of the constituents of the medium so that the best environmental conditions for penicillin production were obtained. Peptone and tryptone were unable to supply the essential elements required by the mold. Therefore a salt mixture was added to prepare a complete medium with these nutrients. Glucose was found to be necessary when media containing yeast extract or fish solubles were used. Lactose at a 5 per cent level was found to be sufficient to meet the needs of the mycelium during the penicillin production phase. The optimum concentration of the nitrogenous nutrient was determined in a medium containing 5 per cent lactose. It was observed that steep liquor, cottonseed meal, soybean meal, and distillers' solubles were the best of the organic nutrients studied.

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Species of *Propionibacterium* Associated with Zapatera Spoilage of Olives

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Characteristics of the undesirable malodorous fermentation of brined olives known as "zapatera" have been described in some detail by Ball (1938), Cruess (1924), Delmouzos, *et al.* (1953), Kawatomari and Vaughn (1956), Vaughn (1946), and Vaughn *et al.* (1943). The first off-odors to appear have been called "cheesy" or "sagey" but, as the spoilage progresses, they eventually develop into an unforgettable, foul, fecal stench. There is a continuous loss in acidity as the spoilage develops. As shown by Delmouzos *et al.* (1953), part of the odor results from the volatile acids developed in the spoiled brine samples. These include formic, propionic, butyric, valeric, caproic, and caprylic acids, together or in various combinations. In contrast, the normal brines contain acetic, lactic and sometimes succinic acids, but none of the more odoriferous volatile acids were found in the spoiled samples.

Recently, Kawatomari and Vaughn (1956) were able to associate various species of *Clostridium* with zapatera spoilage. However, none of the cultures studied produced propionic acids under the conditions tested. Therefore, an additional study was initiated to determine whether species of *Propionibacterium* might also be associated with the spoilage. The characteristics of species of propionic acid bacteria actually associated with zapatera spoilage of different kinds of brined olives are described below.

Experimental Methods

Sources of cultures. In addition to the 54 samples previously examined by Kawatomari and Vaughn (1956), 33 more suspected samples of zapatera olives were tested in this study. The 33 new samples were obtained from Californian, Grecian, Algerian, and Spanish sources. The foreign samples were collected through the courtesy of a number of importers. Additional samples from California included brined olives to be used for preparation of California canned ripe olives as well as Sicilian and Spanish type green fermented olives. The imported samples comprised brined, ripe (mature and colored) olives from Greece, Spanish type green fermented olives from Algeria and Spanish green fermented olives.

Sixty-eight cultures of propionic acid bacteria were isolated from these samples.

Enrichment of the cultures. The enrichment medium, patterned after that of van Niel (1928), had the following composition: yeast extract (Difco), 1 g; sodium lactate, 2 g; and distilled water to make 100 ml. Lactic acid (USP, or reagent grade) neutralized with 10 per cent NaOH to pH 7.0 before sterilization was used in place of commercial sodium lactate. The latter apparently still contained some of the free acid and on sterilization the pH of the medium was found to be too low. Unless a small amount of calcium carbonate was added, the pH of the lactate medium after sterilization at 121 C for 15 min ranged between 5.2 and 5.4. The enrichment medium, without added carbonate, was sterilized by means of a Seitz filter that had been sterilized at 121 C for 45 min. Tubed media were inoculated, sealed with vaspar and incubated at 30 C. If sufficient numbers of propionic acid bacteria were present, a marked turbidity and gas, as indicated by the upward displacement of the vaspar plug, developed in either of the enrichment media within 5 to 7 days. With suspected samples (incipient spoilage), the incubation period was extended to between 15 and 20 days. In contrast, controls and those tubes inoculated with healthy (normal) brines remained clear for over 2 months of incubation at 30 C.

Between 3 and 5 consecutive transfers were made after gas formation and turbidity were observed in the first enrichment tubes. In general, 5 consecutive transfers were required to permit the propionic acid bacteria to dominate the microbial populations in either of the enrichment media. When growth and gas production were uniform through at least 2 consecutive transfers an attempt was made to isolate and purify the bacteria that had predominated the enrichment process.

Purification of the cultures. The following medium was used for isolation of pure cultures of the propionic acid bacteria: yeast extract (Difco), 1 g; glucose, 2 g; calcium carbonate, 2 g; agar, 1.5 g; and distilled water to make 100 ml. This medium was sterilized by autoclaving at 121 C for 20 min. Plates of this medium were streaked and incubated at 30 C in anaerobic jars (McIntosh-Fildes type[1]) either in an atmosphere of 95 per cent nitrogen and 5 per cent CO_2 or in air displaced with about 5 per cent CO_2. Both methods of incubation gave good results.

After incubation for 7 to 15 days, characteristic hemispherical colonies had developed on the surface of the medium. These colonies (all well isolated) varied in diameter from 1 to 3 mm. All had a creamy, butyrous texture and were whitish or tan in color. These primary isolates were purified by 3 or 4 restreakings on the glucose agar until the same homogeneous colony types appeared on each consecutive plating.

[1] Arthur H. Thomas Co., Philadelphia, Pennsylvania.

Sixty-eight gram positive, catalase positive pure cultures were obtained for study. All of these isolates were allocated to the genus *Propionibacterium* because each formed acetic and propionic acids as well as carbon dioxide in the lactate medium. The organic acids were determined by paper chromatography (Brown and Hall, 1950; Lugg and Overell, 1948). Carbon dioxide was measured manometrically.

Three kinds of isolates were recognized. Additional study of the 68 isolates revealed three groups which might merit speciation.

The choice of criteria used for identification of the cultures was based on the studies of van Niel (1928) and von Janoschek (1944) as summarized by van Niel in *Bergey's Manual of Determinative Bacteriology* (1948). Species allocation was based on significant differences in cell and colony size, colony color, degree of anaerobiosis each culture tolerated, and ability to ferment carbohydrates. These compounds (sugars, glucosides, and polyalcohols), in concentrations of 2 per cent, were contained in a basal medium of 1 per cent yeast extract (Difco) in distilled water. The finished media, in 15 ml portions, were adjusted to pH 7.2 before sterilization at 121 C for 15 min. The compounds subject to hydrolysis under such conditions were sterilized by Seitz filtration. After inoculation, the tubes containing the various carbohydrates were incubated at 30 C for 15 days. Then 10 ml aliquots were titrated in 50 ml of distilled water to the phenolphthalein end point with 0.1 N NaOH. Controls included inoculated tubes containing only yeast extract, plus uninoculated tubes with each carbohydrate in yeast extract.

Control cultures obtained from Professor C. B. van Niel were used throughout the study. These included the species *Propionibacterium freudenreichii*, *Propionibacterium jensenii*, *Propionibacterium pentosaceum* and *Propionibacterium zeae*.

Characteristics of the Isolates

In many respects the isolates were very similar. Cells of all cultures had metachromatic granules. They were nonmotile and without endospores. "Involution" cell forms were irregular, clubshaped, branched rods. When grown aerobically in unbuffered media, the involution forms were frequently of a shape reminiscent of rattlesnake rattles (see figure 1). None of the cultures produced indol, reduced nitrates, or liquefied gelatin.

The important differences that could be used for separation of the cultures into species are shown in table 1. All zapatera cultures fermented L-arabinose, glucose, fructose, galactose, sucrose, lactose, maltose, raffinose, aesculin, salicin, glycerol, and mannitol, but did not cause significant acid production from dextrin, dulcitol, glycogen, inulin, or starch.

P. zeae, represented by 2 similar but distinguishable forms, was recovered most frequently from the spoiled

brines. As shown in the table, the Type I cultures, including 32 isolates, had characteristics similar to those of the control culture of the species received from Professor van Niel. However, the 14 Type II isolates of *P. zeae* differed in several characteristics. These latter cultures grew somewhat better under aerobic conditions, but produced little more than half as much titratable acidity from the compounds attacked (*l*-arabinose excepted) as did the more typical Type I isolates. The smaller Type I cells (0.6 to 0.7μ by 1.5 to 1.8μ) did not settle out in 15 days but retained a relatively uniform turbidity throughout the lactate medium whereas the larger cells of the Type II isolates (0.8 to 1.0μ by 1.8 to 2.0μ) had settled enough to leave the upper third of the medium clear. In contrast, the cells of the *P. pentosaceum* isolates, largest of the three (0.8 to 1.0μ by 3.0 to 3.6μ), had completely settled in 15 days, leaving a clear supernatant medium with a thick, slimy sediment.

As shown in the table, the differences in fermentation of the pentoses also served to separate the 2 types o

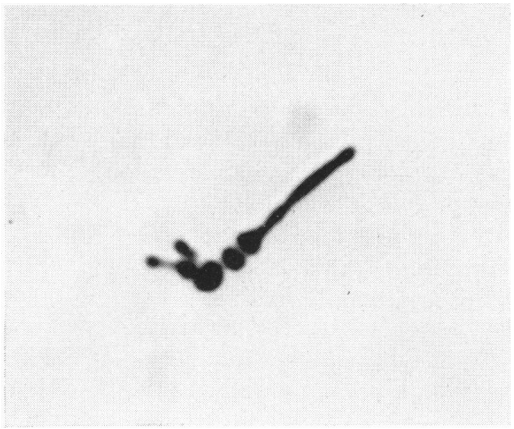

FIG. 1. Characteristic rattleshaped involution form of *Propionibacterium zeae* grown aerobically in unbuffered glucose broth.

P. zeae from the *P. pentosaceum* isolates. However, it is to be stressed that the authors do not consider the slight differences in characteristics observed for the *P. zeae* cultures sufficient to merit separate species allocation. Therefore, under the conditions of these experiments, it would be possible to associate only 2 species of *Propionibacterium* with the malodorous zapatera spoilage of olives. However, before this supposition could be substantiated, it was also necessary to determine the tolerance of the isolates to salt and changes in pH values as well as their ability to grow and produce propionic acid in sterile olive brines.

Maximum salt tolerance. As summarized by Kawatomari and Vaughn (1956), most olives are fermented in brines containing about 5 to 8 per cent salt, and, after fermentation, may be stored in brines containing as much as 15 per cent. Therefore, it was desirable to determine the salt tolerance of the cultures in order to associate more firmly the propionic acid bacteria with zapatera spoilage.

To test the tolerance of the bacteria to salt each isolate was inoculated into the lactate enrichment medium with 0.1 per cent added sodium chloride (the complete medium at pH 6.5 sterilized by Seitz filtration). After this slight adaptation the cultures were transferred to the same basal medium with 4 per cent NaCl; when growth was observed at this concentration, a portion of the culture was transferred to the medium containing 5 per cent. This adaptation technique was continued with increasing concentrations of salt in 1.0 per cent increments until the cultures failed to grow. At this point 0.5 per cent increases in salt were tested with each culture. When growth was not observed within a maximum of 25 days' incubation at 30 C, negative results were recorded.

All of the bacteria (zapatera and control cultures alike) grew in the lactate medium containing 4.0 per cent salt. However, as shown in table 1, the cultures

TABLE 1. *Characteristics of species of Propionibacterium from "zapatera" olive brines**

Number of Cultures	Colony Color	Aerobic Growth	Diagnostic Carbohydrate Fermentations						Species Allocation
			D-Arabinose	L-Arabinose	D-xylose	Glucose	Sucrose	L-Rhamnose	
			Average ml 0.1 N NaOH to neutralize 10 ml of culture						
Isolates from zapatera brines									
32	Cream	Poor	0	4.8	0	9.6	11.2	4.6	*P. zeae* (Type I)
14	Cream	Fair	0	4.2	0	5.8	5.9	4.2	*P. zeae* (Type II)
22	Cream	Fair	13.4	9.3	2.7	12.5	10.6	4.8	*P. pentosaceum*
Pure culture controls									
1	Dirty cream	Scant	8.3	0	0	7.8	0	0	*P. freudenreichii*
1	Cream	Poor	0	6.1	0	10.5	14.2	5.1	*P. zeae*
1	Orange-yellow	Fair	0	5.8	0	14.1	9.6	0	*P. jensenii*
1	Cream	Good	6.7	2.8	3.5	4.4	4.9	4.2	*P. pentosaceum*

* All characteristics observed after 15 days incubation at 30 C.

identified as *P. pentosaceum* and *P. zeae* (Types I and II) were the most salt tolerant. The former species grew in the presence of 7.0 per cent salt whereas the latter two types tolerated 7.5 per cent. The control cultures of *P. pentosaceum* and *P. zeae* also were more salt tolerant than those of the species *P. freudenreichii* and *P. jensenii*.

Minimum pH tolerance. In order to avoid zapatera spoilage, it has been common practice in the California olive industry to attempt to control the fermentation until the pH of the brine has decreased to about 3.8. Kawatomari and Vaughn (1956) already have shown that both pH and salt tolerance are involved in control of the clostridia associated with zapatera. Therefore, it was desirable to determine the minimum pH value tolerated by the propionic acid bacteria already provisionally associated with the spoilage.

The effect of pH on the cultures was tested by growing the isolates in a lactate medium at different pH values between 4.0 and 9.0 at 0.2 unit intervals. The basal medium, similar to the one used for enrichment, made with 2 per cent lactic acid had a pH value of 3.2. The desired pH was obtained by adjustment of the medium with 10 per cent NaOH. Then it was sterilized by Seitz filtration and tubed under aseptic conditions in sterile tubes.

An adaptation technique was used for determining the pH range for growth of the cultures. With this method each culture was transferred to the lactate medium with the next higher or lower pH from neutrality until the cultures failed to grow. Negative results were recorded when the cultures failed to grow after 25 days' incubation at 30 C.

As shown in the table the cultures were not able to grow at very low pH values. The minimum values tolerated ranged between 4.8 and 5.2. The maximum pH values for growth ranged between 7.2 and 8.0. For optimum growth in the medium the pH values should be between 6.5 and 7.0. From these observations it appears that any involvement of the propionic acid bacteria in zapatera spoilage must be limited to conditions of low acidity where the pH of the brine is 4.8 or above.

Production of propionic acid in olive brines. Demonstration that the propionic acid bacteria could produce propionic acid in olive brines was required if they were to be associated with zapatera spoilage. Preliminary experiments with normal commerical and laboratory fermented olive brines were unsuccessful. The cultures all failed to grow in brines adjusted to pH 5.5 before sterilization by Seitz filtration. However, when these partially neutralized brines were supplemented with 0.5 per cent yeast extract (Difco) or 0.5 per cent yeast extract plus 0.2 per cent glucose the cultures all grew and produced propionic acid in the brines. Furthermore, a definite "cheesy" odor was produced although it was masked somewhat by the "scorched" odor developed when the brines were adjusted to pH 5.5 before sterilization.

The relatively complex nutritional requirements of the propionic acid bacteria are well known (Delwiche, 1949). Therefore, on the basis of these past experiences, it might have been predicted that the zapatera isolates of propionic acid bacteria would not grow in normal, readjusted sterile brines without addition of supplementary nutrients, even though an abundance of lactate was available. Kawatomari and Vaughn (1956) also experienced such a difficulty with species of *Clostridium* that were grown in similar olive brines. However, until now, it has not been possible to elucidate all of the factors which predispose olive brines to zapatera spoilage. It can only be speculated that unknown, nutritive accessory compounds are not readily available to the bacteria; are present in insufficient quantities; or are completely absent from the brines. The possibility also exists that inhibitory substances are formed or destroyed by neutralization and heat sterilization of the brines or that essential compounds are removed during filtration sterilization.

Discussion

The association of the propionic acid bacteria with zapatera spoilage of olive brines helps to clarify the etiology of this malodorous fermentation. It is believed that the propionic acid found by Delmouzos *et al.* (1953) to be present in most zapatera brines is produced by 2 species, *P. pentosaceum* and *P. zeae*, the only types of propionic acid bacteria isolated from the spoiled brines involved in this study. These bacteria grown in pure culture in supplemented olive brines produced propionic acid and developed a "cheesy" odor, the first abnormal odor detected in cases of zapatera spoilage. However, it is certain that if the malodorous spoilage progressed no further than the fermentation of lactate by the propionic acid bacteria, it would be so minor as to be classed as inconsequential in commerce.

If the propionic acid bacteria appear first in the sequence of microorganisms responsible for the development of zapatera they may play a major role by causing an increase in pH so that the species of *Clostridium* can produce components of the more odoriferous parts of the fermentation. This is conjectural because Kawatomari and Vaughn (1956) already have shown that many species of *Clostridium* are as tolerant to low pH as are the two species of *Propionibacterium* described here. Furthermore, yeasts from olive brines cause lactate decomposition under aerobic conditions according to Mrak, *et al.* (1956). It will be possible to delegate relative responsibility only when more is known of the significance of other microorganisms in the zapatera fermentation. These unknown microorganisms may

contribute accessory growth factors needed by the species of *Clostridium* and *Propionibacterium* or abnormal volatile compounds that contribute to the complex of odors characteristic of zapatera spoilage.

Nothing is known of the accessory growth factors that may be available in the brines. Soriano and Soriano (1946) and Soriano (1955) have claimed that the sulfate reducing bacterium *Desulfovibrio desulfuricans* is the cause of zapatera spoilage. Nevertheless, hydrogen sulfide does not appear to be a significant constituent of such spoilage. None of the zapatera olives and brines examined by Vaughn and co-workers has ever had a pronounced hydrogen sulfide odor. Furthermore, three separate exploratory studies failed to yield a single positive enrichment for *D. desulfuricans* from the samples investigated by Kawatomari and Vaughn (1956) plus the additional ones used in this study (Vaughn *et al.*, 1956, Unpublished Observation). However, the possibility still exists that other microorganisms contribute to the malodorous volatile compounds involved in the spoilage. Levin and Vaughn, (1956, Unpublished Observation) have found bacteria capable of lipolytic breakdown of olive oil to be present in zapatera brines. Experiments in progress should eventually determine the relationship of these fat splitting bacteria to zapatera spoilage.

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Summary

The characteristics of 68 cultures of *Propionibacterium* isolated from "zapatera" olive brines are described. Two species, *Propionibacterium pentosaceum* and *Propionibacterium zeae*, are identified. The 46 isolates of the species *P. zeae*, represented by 2 types, predominated among the cultures isolated in this study. The 2 types differed in that the 32 Type I cultures produced more acid from glucose and sucrose than did the 14 Type II isolates. Variability in other characteristics of these 2 types of *P. zeae* was so slight that separate species allocation for either type was not warranted.

The isolates of *P. pentosaceum* and *P. zeae* produced propionic acid and a "cheesy" odor in olive brines when suitable conditions were maintained for growth of the cultures. Therefore, the propionic acid found in zapatera brines may be produced by propionic acid bacteria. However, the association of species of *Propionibacterium* with this malodorous fermentation still does not provide a complete explanation of the etiology of the spoilage.

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