minimal salts medium
containing two carbohydrates and three cyclitols
as carbon sources.   Column temperature was 145°C
for 8 minutes, then with heating at 4°C/min., to
160°C.   The change in baseline after 9'30" is a
reflection of the increase in column temperature.
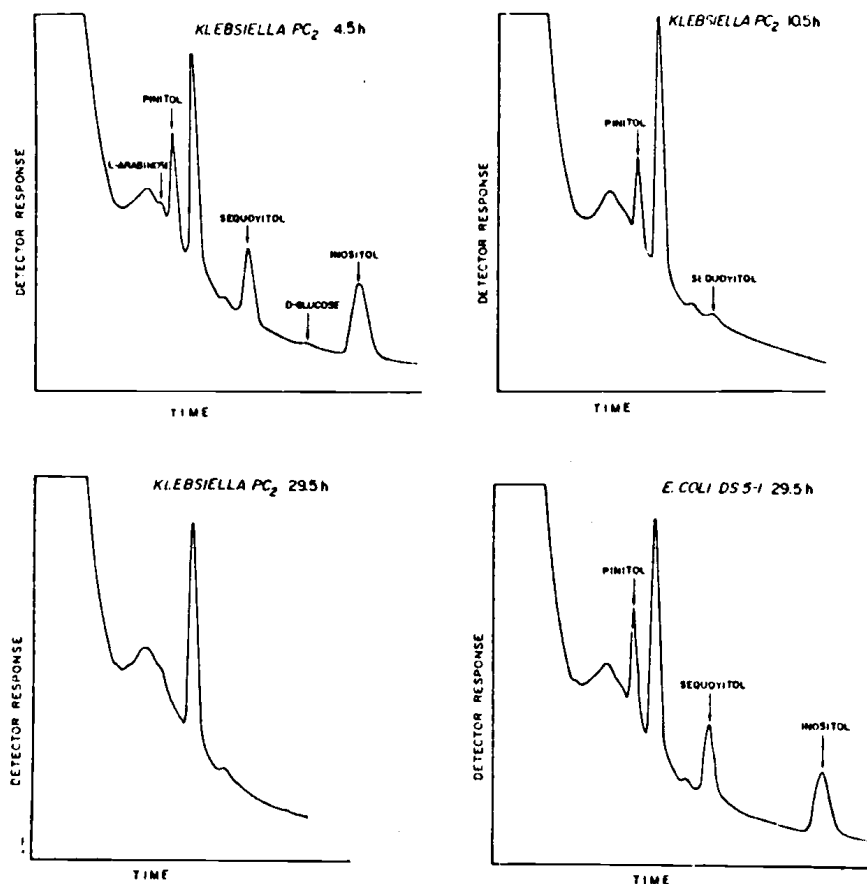
Figure 5. Progressive carbohydrate and cyclitol utilization by <u>Klebsiella</u> strain PC$_2$, an isolate from redwood tank water, with <u>E</u>. <u>coli</u> DS5-1 for comparison. Column temperature conditions as described for Figure 4.

APPENDICES

Appendix 1

Utilization of Myo-Inositol Hexaphosphate by

Gram-Negative Bacteria

A considerable amount of the myo-inositol in nature is

in the form of inositol polyphosphates. Prominent among

these phosphates is inositol hexaphosphate, also called

phytic acid (Figure 1), which is a major constituent of

plant material such as grains and seeds, where it is present

as a mixed calcium-magnesium salt (2,13). Phytate has been

shown to be the major storage compound for phosphorus and

inositol in many plants, including carrots, parsnips, and

potatoes. Up to 90% of the total phosphorus in grains and

seeds may be in this form. Phytate is also a significant

component of soil organic material (2). During germination

in plants, phytic acid undergoes a stepwise dephosphorylation

to yield free myo-inositol, which is incorporated into cell

wall constituents (13). This activity drops rapidly

after germination. Synthesis of phytate involves a stepwise

phosphorylation of inositol in the presence of an inositol

kinase and a series of phosphorylated inositol kinases (2).

The first report of phytase activity in a

microorganism was made in 1911, with a culture of the fungus

Aspergillus niger. Later studies confirmed this activity

with other isolates of Aspergillus (4). Shieh and Ware,

working with a culture of Aspergillus niger, showed that

this organism produced an extracellular phytase which hydrolyzed phytate in acidic solutions (14). Similar results were obtained by Irving and Cosgrove (9).

Greaves, Anderson, and Webley in 1967 described the isolation of a phytase from Aerobacter aerogenes (5). They found that the enzyme activity was located in the insoluble debris obtained after sonication of the cells. Maximum hydrolysis of phytate was obtained with a pH of between 4 and 5 and an optimum temperature of 45°-50°C. The enzyme was inhibited by high substrate concentration and by inorganic phosphate.

In 1970, Cosgrove isolated a soil bacterium with intracellular phytase activity, using enrichment techniques (4). Subsequent identification of this organism placed it in the genus Pseudomonas. Its phytase activity exceeded that of the cultures used by Greaves, and it had a different specificity. The Pseudomonas enzyme was capable of hydrolyzing a wider variety of phytate isomers and derivatives. In addition, the end products of the enzyme reactions differed between the Aerobacter and Pseudomonas phytases.

A related study by Cosgrove proposed that at least two phytases exist in biological systems: one mainly in plants and the other in microorganisms (3). The plant phytase apparently removes the 6-phosphate from inositol hexaphosphate; the microbial phytase removes the 1-phosphate.

Utilizing this isolate of <u>Pseudomonas</u> and its phytase, Irving and Cosgrove have made perhaps the most extensive investigation of bacterial degradation of inositol hexaphosphate (7). The pH optimum of this enzyme was found to be 5.5 at 40°C. It was inhibited to about the same degree by both 1.0mM (+) tartrate and 1.0mM EDTA. The <u>Pseudomonas</u> phytase was also inhibited by fluoride ion. Since the enzyme was present in growth media lacking inositol hexaphosphate, it was assumed that enzyme production does not require induction by substrate. In an accompanying article, Irving and Cosgrove proposed a simple model for the active center of the enzyme, which explained how their <u>Pseudomonas</u> phytase presumably attaches to its substrate and initiates the cleavage of phosphate groups from the phytate molecule (8).

Since the early 1970's, there has been little published work on microbial interactions with phytate. However, it remains a major component of plant material, whose link with microbial ecology is still poorly understood.

In this study, members of several genera of gram-negative bacteria were screened for possible phytase activity. The sources of the cultures are listed in Chapter 2. The medium used contained calcium phytate as the sole source of carbon and phosphorus and was adjusted to pH 5.5. Cleavage of phytate with the release of inorganic

phosphate was monitored, and viable counts were determined on plate count agar (Difco).

Calcium phytate (practical grade) was obtained from Sigma. Purification of this material was accomplished using the method as described by Cosgrove (2), with some modifications. After the ammonia precipitation step in the cited procedure, the precipitate was dissolved in 0.5M HCl and portions of 10% $FeCl_3$ in distilled water were added, the resulting precipitate centrifuged, and the supernatant treated with small portions of 10% $FeCl_3$ until no more precipitate appeared. This avoided the addition of excessive $FeCl_3$. The precipitate was washed twice with large volumes of 0.15M HCl, then washed twice and resuspended in distilled water. If upon the addition of 5% NaOH to a portion of the preparation a yellow-orange color appeared, the washing steps were repeated. Portions of 5% NaOH were added just until a brown precipitate appeared. The precipitate was removed by centrifugation and additional 5% NaOH was added to the supernatant until no additional precipitate formed. The precipitate was discarded, and the supernatant adjusted to pH 7.0 with HCl. Portions of 20% calcium acetate were added until no additional white precipitate (calcium phytate) formed. The precipitate was washed three times with methanol, and air dried to a white powder.

Analysis (see below) of the final product showed that only 0.2% inorganic phosphate was present. Comparison of inorganic phosphate amounts after cleavage with phytase (Sigma) of the purified phytate and of inositol hexaphosphate (Calbiochem, >99% pure) further verified purity of the calcium phytate.

The growth medium for the phytase activity assay contained the following ingredients, per liter: NH_4Cl, 2g; NaCl, 5g; KCl, 0.37g; $MgCl_2$, 0.01g; Na_2SO_4, 0.026g; $FeCl_3$, 0.1g; and calcium phytate (purified) 1g, in tap water (200 ml) and distilled water (800 ml). This medium was adjusted to pH 5.5 with HCl. Cultures to be tested were grown overnight in nutrient broth, centrifuged, and washed twice in 0.01M tris (hydroxymethyl aminomethane) buffer, diluted to 10^{-5}, and inoculated into the medium.

Inorganic phosphate was determined using the procedure of Martin and Doty[11], with modifications as suggested by Irving and Cosgrove[6]. Readings were made at 725 nm on a Gilford Recording Spectrophotometer. A standard curve for inorganic phosphate was used to convert absorbance readings to amount of inorganic phosphate present (Figure 2).

A significant increase in bacterial density in the phytate medium over that in the basal solution minus calcium phytate, along with an increase in the amount of inorganic phosphate present in the medium was taken as

evidence of phytase activity. Incubation of the medium was at 35°C (30°C in the case of Yersinia and Erwinia) with shaking. Samples for inorganic phosphorus determinations and for plate counts were taken at inoculation and at 72 hours.

From the data in Table 1, it appears that the Klebsiella and Enterobacter cultures tested possess some degree of phytase activity. All three Klebsiella cultures attained significantly higher cell densities in the phytate medium than either the Enterobacter or Serratia isolates. Based on this survey, phytase activity is undetermined, but doubtful, in the strain of Serratia marcescens examined. Of the other gram-nagative bacteria tested, the Citrobacter, Pseudomonas, and possibly Escherichia cultures possess some phytase activity; however, none liberated as much inorganic phosphate as did Klebsiella. The Erwinia and Yersinia isolates tested attained cell densities of approximately 10^7/ml in the phytate medium, but the amount of inorganic phosphate present was no greater than that of the uninoculated control. Perhaps these cultures incorporated the released phosphate from phytase activity into cell material, and in doing so rendered it undetectable by the assay procedure. Uninoculated control flasks containing phytate demonstrated that little inorganic phosphate was released independent of microbial activity.

If an organism was to be chosen for an attempt at phytase isolation and purification, based on this study one of the Klebsiella cultures would be first choice. If the phytase activity in Klebsiella and Enterobacter demonstrated in these experiments occurs in the natural environment, it could help explain the presence of these organisms on seeds and roots (1,10,12).

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Table 1. Utilization of inositol hexaphosphate

Experiment 1	medium	at inoculation		at 72 hours	
		viable cells/ml	inorganic PO_4 µg/ml	viable cells/ml	inorganic PO_4 µg/ml
Klebsiella					
ATCC 13883	phytate	1.9×10^1	6.0	4.5×10^7	16.4
	basal	1.9×10^1	2.2	1.6×10^5	2.4
CM$_1$1 (water)	phytate	4.8×10^1	6.0	3.6×10^7	17.0
	basal	4.8×10^1	2.2	1.5×10^4	2.4
PSB49-1 (mastitis)	phytate	2.0×10^1	6.0	4.4×10^7	18.0
	basal	2.0×10^1	2.2	3.7×10^5	2.4
Enterobacter aerogenes ATCC 13048	phytate	3.6×10^1	6.0	1.6×10^5	15.4
	basal	3.6×10^1	2.2	3.0×10^2	2.2
Serratia marcescens OSU	phytate	5.4×10^0	6.0	1.9×10^6	9.0
	basal	5.4×10^0	2.2	1.9×10^6	2.4
phytate medium (uninoculated)			6.0		8.0
basal medium (uninoculated)			2.2		2.2

Experiment 2	medium				
Citrobacter freundii OSU	phytate	6.0×10^2	7.6	6.0×10^6	11.5
	basal	6.5×10^2	2.7	1.6×10^5	2.2
Erwinia carotovora ICPB EC153	phytate	4.1×10^2	7.6	1.5×10^7	9.3
	basal	5.6×10^2	2.7	8.3×10^4	2.7
Escherichia coli DS5-1 OSU	phytate	8.1×10^2	7.6	5.9×10^5	10.4
	basal	8.4×10^2	2.7	2.1×10^3	2.7
Proteus vulgaris OSU	phytate	5.7×10^2	7.6	5.8×10^5	8.7
	basal	5.3×10^2	2.7	1.6×10^6	2.7
Pseudomonas aeruginosa OSU	phytate	1.3×10^3	7.6	5.0×10^7	12.5
	basal	1.7×10^3	2.7	5.8×10^4	2.7
Salmonella enteritidis ser. paratyphi B OSU	phytate	1.3×10^2	7.6	9.6×10^5	9.8
	basal	1.5×10^2	2.7	1.2×10^5	2.2
Shigella dysenteriae OSU	phytate	1.5×10^2	7.6	5.0×10^0	8.2
	basal	1.7×10^2	2.7	5.0×10^0	2.7
Yersinia enterocolitica 867	phytate	3.1×10^2	7.6	9.9×10^6	9.8
	basal	3.6×10^2	2.7	4.6×10^2	2.7
phytate medium (uninoculated)			7.6		9.8
basal medium (uninoculated)			2.7		2.7
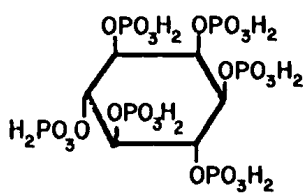
MYO-INOSITOL HEXAPHOSPHATE

Figure 1.   Structure of myo-inositol hexaphosphate
            (phytic acid).
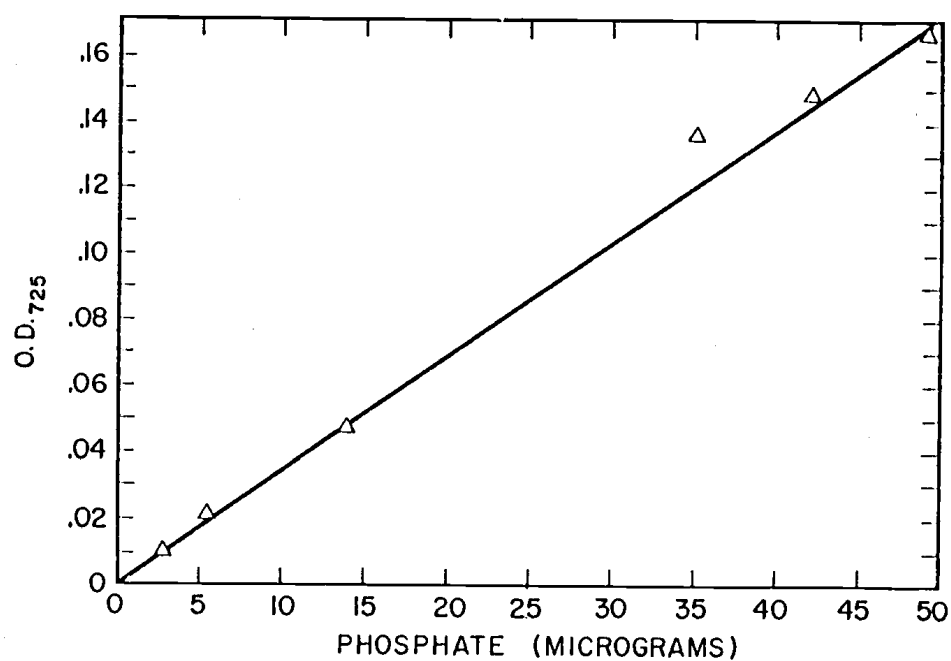
Figure 2.   Plot of inorganic phosphate vs. absorbance at
725 nm.

Appendix 2

Antibiotic Resistance Transfer Between <u>Klebsiella</u> in

Aqueous Extracts of Redwood Sawdust

The transfer of antibiotic resistance with <u>Klebsiella</u>

strains of clinical origin has been previously demonstrated,

usually with <u>Escherichia</u> <u>coli</u> recipients (3,11,20). This

transferable resistance is mediated by R-plasmids, which are

extrachromosomal elements that are found throughout the

<u>Enterobacteriaceae</u> (3,13). R-plasmids have become an

increasingly serious complication in the treatment of

nosocomial diseases caused by <u>Klebsiella</u>, and have contributed

to the rapid rise in the numbers of multiply antibiotic

resistant <u>Klebsiella</u> (9,11).

A large number of clinical <u>Klebsiella</u> have been shown

to harbor resistance to two or more antibiotics (1,3,9).

A previous investigation reported that up to 80% of the

antibiotic resistant <u>Klebsiella</u> isolated from sewage are

capable of transferring this resistance during conjugation

(20). Another study has documented nosocomial outbreaks of

infection caused by antibiotic resistant <u>Klebsiella</u> where

up to 90% of the resistant isolates tested possessed

R-plasmids (9). Since <u>Klebsiella</u> is an opportunistic

pathogen of importance, involved in nosocomial infections

(5,10,19), the proliferation of multiple antibiotic

resistance in this organism is a serious problem.

Klebsiella have also been isolated from many botanical habitats, often in densities of 10^5 cells/ml or 10^5 cells/ gram or greater (7,12). In light of the association of these opportunistic pathogens with botanical material, the present experiments were conducted in an attempt to demonstrate transfer of multiple antibiotic resistance between strains of Klebsiella in a "natural" botanical environment (aqueous extracts of redwood sawdust). Cell densities of Klebsiella in this medium are similar to those encountered in the environment (16). Klebsiella strains used were of environmental as well as clinical origin.

The source and origin of the Klebsiella used in these experiments are listed in Table 1. Potential donors and recipients were chosen and randomly paired based on information obtained in an investigation of which the information presented here is a part (17). Twenty-one donor/ recipient pairs were examined. Recipient cultures used were either resistant to no antibiotics or to ampicillin only. Recipients and donors were streaked on MacConkey agar (Difco) containing known concentrations of antibiotics, to determine the minimum inhibitory concentrations and the concentrations of antibiotics to use in the conjugal matings.

Spontaneous nalidixic acid resistant mutants for use as recipients were selected in the following manner: cultures were grown for 12h in penassay broth (Difco) at

35°C to reach high cell densities. Nalidixic acid (Sigma) was then added to give a final concentration of 40 µg/ml. The cultures were incubated 6 to 12 additional hours, centrifuged, resuspended in 1 ml of sterile 0.01M tris (hydroxymethyl aminomethane) buffer and plated onto MacConkey agar containing 40 µg/ml nalidixic acid.

Single resistant colonies were picked and restreaked onto MacConkey agar with nalidixic acid. After three single colony transfers, the cultures were tested biochemically, to confirm identity with the parent culture, according to the methods of Edwards and Ewing (8). Testing of cultures for independence on drug free medium was done by streaking onto glucose basal salts agar plates.

Aqueous extracts of redwood sawdust were prepared by adding 2.0 grams of sterile sawdust to 1200 ml sterile double distilled water. This suspension was allowed to stand at room temperature for 2 days while nutrients were leached from the sawdust; at this time sterile 0.1% sodium hydroxide was added to neutralize the extract. Recipients and donors were grown overnight at 35°C in an incubator shaker in penassay broth (recipients with 40 µg/ml nalidixic acid) and then diluted to 10^{-2} in sterile tris buffer. The extract was inoculated with a recipient to give initial densities of 10^3 to 10^4 cells/ml. Donors were added a few minutes later at a similar concentration. After three

days of static incubation at room temperature, donors and recipients were enumerated on MacConkey agar, and recipients on MacConkey agar containing 40 µg/ml of nalidixic acid. In order to recover transconjugants, 500 ml of extract was passed through Gelman 0.45 µm membrane filters and placed onto MacConkey agar with nalidixic acid and the other appropriate antibiotics. All plates were incubated at 35°C.

All presumptive transconjugants were picked from membrane filters onto media containing antibiotics to determine which markers were transferred. After verifying antibiotic resistance, transconjugants were inoculated into biochemical media as recommended by Edwards and Ewing for identification and comparison with reactions obtained with non-resistant recipient cultures. Frequencies of antibiotic resistance transfer were reported as transconjugants per recipient.

The results of the experiments in sawdust extracts are presented in Table 1. Of the 21 donor-recipient pairs tested, 8 pairs yielded transconjugants. Resistance to kanamycin, neomycin, streptomycin, and chloramphenicol was transferred. Transfer frequencies were reported only when two or more markers were transferred, since spontaneous mutations to one marker could be suspected with frequencies in the 10^{-8} range. In some cases, resistance to three markers was detected. Transfer frequencies for multiple

antibiotic resistance transfer ranged from 2.5×10^{-6} to 2.8×10^{-8}. Two donors, SL5 and UG28, were involved in all mobilization of resistance in redwood extracts.

Donor and recipient densities at the time of sampling were generally about 10^5 cells/ml. These lower densities at least partially explain the lower frequencies of transfer obtained in these experiments as compared to those in the test tube (17).

It is possible that, although they are low, similar frequencies of transfer could occur environmentally under certain conditions. Bacteria bearing antibiotic resistance mediated by R-plasmids have been isolated from rivers, seawater, and other environmental sources (2,6). If environmental transfer of resistance does occur, it may have a role in the perpetuation of resistant strains in botanical habitats.

Pathogenic strains of Klebsiella have been shown to multiply rapidly on botanical material, including vegetables that are consumed raw by humans (12), so theoretically it is possible that some environmental Klebsiella could originally have been of clinical origin. On the other hand, environmental Klebsiella have been shown to be potentially as pathogenic as clinical Klebsiella, based on virulence tests in mice (4). In any event, antibiotic resistant Klebsiella present in high cell densities

on vegetables or other botanical material, regardless of

origin, can be a potential health hazard. They could have

a selective advantage over other strains upon the

initiation of antibiotic therapy. These organisms could be

introduced into debilitated or compromised patients via

intestinal colonization (14); it has been shown that

Klebsiella can be isolated from several types of vegetables

consumed raw by patients in hospitals (15,21). Klebsiella

have also been found associated with flowers in patients'

rooms (18). Such sources of Klebsiella have been linked

with colonization and subsequent disease (14).

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Table 1. <u>Klebsiella</u> isolates used in antibiotic resistance transfer
experiments.

Source of strain	Origin	Cultures
	Recipients	
Mastitis	Pennsylvania State University	PS401LR
	University of Guelph	UG5
		UG13
Water	Oregon public water systems	$DS_2 1$
		$CM_1 1$
		$JH_4 2$
	Redwood tank water, Oregon State Univ.	PC_2
Human Medical	Mercy Hospital, Pittsburgh, PA	$MH 29$
		MH31
	University of Oregon Medical	UO10945
	School, Portland, OR	UO10994
	University of Texas Medical Center, Houston, TX	UT1003
Vegetable	Mercy Hospital, Pittsburgh, PA	MH24
		MH23
		MH14
	Oregon State University	V236
		V111
Pulp Mill	B. C. Research, Vancouver, B.C.	BC1877
		BC1827
Other	Redwood, OSU	E44.5-1
	Donors	
Mastitis	University of Guelph	UG28
Water	Redwood tank water, OSU	5
		13
		K_2
Human Medical	University of Texas Medical Center, Houston, TX	UT465
	St. Luke's Hospital, Duluth, MN	SL5
Vegetable	Mercy Hospital, Pittsburgh, PA	MH10
	Oregon State University	V125
Textile Mill	EPA	K54

Table 2. Antibiotic resistance transfer frequencies between <u>Klebsiella</u> in aqueous extracts of redwood sawdust.

Recipient	Donor	Donor Marker	Donor count at sampling	Recipient count at sampling	Transfer frequency[a]
PS401LR	SL5	kn(20) nm(20)[b] kn nm str (30)	4.2×10^5	4.8×10^5	4.2×10^{-7} 4.2×10^{-7}
UG13	SL5	kn(20) nm(20) kn nm str (30)	7.6×10^5	5.4×10^5	8.5×10^{-8} 5.2×10^{-8}
DS$_2$1	UG28	str(30)chl(40)	5.8×10^5	3.0×10^4	6.7×10^{-8}
CM$_1$1	UG28	str(30)chl(40)	5.9×10^5	2.5×10^5	4.0×10^{-7}
PC$_2$	SL5	kn(20) nm(20) kn nm str (30)	3.6×10^5	6.4×10^5	3.4×10^{-8} 2.8×10^{-8}
MH29	SL5	kn(20) nm(20) kn nm str (30)	8.0×10^3	4.5×10^5	2.1×10^{-7} 5.8×10^{-8}
UO10994	SL5	kn(20) nm(20)	2.4×10^5	8.0×10^2	2.5×10^{-6}
V111	UG28	str(30)chl(80)	3.7×10^5	2.3×10^5	2.2×10^{-6}

a - expressed as transconjugants/recipient
b( ) - antibiotic resistance in µg/ml

Appendix 3

Cyclitol Concentrations in Water Samples from the

1,000 Gallon Experimental Redwood Reservoir

Ten water samples were taken at intervals throughout

the experimental period from the 1,000 gallon redwood

reservoir (Figure 1) described in Chapter 1. These samples

were frozen at -17°C until analysis for cyclitol

concentrations.

Cyclitol concentrations were determined by gas

chromatography. The methods used in these experiments are

described in Chapter 3.

Figure 2 illustrates the cyclitol profile in tank

water taken on 10/27/75 (4 day retention), during the first

month of tank operation. Inositol was not detectable;

pinitol and sequoyitol were present in concentrations of

20 μg/ml and 6 μg/ml, respectively.

On 11/10/75 (4 day retention) pinitol was present at

a 13 μg/ml concentration and the sequoyitol concentration

was 4 μg/ml. Comparison with the earlier data reveals the

decrease in cyclitol concentrations with time as water is

circulated through the tank and water soluble extractives

are leached from the wood.

By 2/7/76 (12 day retention), cyclitol concentrations

in the tank water were quite low (Figure 3). Pinitol had

a concentration of 6 μg/ml, and sequoyitol, 3 μg/ml.

Almost three months later, cyclitols had decreased to below
detectable levels (Figure 4). No cyclitols were found in
the six subsequent samples.

Therefore, it appears that cyclitols, which are used
as carbon sources by Klebsiella, can be removed from redwood
tanks within several months after filling provided that
water is circulated continuously through the tank. It
should be noted, however, that this reservoir received two
soda ash treatments to promote leaching of water soluble
extractives (which includes cyclitol compounds) before any
of the samples analyzed here were taken. In addition, larger
reservoirs may take longer to age than smaller tanks of
1,000 gallons or less.

Figure 1.   1,000 gallon experimental redwood reservoir.
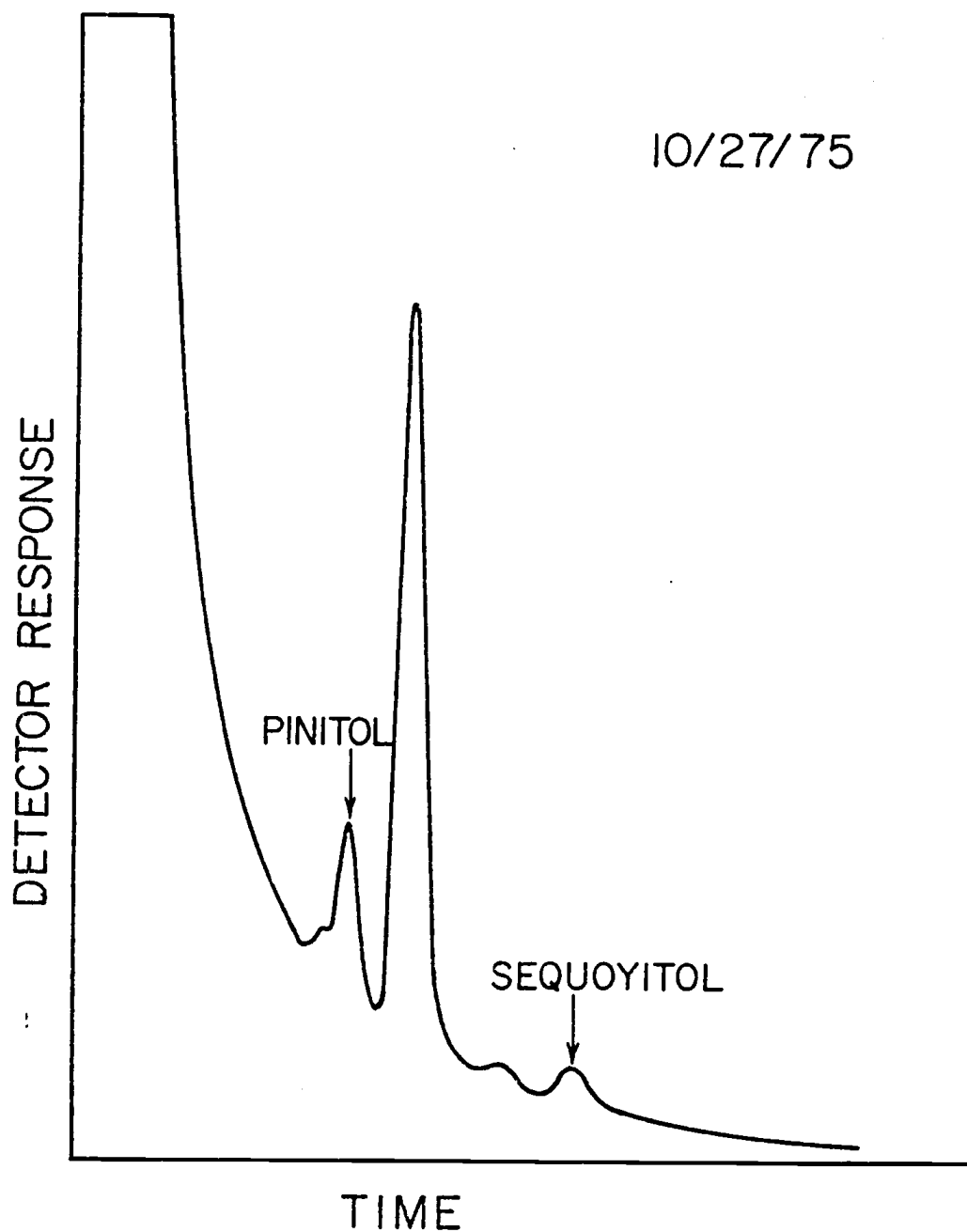
Figure 2. Gas chromatographic analysis of a sample taken from the experimental redwood water reservoir on 10/27/75. Column temperature was 160°C.
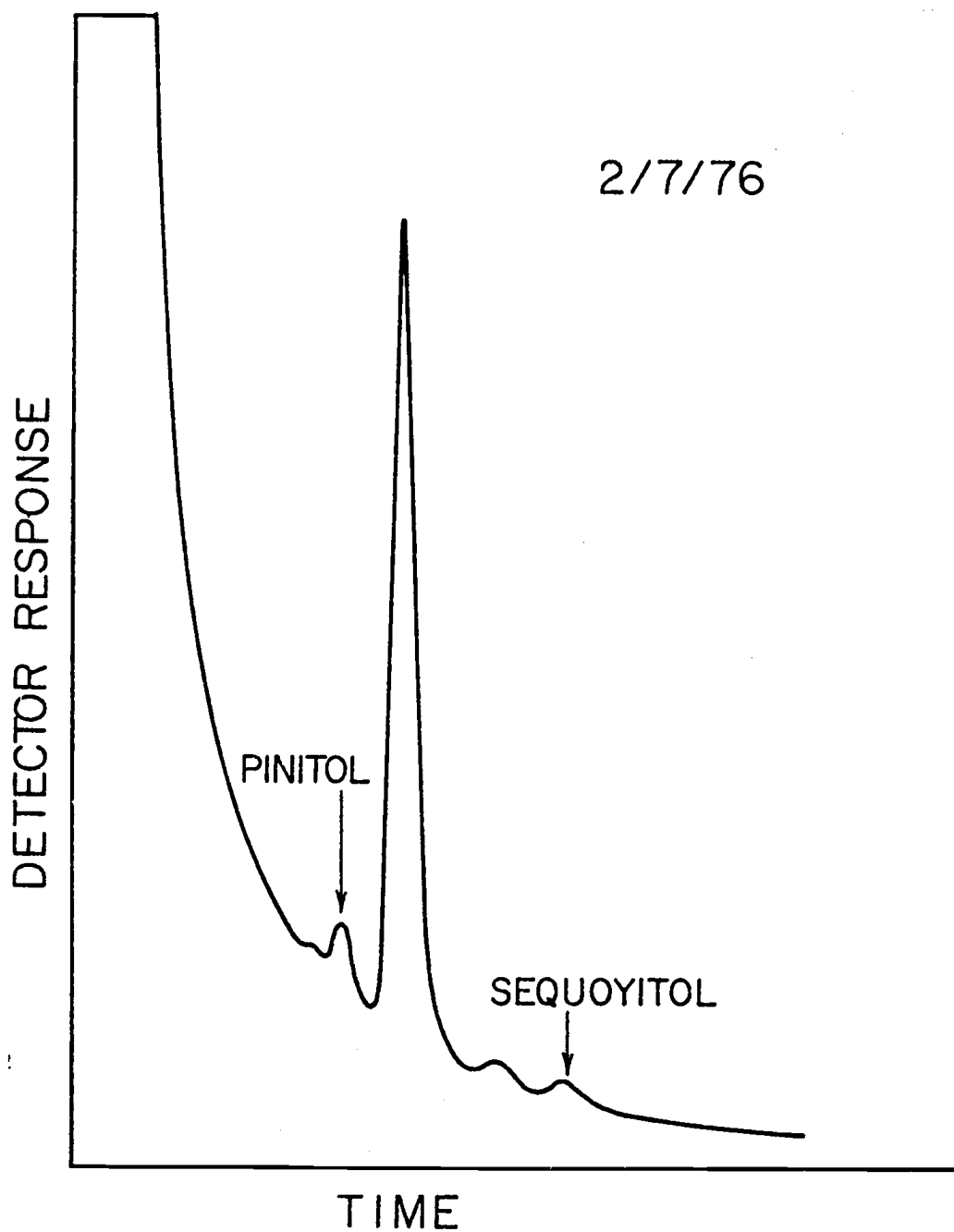
Figure 3. Analysis of sample taken from the experimental
reservoir on 2/7/76. Column temperature was
160°C.
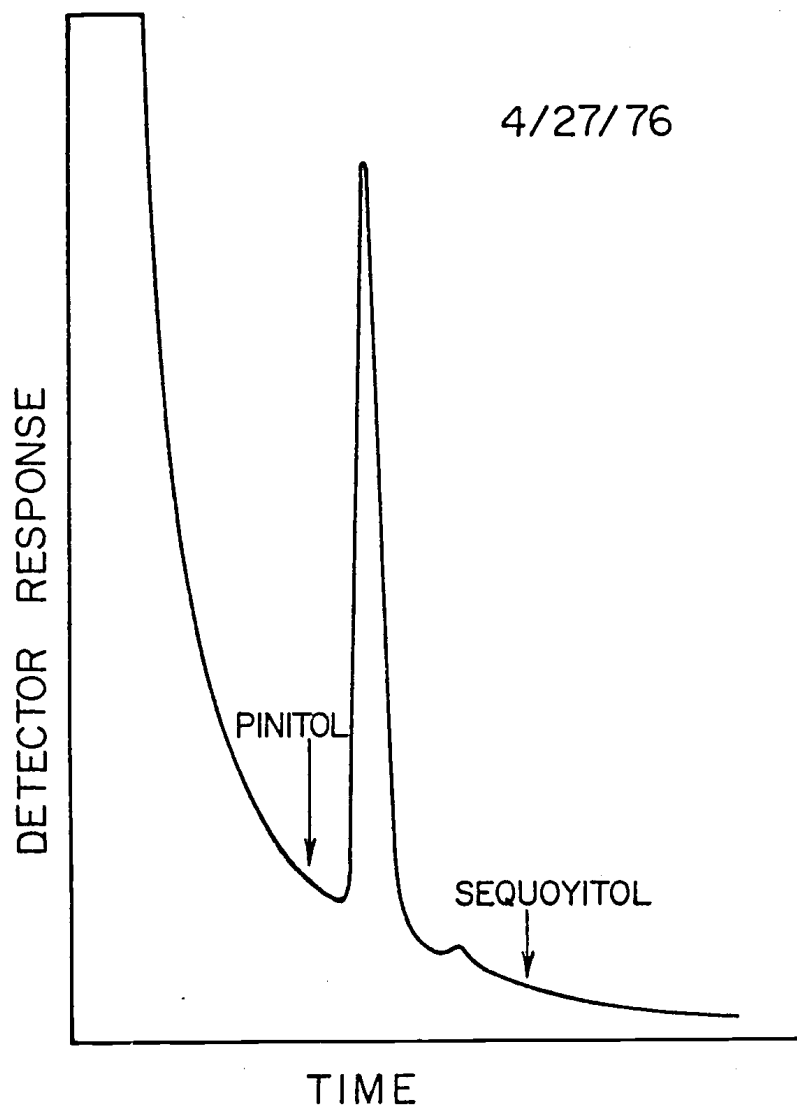
Figure 4. Analysis of sample taken from the experimental
           reservoir on 4/27/76. Column temperature was
           160°C.

Growth Characteristics of Gram-Negative Bacteria

in Aqueous Sawdust Extracts

The information contained in this appendix is related
to the studies described in Chapter 2. The four tables
here were deleted from the chapter, since it was written in
manuscript form. Materials and methods for these
experiments are described in Chapter 2.

Table 1 gives the results of an experiment conducted
to determine the concentration of redwood extract that
would support growth, yet not contain sufficient tannin and
polyphenolic compounds to be growth inhibitory. The data
showed that a concentration of 0.25 g/600 ml water was the
best to use in the experiments described in Chapter 2,
since it was the lowest concentration that supported good
growth of Klebsiella. This concentration of redwood
extract also approximates that of redwood tank water. The
Escherichia coli isolate tested did not increase in density
at most concentrations.

Table 2 contains the results of growth experiments in
aqueous extracts of fir sawdust with several genera of
gram-negative bacteria. None of the strains tested were
able to significantly increase in cell density in fir
extracts. Presumably the high density of indigenous flora
contributed to the inability of these organisms to grow,

since sterile fir extract will support the growth of at least some Klebsiella isolates.

Table 3 gives the data obtained with similar experiments in extracts of pine. Most of the Klebsiella strains tested grew to densities exceeding 10^5 cells/ml. Other gram-negative bacteria were also capable of significant levels of growth. The lack of competition from indigenous bacteria during the first two weeks after inoculation very likely contributed to the ability of the inoculated organisms to multiply in the extracts. No cyclitol compounds were detected in either pine or fir extracts, so carbohydrates probably were the carbon source utilized by the inoculated organisms.

There are eight carbohydrates present in aqueous extracts of redwood, although total carbohydrate concentration is only 0.5% of the extract (1). Table 4 contains data on the utilization of these redwood carbohydrates by gram-negative bacteria. The carbohydrates were supplied at a 1% concentration in phenol red broth base. Incubation was at 35°C, except for the Erwinia and Yersinia cultures which were incubated at 30°C. The Klebsiella isolates tested fermented all 8 carbohydrates tested (determined by acid production in the broth base). Most of the other strains tested were capable of degrading the majority of the carbohydrates, with the exception of

the <u>Pseudomonas</u>, <u>Proteus</u>, <u>Salmonella</u> <u>typhi</u>, and <u>Shigella</u>

cultures. The results of this experiment, along with

those obtained in Chapter 2, indicate that carbohydrate

utilization in extracts of redwood probably is not a factor

in the colonization of aqueous redwood extracts by gram-

negative bacteria in the presence of indigenous flora.

Literature Cited

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    on tree properties. I. California redwood (Sequoia
    sempervirens). J. Inst. Wood Sci. 8:14-34.

Table 1. Growth in various concentrations of aqueous extracts of redwood sawdust.

Culture	Grams of redwood/ 600 ml H_2O	Viable cells/ml, days after inoculation				
		0	2	6	14	31
Klebsiella PSB49-1	0	5.5×10^2	4.5×10^2	4.0×10^2	2.6×10^2	4.7×10^3
	.0625	5.4×10^2	5.1×10^2	3.3×10^2	5.0×10^1	1.0×10^1
	.125	3.5×10^2	3.7×10^2	1.3×10^3	3.1×10^2	4.4×10^2
	.25	4.0×10^2	7.5×10^2	8.2×10^5	5.7×10^5	8.1×10^5
	.50	4.2×10^2	1.0×10^4	5.3×10^5	5.0×10^5	5.0×10^5
	1.0	4.6×10^2	2.1×10^4	8.4×10^5	6.7×10^5	7.5×10^5
	5.0	4.2×10^2	6.8×10^4	1.3×10^5	1.4×10^5	1.3×10^5
Escherichia coli DS5-1 OSU	0	6.0×10^2	4.9×10^2	2.1×10^2	1.5×10^2	2.4×10^2
	.0625	6.3×10^2	5.6×10^2	3.4×10^2	2.9×10^2	1.0×10^2
	.125	5.8×10^2	5.8×10^2	3.6×10^2	1.7×10^2	1.0×10^1
	.25	4.5×10^2	5.0×10^2	1.8×10^2	1.0×10^2	$< 1.0 \times 10^1$
	.50	5.8×10^2	4.1×10^2	9.0×10^1	8.0×10^1	1.0×10^1
	1.0	6.2×10^2	7.0×10^2	6.8×10^3	1.6×10^3	5.0×10^2
	5.0	4.6×10^2	1.8×10^2	6.0×10^1	6.0×10^1	$< 1.0 \times 10^1$

Table 2. Growth in aqueous extracts of fir sawdust.

Klebsiella	Origin	Viable cells/ml, days after inoculation				
		0	2	6	14	31
PSB49-1	bovine mastitis	3.0×10^2	3.4×10^2	2.8×10^2	1.9×10^2	6.0×10^1
PS401LR	bovine mastitis	1.9×10^2	3.4×10^1	4.0×10^2	6.0×10^1	1.1×10^2
MSU1684	bovine mastitis	1.2×10^2	3.0×10^2	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$	$< 1.0 \times 10^2$
SL-1	human medical	4.8×10^2	3.8×10^2	6.2×10^2	8.2×10^2	1.2×10^2
UT1500	human medical	3.5×10^2	3.5×10^2	3.6×10^2	2.6×10^2	9.0×10^1
UO10994	human medical	3.2×10^2	4.7×10^2	2.6×10^1	1.7×10^2	$< 1.0 \times 10^1$
UO11065	human medical	1.3×10^2	4.5×10^2	7.0×10^1	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
MH31	human medical	4.7×10^2	5.2×10^2	2.5×10^2	1.0×10^2	$< 1.0 \times 10^1$
PC_2	redwood tank water	5.5×10^1	4.3×10^2	2.9×10^2	2.4×10^2	4.0×10^1
$JH_4 2$	drinking water	6.0×10^2	1.2×10^2	1.2×10^2	6.0×10^1	4.0×10^1
$K_2 4$	redwood	3.3×10^2	3.1×10^2	1.5×10^2	6.0×10^1	$< 1.0 \times 10^1$
13882		4.4×10^2	4.8×10^2	3.6×10^2	1.7×10^2	4.0×10^1
13182		1.6×10^2	5.3×10^2	2.0×10^2	2.0×10^2	7.0×10^1
13883		1.3×10^2	3.2×10^2	1.4×10^2	1.0×10^1	$< 1.0 \times 10^1$
Enterobacter aerogenes 13048		2.0×10^2	2.9×10^2	2.3×10^2	8.0×10^1	2.0×10^1
Enterobacter cloacae OSU		3.6×10^2	3.6×10^1	1.9×10^2	6.0×10^1	$< 1.0 \times 10^1$
Enterobacter agglomerans ICPB EH 103		1.8×10^2	5.0×10^1	3.6×10^2	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
Serratia marcescens OSU		2.6×10^2	5.6×10^3	1.6×10^2	5.0×10^1	$< 1.0 \times 10^1$
Serratia liquefaciens 14460		5.6×10^2	8.2×10^2	6.9×10^2	7.0×10^1	$< 1.0 \times 10^1$
Erwinia carotovora ICPB EC 153		6.0×10^2	1.2×10^2	2.0×10^1	1.0×10^1	$< 1.0 \times 10^2$
Yersinia enterocolitica 867		2.0×10^2	8.8×10^2	1.4×10^2	6.0×10^2	2.7×10^2
Pseudomonas aeruginosa OSU		5.3×10^2	3.6×10^2	3.0×10^2	2.6×10^2	9.0×10^2
Citrobacter freundii OSU		6.4×10^2	5.3×10^2	3.4×10^2	1.2×10^2	1.4×10^2
Escherichia coli DS5-1 OSU		4.7×10^2	3.5×10^2	3.8×10^2	1.3×10^2	$< 1.0 \times 10^1$
Shigella dysenteriae OSU		2.7×10^2	2.2×10^2	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
Salmonella typhi OSU		2.6×10^2	6.0×10^1	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
Salmonella enteritidis ser.paratyphiB OSU		7.0×10^2	1.2×10^2	$< 1.0 \times 10^2$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
Proteus vulgaris OSU		2.5×10^2	4.7×10^5	1.3×10^6	$< 1.0 \times 10^6$	$< 1.0 \times 10^1$
Indigenous flora (uninoculated)		2.9×10^2	5.0×10^5	2.2×10^6	1.1×10^6	3.5×10^6

Table 3. Growth in aqueous extracts of pine sawdust.

Klebsiella	Origin	Viable cells/ml, days after inoculation				
		0	2	6	14	31
PSB49-1	bovine mastitis	4.0×10^2	5.1×10^2	3.2×10^4	1.9×10^5	2.1×10^5
PS401LR	bovine mastitis	4.5×10^2	1.9×10^3	6.2×10^5	5.7×10^5	3.8×10^5
MSU1684	bovine mastitis	2.8×10^2	1.7×10^2	2.0×10^2	1.0×10^1	$<1.0 \times 10^1$
SL-1	human medical	1.9×10^2	3.7×10^3	5.3×10^4	5.7×10^5	3.0×10^5
UT1500	human medical	3.3×10^2	1.2×10^2	7.4×10^5	6.4×10^5	8.8×10^5
U010994	human medical	2.8×10^2	2.7×10^2	1.4×10^4	4.6×10^5	3.8×10^5
U011065	human medical	2.6×10^2	2.9×10^2	4.0×10^2	1.2×10^5	2.8×10^4
MH31	human medical	2.9×10^2	3.1×10^2	3.9×10^6	5.0×10^5	5.4×10^5
$JH_4 2$	redwood tank water	2.2×10^2	2.0×10^6	3.8×10^6	3.5×10^6	3.6×10^6
$PC_4 2$	drinking water	7.1×10^2	8.5×10^2	1.2×10^3	1.4×10^3	6.2×10^2
$K_2 2$	redwood	3.5×10^2	7.2×10^2	5.4×10^5	7.0×10^5	6.1×10^5
13882		4.3×10^2	6.6×10^1	5.1×10^3	7.4×10^5	1.2×10^5
13182		1.0×10^1	9.0×10^1	1.8×10^3	5.5×10^4	2.5×10^6
13883		6.0×10^1	3.0×10^1	6.2×10^2	1.1×10^3	1.0×10^3
Enterobacter aerogenes 13048		5.0×10^1	5.0×10^2	1.8×10^2	4.5×10^3	2.8×10^3
Enterobacter cloacae OSU		8.0×10^2	3.8×10^4	1.5×10^2	2.0×10^1	$<1.0 \times 10^1$
Enterobacter agglomerans ICPB EH 103		1.2×10^2	2.1×10^4	8.4×10^5	8.8×10^5	5.8×10^4
Serratia liquefaciens 14460		8.7×10^2	1.2×10^3	1.1×10^6	9.9×10^5	3.3×10^5
Serratia marcescens OSU		1.2×10^2	2.2×10^2	2.4×10^3	2.4×10^4	1.4×10^4
Erwinia carotovora ICPB EC 153		6.0×10^2	6.7×10^2	2.2×10^6	5.3×10^5	5.3×10^5
Yersinia enterocolitica 867		3.0×10^2	1.9×10^2	2.5×10^4	4.0×10^3	3.0×10^4
Pseudomonas aeruginosa OSU		5.0×10^2	6.0×10^2	8.0×10^4	1.3×10^5	8.4×10^4
Citrobacter freundii OSU		4.0×10^2	3.0×10^2	4.0×10^4	1.1×10^5	1.6×10^5
Escherichia coli DS5-1 OSU		6.1×10^1	3.8×10^2	3.1×10^2	1.0×10^2	$<1.0 \times 10^1$
Shigella dysenteriae OSU		8.0×10^1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Salmonella typhi OSU		7.0×10^2	6.0×10^2	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Salmonella enteritidis ser.paratyphi B OSU		2.5×10^2	1.2×10^2	$<1.0 \times 10^2$	$<1.0 \times 10^1$	$<1.0 \times 10^1$
Proteus vulgaris OSU		1.4×10^2	4.4×10^2	6.0×10^2	$<1.0 \times 10^4$	$<1.0 \times 10^5$
Indigenous flora (uninoculated)		2.0×10^1	$<1.0 \times 10^1$	3.0×10^3	1.0×10^4	2.8×10^5

Table 4. Utilization of carbohydrates present in redwood by gram-negative bacteria.

	L (+) arabinose	D (-) fructose	D (+) galactose	D- glucose	raffinose	α-L- rhamnose	sucrose	D-xylose
Klebsiella PSB-49-1	+g[a]	+g	+g	+g	+g	+g	+g	+g
PS401LR	+g	+g	+g	+g	+g	+g	+g	+g
MSU1684	+g	+g	+g	+g	+g	+g	+g	+g
SL1	+g	+g	+g	+g	+g	+g	+g	+g
UT1500	+g	+g	+g	+g	+g	+g	+g	+g
U010994	+g	+g	+g	+g	+g	+g	+g	+g
U011065	+g	+g	+g	+g	+g	+g	+g	+g
MH31	+g	+g	+g	+g	+g	+g	+g	+g
PC$_2$	+g	+g	+g	+g	+g	+g	+g	+g
JH$_4$2	+g	+g	+g	+g	+g	+g	+g	+g
K2	+g	+g	+g	+g	+g	+g	+g	+g
ATCC13882	+g	+g	+g	+g	+g	+g	+g	+g
ATCC13182	+g	+g	+g	+g	+g	+g	+g	+g
ATCC13883	+g	+g	+g	+g	+g	+g	+g	+g
Enterobacter aerogenes ATCC 13048	+g	+g	+g	+g	+g	+g	+g	+g
Enterobacter agglomerans ICPB EH103	+	+	+	+	-	+	+	+
Enterobacter cloacae OSU	+g	+g	+g	+g	+g	+g	+g	+g
Serratia liquifaciens ATCC 14460	+	+	+	+	+	+	+	+
Serratia marcescens OSU	-	+	+	+	-	-	+	-
Erwinia carotovora ICPB EC153	+	+	+	+	+	+	+	+
Yersinia enterocolitica 867	+	+g	+g	+	-	+g	+g	+g
Pseudomonas aeruginosa OSU	-	-	-	+	-	-	-	+
Citrobacter freundii OSU	+g	+g	+g	+g	-	+g	-	+g
Escherichia coli DS5-1 OSU	+g	+g	+g	+g	+g	+g	+g	+g
Shigella dysenteriae OSU	-	+	+	+	-	-	-	-
Salmonella typhi OSU	-	+	+	+	-	-	-	+
Salmonella enteritidis ser. paratyphi B OSU	+g	+g	+g	+g	-	+	-	+g
Proteus vulgaris OSU	-	+	+g	+g	-	-	-	+g

[a]g=gas production