California State University at Northridge

THE DISTRIBUTION AND PHYSIOLOGY OF *SPOROSARCINA UREAE*

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Biology

by

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Abstract

Fifty-one strains of Sporosarcina ureae were isolated from 198 soils by plating diluted soil samples onto a complex agar medium containing 1.5% filter sterilized urea. S. ureae was found to be widely distributed in the United States and in various parts of the world. The primary habitat of this organism appears to be concentrated in certain urban soils closely associated with the activities of man and especially of dogs. However, a variety of soils cannot support growth of S. ureae. This organism was not found in soils obtained from zoos, corrals, stables, grazing lands, deserts, or from cultivated fields. Nor was it found in salt water samples.

Sixty-one strains of this organism were tested for their ability to utilize 45 different organic compounds as sources of nitrogen, carbon and energy, or as growth factor requirements. The vast majority of strains required only ammonium salts for a nitrogen source but all of the organisms showed limited ability to metabolize carbon sources. Sugars are not utilized or are utilized very poorly, and glucose could not be attacked by any strain. Acetate (or butyrate) and glutamate are apparently universal substrates. The overall metabolic pattern was one of random variation but there was a clustering of strains according to growth factor requirements. The pattern that emerged was that of gradation in growth factor requirements ranging from organisms which required no growth supplements to those organisms whose nutritional requirements are complex.

In general, the ecology, morphology, cultural characteristics, or biochemical reactions revealed no consistent differences that could be correlated with nutritional differences. Nor did this study reveal a constellation of differential characters that would permit recognition of additional species of Sporosarcina. The results did suggest that the leading criterion for species identification and classification may be the nutritional differences shown by this organism.

1. INTRODUCTION

Sporosarcina ureae is a motile bacterial coccus which occurs in regular packets of two, four, or eight cells, and produce highly refractile, heat resistant endospores. Motility among cocci is rare enough, but it is the presence of endospores that makes this organism unique among cocci, since all other spore forming bacteria are rods, assigned taxonomically to the genera Bacillus and Clostridium in the family Bacillaceae.

Until very recently, this organism was generally known as Sarcina ureae. The seventh edition of Bergey's Manual of Determinative Bacteriology (1957), classified this coccus as a member of the family Micrococcaceae, along with other gram positive cocci. It shared the generic name, Sarcina, with both aerobic and anerobic non-sporulating cocci on the basis of the type of cell division and cell adhesion; packets are formed from division of the cell in planes perpendicular to one another. However, the Family Micrococcaceae has recently been redefined (Canole-Parola, Mandel, and Kupfer, 1967; Canole-Parola, 1970; Rogosa, 1971a, 1971b). In the current revision, the aerobic, non-sporulating packet forming species, commonly known as Sarcina have been placed in the genus Micrococcus with certain other non-packet forming, gram positive cocci. The genus designation, Sarcina, now refers solely to those species that are anerobic, gram positive packet formers, of which there are currently only two representative species, Sarcina maxima and Sarcina ventriculi. Nevertheless, because of the current understanding of the generic term, Sarcina, and its

popular usage in all but the most recent literature, in this work I will use this term when referring to those bacterial species that are gram positive, non-sporulating, aerobic or anerobic packet formers.

The earliest descriptions of organisms resembling S. ureae included sarcinae which were motile but did not form spores, or sarcinae which formed spores but were not motile. Maura, in 1892, first described a motile, orange-pigmented species which he named Sarcina mobis (Hucker and Thatcher, 1928). In 1898, Sames isolated a motile grayish-white pigmented Sarcina species which was later placed in a separate genus on the basis of its motility by Migula, and named Planosarcina samesii (Hucker and Thatcher, 1928). Neither Maura nor Sames mentioned the presence of spores. The first description of a spore forming coccus was noted by Hauser in 1887 and was mentioned by Lehmann and Neumann in 1927 (Gibson, 1935), although neither worker commented on the motility of this coccus.

The earliest descriptions of Sarcina species which were both motile and able to produce spores were by Beijerinck in 1901, and by Ellis in 1902. Beijerinck noted that the heat resistant bodies of his isolate survived exposure to 90° C for ten minutes but he did not believe that typical endospores were present. Beijerinck named this organism Planosarcina ureae because of its ability to decompose urea. The name was changed to Sarcina ureae by Lohnis in 1911 (Gibson, 1935), and, as already noted, this name was retained for many years.

Following Beijerinck's work in 1901, no additional isolate of

this organism was noted for several decades. In 1935, Gibson described the ecology, morphology, and physiology of a number of strains of S. ureae which he isolated from soils. He stated that the spores formed are true endospores for they survived exposure to 65° C for 15 minutes. In 1946, Wood isolated a motile spore forming coccus from sea water which could survive a treatment of 99.5° C for 3 minutes.

The few studies undertaken in recent years have been primarily concerned with the structure and physiology of the spore and the relationship of this organism to Bacillus species. Mazanec, Kocur, and Martinec (1965), studied the ultra structure of spores of S. ureae with electron microscopy and found them to be similar to spores of Bacillus species in size, shape, and morphology. The spore is composed of an outer coat of several layers, a cortex, a spore wall, and cytoplasm. Spores of S. ureae are also similar to spores of Bacillus species because both species show the same degree of heat resistance and both contain dipicolinic acid at the same concentrations (Thompson and Leadbetter, 1963). In addition, germination has been observed to be comparable to germination in Bacillus species (Thompson and Leadbetter, 1963; Iandolo and Ordal, 1964). A number of workers have investigated the DNA base composition of S. ureae (MacDonald and MacDonald, 1962; Auletta and Kennedy, 1966; Rosypalová, Boháček, and Rosypal, 1966; Venner, 1967; Boháček, Kocur, and Martinec, 1968). The results of these investigations indicate that the G+C content of the DNA is within the range reported for Bacillus species, but quite dissimilar

from that of sarcinae. S. ureae differs from Bacillus, however, in the composition of the vegetative cell wall. The chief amino acids present in S. ureae are alanine, glutamic acid, lysine, and glycine. Diaminopimelic acid and sugars, which are found in cell walls of Bacillus species, are absent from walls of Sporosarcina (Salton, 1964; Cummins and Harris, 1956).

It is evident that Sporosarcina ureae has characteristics in common with both the aerobic packet forming bacteria in the Family Micrococcaceae and with Bacillus species in the Family Bacillaceae. The vegetative cells are like those of sarcina; their spherical shape and their method of cell division is a stable characteristic, and S. ureae closely resembles sarcinae and micrococci in the composition of the vegetative cell wall. The production and nature of the endospores, motility, and base composition of the DNA is a link this organism has with the genus Bacillus. The eighth edition of Bergey's Manual of Determinative Bacteriology which soon is due to appear, has acknowledged that the spores formed by this coccus are true endospores and has placed S. ureae with the Bacillaceae in a separate genus, Sporosarcina. The legitimate nomenclature is Sporosarcina ureae (Beijerinck) Kluyver and van Niel.*

Little is known of the ecology of this organism. No worker since Gibson's study in 1935, has described the isolation or distribution of more than single strains of this organism. Specifically, I have attempted to isolate strains of S. ureae in a more systematic fashion, to investigate not only the distribution

* Personal communication with T. Gibson.

of this organism but also its habitat or habitats. Accordingly, Part I of this paper is devoted to the ecology of Sporosarcina ureae.

An additional purpose of this study was to investigate the nutritional requirements and to compare the morphology and physiology of freshly isolated strains, as well as pure cultures of Sporosarcina supplied by other laboratories, to determine if a spectrum of activities or a pattern emerges among these strains which could serve to delineate species or biotypes. The results of this work (Part II) extend and complement earlier work which examined and compared the morphology, physiology, and biochemistry of between nine and fifteen strains of S. ureae (Gibson, 1935; MacDonald and MacDonald, 1962; Kocur and Martinec, 1963).

Part I

The Isolation and Distribution of *Sporosarcina ureae* in Soils.

2. ISOLATION OF SPOROSARCINA UREAE

With one exception, all isolated strains which conceivably resemble S. ureae have been isolated from soils. Several descriptions of sarcinae are found in the earlier literature. In 1927, Lehmann and Neumann (Gibson, 1935) described an organism they termed Sarcina pulmonium which decomposes urea, forms spores, and was apparently isolated from the human respiratory tract, chiefly in cases of phythisis. Such a habitat would imply that this organism possesses a higher optimum temperature than S. ureae; the optimum temperature for growth of S. ureae is between 22° C and 30° C. A motile, urea-decomposing Sarcina species was isolated from liquid manure by Sames in 1898 (Gibson, 1935). It grew on alkaline media rather than on neutral substrates and seems to correspond to S. ureae except that the production of spores was not observed. Wood's isolate (1946), appears to be the only Sporosarcina ureae strain to have been isolated from sea water; this isolate was obtained from surface water approximately five miles east of Jibbon Cape in Australia.

Gibson (1935), isolated fifteen strains of S. ureae from rich garden soil and cultivated field soils. Other workers also used various soils for isolations. Surprisingly there is little information published on the procedure for isolating S. ureae from soils. Beijerinck (1901), used beef broth containing 10% urea for selective culturing of the isolate he obtained from garden soil. The most complete study of the isolation procedure of S. ureae is Gibson's

work (1935). He was able to isolate a number of strains by directly plating soil dilutions on a meat extract-peptone medium containing 10% ureae; pure cultures were obtained by isolating and replating colonies which developed on the plates. Without success, Gibson repeated Beijerinck's isolation procedure by using urea broth as the selective medium. Gibson inoculated the broth with varying amounts of soil, having previously estimated the number of S. ureae cells present per gram by plating measured dilutions. The enrichment cultures were incubated at varying temperatures but Gibson was not able to detect this organism when the cultures were plated. He suggested that the difficulty that he and other workers have had in repeating Beijerinck's isolation method is due to the unsuitability of urea containing solutions for selective cultures.

This study reports the isolation of 51 strains from 198 soil samples representing a variety of soil types. Generally only one strain was isolated from any one soil type; however, several representatives from a single soil type were included when these strains showed obvious differences in morphology or in pigmentation. The origin of all organisms described in Part I and Part II of this study is given in Table 5, Part I.

3. METHODS AND MATERIALS

I Media for maintenance and for isolations

a. Maintenance medium

Tryptic soy-yeast agar (TSY) was the principal medium employed for maintenance of cultures of S. ureae on plates and on slants. Cultures of S. ureae maintained on slants were refrigerated until transferred to fresh TSY. Transfers were made every 3 months during the first year following their isolation, and then every 6 months thereafter. All the cultures of this organism, in fact, could be readily revitalized after being maintained on slants for a year or longer without being transferred in the interim. The composition of the basic TSY medium was as follows.

(Per liter of deionized water):

27.5	g	Difco tryptic soil broth
5.0	g	Difco yeast extract
5.0	g	Difco glucose
15.0	g	Bacto agar

Glucose was omitted later from the maintenance medium with no change in the ability of the medium to support growth. The pH, which was approximately 7, was not adjusted.

b. Isolation media

For isolations, sufficient IN NaOH was added to TSY to give a pH of 8.5 before autoclaving, and filter sterilized urea was added aseptically to the autoclaved complex medium to give a final concentration of 1.0% (w/v). Sea water replaced deionized water on the isolation medium when plating samples from salt water sources.

Other isolation media described in this study included an alkaline yeast extract medium and a nutrient agar medium. Each of these media was supplemented with sufficient IN NaOH to give a pH of 8.5 before autoclaving, and 1.0% filter sterilized urea was added aseptically to the autoclaved media. Alkaline yeast extract medium (per liter of deionized water): 5.0 g Difco yeast extract and 15.0 g. Bacto agar. Nutrient agar medium (per liter of deionized water): 10.0 g. Difco nutrient broth and 15.0 g. Bacto agar. In some cases, the urea-nutrient agar medium was supplemented with 5.0 g. Difco yeast extract.

Pure cultures of S. ureae were incubated at 30° C. For isolations, unless stated otherwise, the temperature of incubation was at 22° C.

II Medium for obtaining spores

All strains of S. ureae were grown in the sporulating medium recommended by MacDonald and MacDonald (1962), and incubated at 22° C. The sporulating medium has the following composition:

	Final concentration in medium: (g/l)
Bacto-agar	30.0
yeast extract	2.0
peptone	3.0
glucose	4.0
malt extract	3.0
K_2HPO_4	1.0
$(NH_4)2SO_4$	4.0
$CaCl_2$	0.1
$MgSO_4$	0.8
$MnSO_4$	0.1
$FeSO_4 \cdot 7H_2O$	0.001
$ZnSO_4$	0.01
$CuSO_4 \cdot 5H_2O$	0.01

The pH was adjusted to 8.8 before autoclaving.

III Morphology

Zeiss or Wild phase contrast microscopes were used for routine examinations and for morphological studies. For isolating colonies of S. ureae from plates containing soil dilutions, and for examination of colony morphology, a Spencer dissecting microscope was used.

IV pH of soil*

For determination of the pH of six soil samples, a slurry, consisting of 1 part of soil and 2 parts of water were prepared, and the pH was determined with a Corning M-12 glass electrode pH meter.

V Urea content of soil*

The amount of urea present in several soil samples was determined by following the method devised and approved by the Association of Analytical Chemists (Methods of Analysis of The Association of Official Analytical Chemists, 1970). The percentage of urea was calculated after measuring the amount of ammonia liberated from the soil by urease hydrolysis.

VI Enrichment and isolation techniques

The plating technique used by Gibson (1935), was essentially the method used in this study to obtain pure cultures. Instead of Gibson's medium of meat extract-peptone, I used tryptic soy broth, glucose, and yeast extract. The urea concentration was also reduced to 1.0% instead of 10% when it became apparent that a urea concentration of 10% was barely tolerated by S. ureae, and good growth with mixed cultures was obtained at the lower urea concentration. Sufficient IN NaOH was added to give a pH of 8.5

* Determined by Truesdail Laboratories, Inc., Los Angeles, California.

before autoclaving. One gram of soil was brought to a volume of 15 mls. with distilled water in a 6 x 5/8" test tube. The slurry was mixed with a Vortex mixer, and 0.1 ml. from a series of dilutions was spread on plates with a sterile bent glass rod. Triplicate plates were prepared at 10^{-1} and 10^{-2} dilutions and duplicate plates were prepared at 10^{-3} and 10^{-4} dilutions. Heavily manured soils invariably produced the greatest numbers of cells on the isolation medium; dilutions of such soils were, in some cases, increased to 10^{-5}. 10^{-1} and 10^{-2} dilutions were spread over the entire surface of one set of plates. Generally the brightly pigmented strains of S. ureae were readily identified by this method, but at these dilutions, colonies become very crowded and growth of S. ureae under these conditions is considerably slower than much of the background growth. Crowding of colonies was lessened by spreading 10^{-1} and 10^{-2} dilutions over approximately one-third of two additional plates and then by streaking from this inoculum with a wire loop onto the remainder of the plates. The less crowded cells on the streaked portion of the plate allowed a substantial increase in colony size, making colonies of S. ureae relatively easy to identify. At the higher dilutions all plates were prepared by spreading the cells over the entire surface. Plates were incubated at 22° C, were examined on the third day and then daily with a dissecting microscope. Prior to three days, most colonies of S. ureae are generally indistinguishable from those of a number of other cell types.

In some cases, the slurry was pasteurized in an attempt to select for spore formers. An isolation procedure which was tried without success involved the pasteurization of the soil slurry for

ten minutes at 80° C before plating but there appeared to be as many cells on the plates with the heat treatment as on the control plates. Thus, there was no advantage in initially pasteurizing the soil. Gibson (1935) also found this procedure offered no advantage in the isolation of S. ureae and ascribed the ineffectiveness of this treatment to the sporeforming bacteria which develop on the plates. I noted that these urea-tolerant or ureolytic Bacillus species can germinate and grow much more rapidly than S. ureae on the TSY-urea plates.

All the strains used for the nutritional study were isolated on the TSY-urea medium described in this chapter. The satisfactory recovery, however, of an additional strain from alkaline TSY (pH 8.2), without the addition of urea, prompted an investigation of the original enrichment procedure under altered environmental conditions. The pH, temperature of incubation, and urea content of the TSY enrichment were varied, and the recovery of S. ureae when plated on the different TSY media was studied. A fresh soil sample known to contain the organism was used in this investigation.

Because the TSY-urea enrichment used for isolating S. ureae also supports a large and varied microbial population, several other enrichments were used to selectively enhance growth of S. ureae. These enrichments include alkaline yeast and alkaline nutrient agar, both supplemented with urea (described in Chapter 3, Part I).

The criteria established for including an isolate in this study as a member of Sporosarcina ureae were gross cell and colony morphology, motility of young cells (ca 12-15 hours) on TSY plates,

and the production of spores on MacDonald and MacDonald's spore medium (1962).

The features of colony morphology found most useful for preliminary selection of colonies from mixed cultures are uniform surface granularity, smoothly opaque interiors, and pigmentation. Even those strains which show little or no pigmentation on TSY rarely appear as devoid of color as do many other organisms of the microbial population. More detailed information on cultural characteristics of S. ureae is found in Chapter 8, Part II.

4. SOIL AND SALT WATER COLLECTIONS

With the object of obtaining as great a variety of organisms as possible, a large number of soils were sampled from a variety of soil types and from widely separated areas. Collections were made from areas of Southern and Northern California, Boston, New Orleans, Japan*, Hawaii*, Wake Island* and Anchorage, Alaska*.

Soils collected from California were examined within several days. Those soils collected from Boston and New Orleans were examined four or five days following their collection. Soils from Japan, Hawaii, Wake Island, and Anchorage, could not be examined for several weeks after collection; such soils which appeared moist were dried by spreading in cardboard containers since moisture retained in soils may have a profound effect in altering the microbial population. These alterations are kept to a minimum when the soil is dried (Alexander, 1971).

Several guidelines were followed when selecting a specific site for soil collections within an area. Some soil samples were taken from cultivated fields and from rich garden soils, since Gibson was able to isolate a number of strains from these soil types. He found that cultivated field soils were a particularly good source for these organisms and reported that about fifty percent of the colonies of S. ureae which developed on plates came from this soil type.

One of the conditions for good growth of this organism in laboratory cultures is alkalinity of the medium. MacDonald (1960), found the pH range for growth is quite wide, between pH 7 and 9.5,

* I am grateful to the Department of Agriculture for permitting the importation of the soil samples.

with the optimum range between pH 8.2 and 8.6. Gibson (1935), was not able to detect any strains of S. ureae from strongly acidic soils of pH 4 to 5.5 which were rich in organic matter but he did obtain isolates from soil where the pH was between 5.9 and 7.3. Accordingly, sites noted for their acidity or alkalinity were included.

Because of the special tolerance of these organisms to urea, samples were taken from sites which were suspected of containing considerable urea--zoos, corrals, and stables. In addition, soils were sampled from areas where domestic animals graze. These included pasture lands occupied by cattle, swine, and sheep. Other sites thought to contain urea because of their use as urination places by dogs, were selected for sampling. For example, soils were collected under park trees or shrubs, (especially in areas bounding dog training centers), beneath shrubs, trees, or light standards along parkways, under trees lining sidewalks in densely populated residential areas, and in yards where dogs are kept. As I shall show, sites presumed or known to be visited by dogs were the most reliable isolation soils.

Collections were also made from unpopulated desert and from mountainous regions for comparison.

Certain practical considerations determined the type of soil samples collected from the various geographical areas--the time available for soil sampling, the accessibility of different soil types, and the population density of areas selected for sampling. Collections from Anchorage, Alaska and Wake Island were of necessity made "on the run." In each case, soil samplings were

taken from within a two to three block radius of the airline terminal. These areas were considered "disturbed" because of the flow of people within a small area, although no dogs were seen, an important consideration as I discuss later.

A variety of soil types were collected from Oahu because the island has densely populated areas as well as accessible sites where soils range from desert type to subtropical and where the pH of such soils is known (U.S. Dept. of Agriculture, Soil Survey Series, 1955). The windward side of the Nuuanu Pali mountain contains less organic matter, in general, than the leeward side of the island near Diamond Head and Kokohead. In addition, the soil at the base of the Nuuanu Pali mountain is considered quite acidic between pH of 4 - 5.5, becoming increasingly more alkaline as one proceeds to the crest, the pH here ranging from 6.5 - 7.3. There is a concomittant change in soil color from brick red to brown, with increased elevation. Soil collections were made from the base of the Pali to the crest, at sites inaccessible by car and free of debris, people, or pets. The soil around Diamond Head and Kokohead, which is at the leeward side of Oahu, is considered rich in organic matter and is considerably more alkaline; the pH ranges between 7 - 8.3. This area lacks the rainfall of the Pali region, consequently the soil and vegetation are desert-like. Soil collections here were also taken from sparsely populated areas. The rest of the soil samplings from Oahu focused on sites presumed to contain urea. These were sites where people and pets congregate, as at parks, beaches, and in the residential areas of Honolulu and its neighboring communities. It should be pointed out that some of the collections

made in these populated areas were from communities where acidic or alkaline soil conditions may also prevail.

No attempt was made to collect soils from "undisturbed" areas in Japan since such areas were not easily accessible in the time available. Soil samples from Japan were collected from parks, schools, shopping centers, and residential areas of Tokyo and its environs. There is a surprisingly large dog population in Tokyo itself despite the scarcity of yard space and the extremely crowded living conditions. Soil was collected from these residential areas, as in yards, along fences, trees, walls and alleys where, it should also be noted, there is no censure nor proscription against men also relieving themselves.

Soil collections from San Diego were limited to the San Diego Zoo and to a small public park on Coronado Island. This park had one feature not encountered at other parks. It provided a drinking fountain for dogs. Several soil samples were collected from this area, where a number of dogs had congregated, as well as from other regions of the park. The soils sampled from the zoo of San Diego* included collections from the following animal compounds: okapi, kangaroo, snake cage (King Tut), llama, monkey, guanaco, horned and hoofed animals, elephant, wallaroo, antelope, rhinoceros, bison, and tapir.

There was a greater diversity in soil types collected in the Los Angeles area than in any other region; these included soil samples from corrals, ranches, stables and grazing lands (sheep, cow, and swine), in suburban Los Angeles, as well as from cultivated fields,

* I am grateful to the zoo director of the San Diego Zoo for allowing us access to the animal compounds.

TABLE 1

The pH and urea content of selected soil samples. Soils 118 and 129 are from bison and camel compounds, respectively.

Soil sample	*S. ureae*	pH	% urea
118	-	9.52	0.0023
129	-	7.78	0.0015
P5	+	7.24	0.0013
P6-A	+	5.70	0.0018
P3	+	6.62	0.0018
P2	+	6.27	0.0046

parks and beaches of Los Angeles and Ventura Counties. Soil samples were also collected from the Los Angeles Zoo at Griffith Park.* Soils were sampled from the following animal compounds: wild horse, tapir, tarpin, bison†, antelope, elephant, onager, Tulli Elk, deer, Indian Rhinoceros, White Rhinoceros, Black Rhinoceros, llama, guanaco, giraffe, and camel†.

Because one strain of this organism had previously been isolated from sea water, collections were also made from surface water along the shore of Southern California and off the pier at Malibu, California. Collections also included samples from salt water aquaria, which contained a variety of arthropods and gastropods.

* I thank the zoo director of the Los Angeles Zoo for allowing us access to the animal compounds.

† These soils were investigated further for pH and urea content (Table 1).

5. RESULTS

The effect of pH, temperature of incubation and urea content on recovery and isolation of S. ureae from soil were defined by plating one freshly collected soil sample (known to contain the organism) under a variety of conditions. These results are shown in Tables 2 and 3.

The data in Table 2 show that TSY at pH 7 without urea was useful only at the 10^{-4} dilution where background was greatly reduced. At lower dilutions the plates became overgrown within two days. Since it is known from plating numerous soil samples that S. ureae is not often found at the 10^{-4} dilution but is easily recognizable at lower dilutions, it is likely that this organism will be missed when plated on this medium. A superficial examination of the microbial population on TSY at pH 7 indicates that the population growing on this medium appears to be quite different (in pigmentation and colony morphology), from populations found on Alkaline TSY with or without urea.

The recovery of S. ureae on alkaline TSY without added urea, though satisfactory, was not as good as in the presence of urea. The background growth of extraneous colonies was no greater on the alkaline TSY medium than on alkaline TSY-urea, but the colonies were much larger and the plates became crowded after two days growth. This made recovery difficult especially at the lower dilutions.

Although S. ureae could be recovered at urea concentrations above 2.0%, growth was increasingly inhibited as the urea content

TABLE 2

The effect of medium composition on recovery of *S. ureae* from soils.

Dilution	TSY										yeast extract	NA*	NA yeast extract
	urea concentration (%)												
	0	0.5		1.0		2.0		3.0	5.0	10.0	1.0	1.0	1.0
	pH	pH		pH		pH		pH	pH	pH	pH	pH	pH
	7	7	8.4	7	8.4	7	8.4	7	7	7	8.0	8.4	8.4
partly streaked 10^{-2}	0,+,0	+,0,+	+,+,0	+,+,0	+,+,+	+,+,0	+,0,0	0,+,0	0,0,0	0,0,0	0,0	0,0,0	0,0,0
10^{-2}	plates over-grown	0,3,3	0,3,5	0,3,4	1,3,5	0,0,3	0,3,4	0,1,1	0,1,2	0,0,0	0,0,1	0,0,0	0,0,1
10^{-3}	0,0,0	0,1,2	0,2,2	0,0,2	1,1,2	0,1,2	0,1,1	0,0,1	0,0,1	0,0,0	0,0,0	0,0,0	0,0,2
10^{-4}	0,2,1	0,0,1	0,0,1	0,0,1	0,1,1	0,0,1	0,0,1	0,0,0	0,0,0	0,0,0		0,0,0	0,1,0
plate count (av.) 10^{-4}	300	182	171	167	158	184	139	104	93	62		194	157

TABLE 3

The effect of environmental factors on recovery of S. ureae from soil on TSY.

Triplicate plates were prepared at each dilution. Plates were examined on the third and fifth day. Numbers indicate the total number of colonies of Sporosarcina which were identified and recovered by the fifth day from each plate. + indicates that at least one colony of Sporosarcina was present on a plate.

TABLE 3

The effect of environmental factors on recovery of S. ureae from soil on TSY.

	urea concentration (%)							
	0				1.0			
	pH				pH			
	7		8.4		7		8.4	
Dilution	22°C	30°C	22°C	30°C	22°C	30°C	22°C	30°C
partly streaked								
10^{-1}	0,0,+	0,0,0	0,+,+	plates overgrown	+,+,+	0,0,1	+,+,+	0,0,1
10^{-2}	plates overgrown		0,1,2	plates overgrown	1,2,4	0,1,1	1,3,5	0,0,0
10^{-3}	0,0,2	0,0,0	0,-,2	plates overgrown	0,2,5	0,1,1	1,2,3	0,0,1
10^{-4}	0,0,1	0,0,0	0,0,0	0,0,1	0,0,1	0,0,1	0,0,0	0,0,0

increased and, more important, S. ureae showed a loss in pigmentation which made identification difficult.

Both alkaline and neutral TSY containing 1.0% urea gave satisfactory recovery of S. ureae at all the dilutions (Table 2). The alkaline medium, however, is more favorable for isolations because it tends to be more inhibitory of other types of organisms, despite the fact that it darkens TSY and makes it more difficult to identify Sporosarcina, especially the non pigmented strains. An alkaline environment appears to inhibit certain troublesome Bacillus species, particularly those whose colony morphology resembles that of Bacillus mycoides. The rhizoidal growth of this organism enveloped several plates of TSY-urea at pH 7 within 2 days, but was not a serious problem on alkaline plates. At a neutral pH, it is possible to recognize and recover S. ureae by the second or third day, but the increased background growth in certain cases, makes this medium unsuitable as a general isolation medium.

The comparative results from one soil sample indicate that the urea concentration has a significant effect in the isolation of this organism, although S. ureae can grow over a wide range of concentrations. The most favorable range for its isolation is at urea concentrations between 0.5 and 2.0%. Concentrations of urea above 3.0% are generally not favorable for its isolation. A superficial examination in this case did not reveal the presence of this organism on plates containing urea concentrations of 10%, but in the past careful perusal of similar plates from other soil samples has revealed them. In general, though, it is difficult to distinguish the differential culture characteristics of this organism

from background growth, because as noted previously, there is a loss of pigmentation of all cells and an inhibition of growth at this urea concentration. Some strains of S. ureae cannot tolerate 10% urea concentrations (see Diagnostic tests, Table 13, Part II), and it is possible that the organism isolated from this soil sample is such a strain.

There appears to be more certainty of finding this organism on evenly spread TSY plates at the low dilution (10^{-2}) than at higher dilutions, particularly at urea concentrations below 3.0%. Similar results were obtained with other soil samples.

The isolation of S. ureae on alkaline-urea yeast extract and on alkaline-urea nutrient agar was not satisfactory. The addition of yeast extract to the nutrient agar medium allowed somewhat better growth, but even at high dilutions, growth was not as good as on TSY-urea. Furthermore, the dark background of the nutrient agar medium made identification of S. ureae difficult.

The incubation temperature of 22° C is far more satisfactory for recovery of S. ureae than is 30° C (Table 3), an observation first noted by Gibson, (1935). Although the optimum temperature for growth of pure cultures of S. ureae is near 30° C (MacDonald, 1960), in mixed cultures the higher temperature appears to favor the growth of a number of other species which quickly swamp the plates at all but at the highest dilutions.

While it appears then, that this organism can be recovered from TSY in the absence of urea or in its presence at concentrations between 0.5% and 5.0%, on both neutral and alkaline pH, factors

such as competition from certain Bacillus species at neutral pH and inhibition of growth of this organism at urea concentrations above 2.0%, make alkaline TSY at comparatively low urea concentrations a more favorable medium for recovery of this organism than others tested. Although the addition of urea to TSY may inhibit growth of some cultures of S. ureae even at concentrations as low as 0.3% (see results with pure cultures), its presence was necessary to inhibit background for isolations.

The various modifications of TSY described in Table 2 were also plated with five pure cultures of Sporosarcina, and growth of these cultures was compared by measurement of relative colony size over a seven day period. In every case, growth was best at pH near neutrality without the presence of urea, and the addition of urea above 3.0% diminished growth considerably. Increasing the pH to 8 or 8.4 gave only slightly better growth than at neutrality, and the addition of 1.0% urea to TSY under alkaline conditions depressed growth of each culture further, though growth was still good. It thus appears that there is no particular advantage in increasing the pH or in adding urea when growing pure cultures on TSY.

When tryptic soy broth and yeast extract are reduced to one fifth their concentration, (TSY/5), pure cultures show only punctiform growth. Growth is considerably enhanced, however, as the pH is increased to 8 without the presence of urea or at pH 7 with the addition of 1.0% - 1.5% urea, but not when both urea and an alkaline environment are provided. Presumably, the buffering capacity

provided by the complex organic substances is considerably reduced by dilution in TSY/5, and the combined effect of proteolysis of urea by these organisms in an environment which is already alkaline initially creates an unfavorably high alkaline environment. Colonies remain punctiform under these conditions and do not show pigmentation. As one would expect, the addition of either tryptic soy broth (1.2%) or yeast extract (0.4%) to TSY/5 greatly enhances growth; these added nutrients not only increase the buffering capacity but also increases the carbon and energy supply. The growth of pure cultures on TSY/5 with 1.0% urea or at alkaline pH was not sufficiently good to warrant investigating this medium further as a possible enrichment for Sporosarcina.

One of the unusual properties of S. ureae determined from the nutritional study is the inability of this organism to utilize glucose. It has been known from published literature that S. ureae cannot ferment glucose. Gibson, (1935), MacDonald and MacDonald, (1962), and Kocur and Martinec, (1963), noted that no acid or gas is produced from glucose and other sugar substrates. This organism however, is a strict aerobe, and thus is not able to catabolize anerobically. Glucose had been routinely included as an energy source for the isolation and maintenance of the isolates, and it is not known what effect the inclusion of glucose may have had on the mixed culture population, and consequently on the isolation of S. ureae. One soil sample used in the original isolations was replated on the isolation medium in the presence and in the absence of glucose. S. ureae was found on both the glucose and non-glucose

plates but because the relative numbers had changed in the interim, these results could not be compared with the original results. A more valid appraisal would be to follow the same procedure with fresh soil samples known to contain S. ureae.

The most probable number of organisms present in samples was determined from each of 24 soil samples by using statistical methods (Halvorson and Ziegler, 1933). By plating measured dilutions of 24 soils, it was calculated that between 3,000 and 66,000 cells of S. ureae were present per gram of soil. This estimate is in agreement with the results obtained by Gibson, (1935). He calculated that between 10,000 and 20,000 cells of this organism were present in the soils he examined.

There is some evidence to indicate that soils which did not reveal S. ureae by the isolation methods used here did, in fact, contain cells of this organism. On one occasion, a broad swipe from densely populated area on a ten fold dilution-plate of soil from the bison compound, (Los Angeles Zoo), did reveal, on microscopic examination, several motile sarcinae. All attempts at subculturing these cocci or in the replating of the bison soil on the isolation medium failed. It is possible that S. ureae is present in some soils at such low concentration that it cannot be recovered by the methods used for its isolation. At the lowest dilution, the few cells of this organism may be swamped by the multitude of fast growing mixed colonies present on these plates.

Although TSY-urea has been a satisfactory medium for isolating S. ureae, neither this medium nor several environmental

variations of this medium is a specific enrichment for this organism. Both neutral or alkaline TSY-urea agar supports a very large and diverse microbial population and many members of this population show a much faster growth rate than S. ureae over a wide range of urea concentrations especially at the lower dilutions where there is considerable competition for substrates. There is a clear need for an elective enrichment that would discourage much of the background growth that occurs at the low dilutions to allow for recovery of this organism from soils where it may be present at very low concentrations.

The results from the soil collections (Table 4, and Fig. 1), clearly show that the overwhelming majority of isolates came from disturbed areas. The term "disturbed" is used to designate those urban areas heavily populated with people and especially with dogs,* as opposed to undisturbed areas such as deserts, mountains, and grasslands. Thirty-nine of 41 soils which contained at least one strain of this organism came from these disturbed areas. Two areas supplied approximately one-third of all soils found to be positive for S. ureae. These sites were dog training centers (which includes one soil sample from grounds of a dog hospital), and yards where dogs are kept. Where the ratio of the available soil to concrete area is small, as along sidewalks, stairs, in shopping centers, or in business districts which often adjoin residential areas, 16 soil samples from 36 collected, or about 44% from this catagory, contained at least one strain of this organism. Park interiors or park-like areas yielded 8 positive soils from 34 collected, (or 23% positives).

* Cats are also a common household pet but their contribution to disturbed soils described is less certain than that of dogs. Nevertheless, they should not be ignored as a potential source of urea in soils, if the presence of urea is important.

TABLE 4

The distribution of Sporosarcina ureae in soils.

The numbers in the table refer to total number of different samples, the results as indicated. + = number of soil samples which yielded at least one strain of S. ureae. - = number of soil samples in which no strains of this organism could be demonstrated.

*. Multiple strains were isolated from some soil samples. In such cases, each additional strain isolated from one soil sample is indicated by *.

†. One positive soil sample, isolated from grounds adjoining a dog hospital (Tokyo University, Tokyo, Japan) was included in this catagory.

TABLE 4

The distribution of Sporosarcina ureae in soils.

Type of Collection areas	Results	Los Angeles	San Diego	Berkeley (Yosemite)	New Orleans	Boston	Anchorage, Alaska	Japan	Hawaii	Wake Island
Dog training area †	+	6						1		
	−	4								
Dogs in yard	+							5**	1	
	−	1						2	1	
Public site	+	1*	2***	2	1	4		5*	1	
	−	1		1	2		2	3	8	3
Park interior	+		1	5***		1		1		
	−	17	1		2			3	3	
Beach	+	2								
	−	2							2	1
Zoo	+									
	−	18	10							
Corral, stable, pasture	+									
	−	13								
Garden	+	2								
	−	17							1	
Cultivated field	+									
	−	6							9	
Desert	+									
	−	7								
Acid or alkaline soil	+									
	−	6								
Salt water	+									
	−	11								
Total	+	11*	3***	7***	1	5		12***	2	
	−	103	11	1	4		2	8	24	4

FIGURE 1

Distribution of S. ureae in soils.

Graphical representation of the data of Table 4. Numbers above bars represent percentage of samples from that area giving positive results.

FIGURE 1

Distribution of S. ureae in soils.

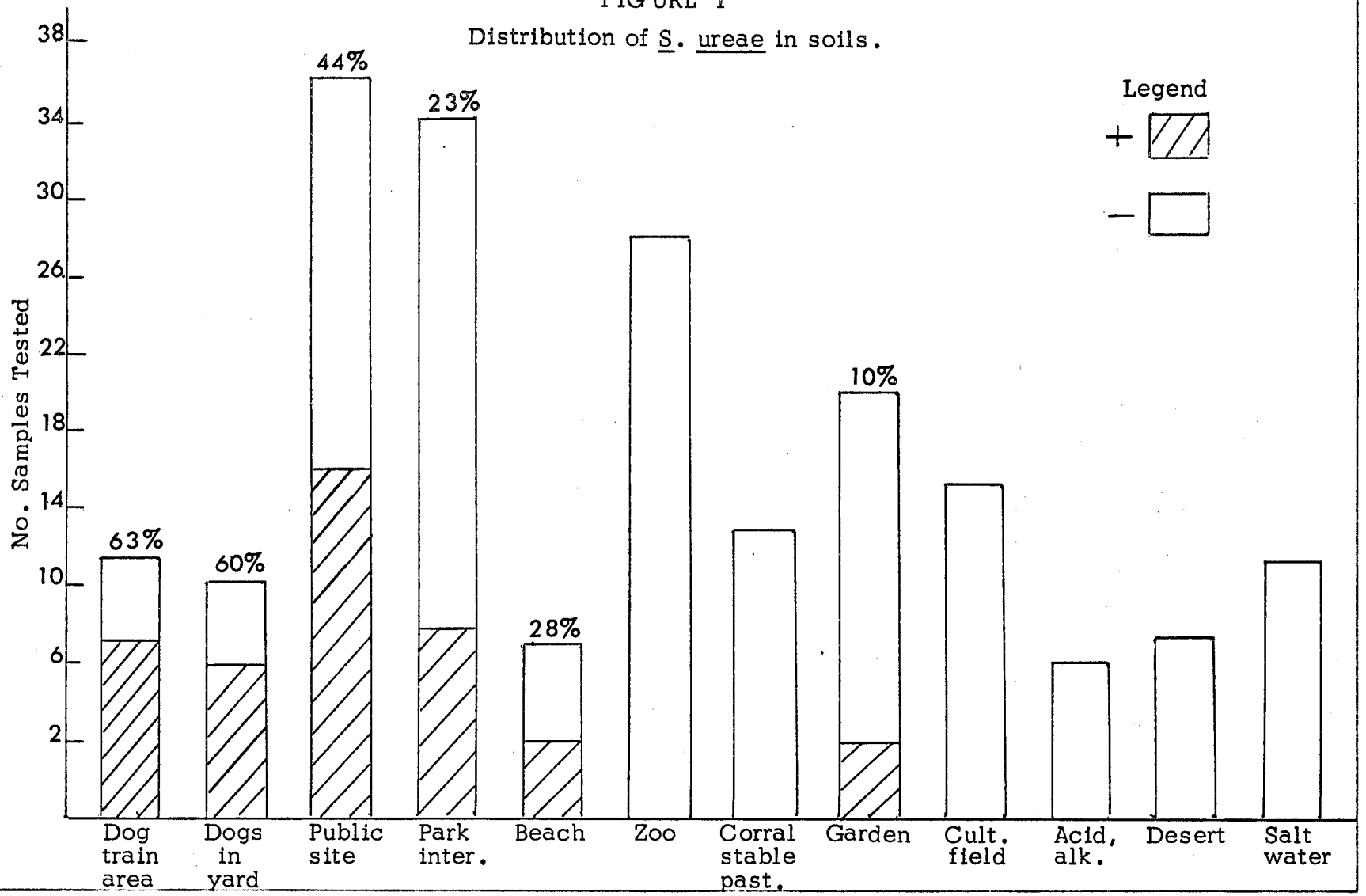

TABLE 5

Sources of Strains Studied

Isolates of Sporosarcina Collected From Soils

Strain	Collection site
P 1	Base of eucalyptus tree at Shadow Ranch Park, a suburban public park in Canoga Park, Calif.
P 2	Base of eucalyptus tree, near Vanowen St., Shadow Ranch Park, Canoga Park, Calif.
P 3	Base of eucalyptus tree near dog training area at Reseda Park, a suburban public park at Reseda, Calif.
P 4	Base of tree near dog training area, Reseda Park, Reseda, Calif.
P 5	Base of magnolia tree in front of Court House, just visited by standard poodle, French Quarter, New Orleans, La.
P 6-A	Base of sycamore tree near dog training center, Griffith Park, Los Angeles, Calif.
P 6-B	Base of tree near dog training center, Griffith Park, Los Angeles, Calif.
P 7	Rich garden soil from base of potted plant, private residence; owners do not keep dogs or cats. Woodland Hills, Calif.
P 8	Base of tree adjacent to curb near tennis courts and dog training center, Griffith Park, Los Angeles, Calif.
P 9	Base of lifeguard tower (sandy soil), a popular surfing area at Malibu, Calif.
P 10	Base of telephone pole adjacent to curb outside entrance to surfing area at Malibu, Calif.
P 11	Base of palm tree adjacent to curb, Coronado Park, a suburban public park on Coronado Island, San Diego, Calif.

TABLE 5 (cont.)

Strain	Collection site
P 12	Base of pine tree, Collie dog tied to tree near dog drinking fountain, Coronado Park, Coronado Island, San Diego, Calif.
P 13	Base of tree near dog drinking fountain, Coronado Park, Coronado Island, San Diego, Calif.
P 14	Base of pine tree, populated campsite, Toulumne Meadows, High Sierras, Calif.
P 15	Base of rose bush, near urine-stained front walk of private residence, Woodland Hills, Calif.
P 16-A P 16-B	Base of oak tree, park-like setting near Strawberry Creek across from LSB Bldg., Univ. of Calif. at Berkeley.
P 17-A P 17-B	Base of tree near Student Union Bldg., Univ. of Calif. at Berkeley.
P 18-A P 18-B	Base of steps leading to terrace of Campanille, Univ. of Calif. at Berkeley.
P 19	Base of eucalyptus tree in Eucalyptus Grove, Univ. of Calif. at Berkeley.
P 20-A P 20-B	Base of oak tree near north entrance to LSB Bldg., Univ. of Calif. at Berkeley.
P 21-A P 21-B P 21-C	Base of tree in front of Student Union Bldg. near Sather Gate, Univ. of Calif. at Berkeley.
P 22	Dog kennel area, private residence, Akasaka District, Tokyo, Japan.
P 23	Accumulated dirt at head of stairs leading from business district to residential area, Akasaka District, Tokyo, Japan.
P 24	Grassy area along curb adjacent to golf driving range, Akasaka District, Tokyo, Japan.
P 25	Accumulated dirt at foot of stairs near Artist's Center Bldg., Akasaka District, Tokyo, Japan.
P 26-A P 26-B	Dirt walkway in business district, Akasaka District, Tokyo, Japan.

TABLE 5 (cont.)

Strain	Collection site
P 27	Kennel area of dog hospital on campus of Tokyo University, Tokyo, Japan.
P 28-A P 28-B	Backyard of private residence where dog is kept, Yokahama, Japan.
P 29	Dirt alley near Maruyama Cho shopping area, Yokahama, Japan.
P 30	Front yard where dog is kept, Kumomi, Japan.
P 31	Front yard where neighbor's dogs are kept, Kumomi, Japan.
P 32-A P 32-B	Public walkway near shopping center, Kumomi, Japan.
P 33	Soil near side wall; man urinating in Hibya Park, Tokyo, Japan.
P 34	Base of shrub near apt., Ala Wai St., Waikiki, Hawaii.
P 35	Base of shrub at back entrance to private residence; dog is kept in back yard, Honolulu, Hawaii.
P 36	Base of tree at top of Beacon Hill, Boston, Mass.
P 37	Base of single tree in sidewalk along Rollins Rd. Evidence of many dogs, Boston, Mass.
P 38	Base of elm tree at the Commons across from Park Church, near subway station entrance. Numerous dogs, Boston, Mass.
P 39	Base of tree in parking lot, Beacon Hill, Boston, Mass.
P 40	Base of single tree in sidewalk along Charles St. Evidence of many dogs. Boston, Mass.
* C 1	Soil, Ithaca, New York
* C 2	Soil, Edinburgh, Scotland
* 204	A. Pijper, Pretoria, South Africa
* 380	Dept. Microbiol., Techn. High School, Delft.

TABLE 5 (cont.)

Strain	Collection site
* 634	J. Evans, Amer. Meat Inst. Found., Chicago
* 684	?
* 752(286)	W. C. Haynes, NRRL,† Peoria, Ill.
* 858(L.E.I.I)	C. B. van Niel, Hopkins Marine Sta., Pacific Grove, Calif.
* 860(L.E.I.I)	C. B. van Niel, Hopkins Marine Sta., Pacific Grove, Calif.
* 981(8691)	Natl. Coll. Indust. Bacteria, Torrey Res, Sta. Aberdeen, Scotland.
* S 1	Soil, Scotland

* These cultures and one soil sample (S 1), were received through the courtesy of Dr. R. E. MacDonald, Division of Bacteriology, College of Agriculture, Cornell University, Ithaca, New York. Strain numbers in parentheses are those of original isolates.

† NRRL, Northern Utilization Research and Development Division, U. S. Department of Agriculture.

From populated beach areas where dogs were also present, 2 samples were positive out of 7 collected. On the other hand, only two strains were found from 19 garden soils and none were obtained from 6 cultivated field soils. The latter results are surprising, since Beijerinck (1901) and Gibson obtained isolates from these sources. Gibson* recently suggested that the soils he successfully sampled (1935) were possibly either pasture soils, or soils which had acquired liberal applications of farm manure. He also noted that a strain was recently isolated from sheep and cattle grazing lands in Scotland. Unaccountably though, in this study, no isolates were found from sheep and cattle grazing areas in the San Fernando Valley, nor, for that matter, were strains found from animal sources other than (apparently) from dogs. Soils from stables or corrals yielded none, nor were any strains isolated from zoo compounds.

* personal communication

6. DISCUSSION

Although Sporosarcina ureae appears to be associated with urea soils because of its presence in places where dogs urinate, the specific role urea plays in the ecology of this organism is not certain. Results of the nutritional study (Part II), indicate that none of the 61 isolates can use urea as a carbon or energy source. However, 60 of these isolates can synthesize urease and so can catalyze the hydrolysis of urea to ammonia and carbon dioxide. As noted previously while alkalinity is not a necessary requisite for good growth on TSY, an alkaline medium appears to enhance growth when nutritional requirements are kept minimal. Due to competitive pressures in soils, growth substrates are most likely minimal. Moreover, S. ureae may have a selective advantage, not only in tolerating certain urea concentrations at elevated pH but also in utilizing the ammonia produced from hydrolysis as a nitrogen source, hence S. ureae gains a competitive advantage by maintaining an alkaline environment favorable for their growth through hydrolysis of urea to ammonia.

An attempt was made to correlate the distribution of S. ureae with environmental factors including the pH and urea content (Table 1). The pH of soil from the bison compound was determined to be 9.5 which is outside the range for growth of S. ureae when cultivated under laboratory conditions (Gibson, 1935; MacDonald, 1960). This high pH may account for the absence of this organism from bison soil but does not explain the presence of isolates P6-A, P3 and P2 from soils where, in each case, the pH is below 6.8

(Table 1). MacDonald (1960), found that pure laboratory cultures cannot tolerate a pH below this level. The apparent discrepencies in these findings point up to a major difficulty encountered when attempting to equate the macroenvironment with the microenvironment. Soils are known for their spatial heterogeneities and multitude of microenvironments. Regions barely microns apart may differ greatly in abiotic factors such as oxygen tension, carbon dioxide, kinds and amounts of nutrients and pH. The slurry method used for measuring the pH of soils must perforce wash out microenvironmental differences existing within the soil matrix, (see Methods and Materials, Part I). Even if the pH of the microhabitats were known, other abiotic factors would also need to be known before a reasonable appraisal of the limiting factors could be made.

When this study was undertaken, the first soils sampled were from areas considered high in urea content, namely from zoos, corrals, and stables. These sites were selected because Beijerinck (1901) and Gibson (1935) succeeded in obtaining isolates by using an enriched medium containing 10% urea. It therefore seems puzzling that not a single soil from 28 zoo samples or from 13 corral and stable sites revealed S. ureae. At Griffith Park Zoo, where most of the samples were collected, the soil is decomposed granite which is replenished continuously because of erosion. The compounds are maintained by removal of fecal matter through daily raking, and disinfectant is applied when concrete feeding areas are washed down occasionally. It is not known whether the

disinfectant is washed onto soil surfaces. It is possible that concentrations of fecal matter are inhibitory to the growth of this organism since no isolates were found in any of the manured areas.

None of the six soils examined showed a urea content above 0.004% (Table 1). It was surprising to find the zoo soils so low in urea concentration--a major reason for selecting such soils was the belief that their urea content would probably be rich. The low concentration of urea may reflect the rapid conversion to ammonia and then to other products since soils contain a number of actively ureolytic organism, but it should also reflect in a higher pH. As with the measurement of pH, the data reflects the macroenvironment not the microenvironment, for the method of calculating the urea content is indirect and crude. (see Methods and Materials, Part I).

It is difficult to understand why this organism was found only in association with dogs and man and not with domestic animals found on grazing lands. Such soils appeared to be lightly manured when compared to soils from zoos, stables, and corrals. Since only five soil samples were collected from pastures, it would be profitable to undertake a larger survey of this soil type.

In conclusion, the results from this study clearly showed that the primary habitat of this organism is associated with certain disturbed soils. This study did not reveal the specific conditions necessary for growth within the disturbed areas, nor is there any clear cut evidence that one geographical region is more favorable than another. The results did show that within these disturbed

regions, Sporosarcina ureae is found in many parts of the world.

This organism was not found in soils obtained from deserts, cultivated fields, mountainous regions, relatively unpopulated alkaline or acidic soils, zoos, corrals, stables, or grazing lands. By the methods used for isolations, no strains of S. ureae were obtained from the salt water sources examined.

Whatever the microhabitat of these organisms has been in their evolutionary history, their primary habitat now appears to be concentrated in certain urban soils in close association with the activities of man and of dogs.

Part II

The Nutrition and Physiology of *Sporosarcina ureae*.

7. METHODS AND MATERIALS

I. Organisms

Fifty-one strains of Sporosarcina ureae isolated in this study (Table 6, Part I) and ten pure laboratory cultures from other sources (Table 6, Part I), were used for the nutritional study.

II. Screening for Nutritional Requirements

The following nutritional requirements were examined:

a. Sources of carbon and energy. Forty-six carbon compounds were tested as the sole source of carbon and energy.

b. Essential growth factors. Vitamins and several amino acids were tested as essential growth factors.

c. Sources of nitrogen. The ammonium and nitrate ions, several amino acids, and several nitrogenous compounds were tested as the sole source of nitrogen.

The usual salts and known traces of elements were added to a synthetic medium (Part III) but the effects of these ionic compounds as nutrients for S. ureae were not investigated. No growth occurred on unsupplemented synthetic medium, therefore the criterion for nutritional requirements was simply the ability to support growth. Growth was not necessarily optimal in the screening media but was always sufficient to indicate the effect of omitting any component(s) from a specific medium.

Before beginning the determinations, it was necessary to check for purity and revitalize each strain. Each culture was streaked on TSY agar, and after rejuvenation, was restreaked on the same medium

for 1-2 successive days prior to being tested on a defined medium.

Cultures were between 16-24 hours old when transferred to the defined medium. Unless stated otherwise, cultures were incubated at 30°C. Extra precautions were taken to avoid contamination by traces of unwanted nutrients (Hutner, Cury, and Baker, 1958), especially when testing vitamins. All glassware was cleaned with laboratory detergent, then rinsed thoroughly in tap water and deionized water. Each test series was run in duplicate or triplicate on the same test media as well as on additional batches of freshly made media. This procedure was helpful as a check against errors made in media preparation. When results were contradictory, tests were repeated several times. Each test series included controls with and without the nutrient to be tested. TSY also served as a control for replica plating (see screening for nutritional requirements with replica plating).

A basal synthetic medium was developed for all nutritional tests. This medium was autoclaved separately at double strength and mixed aseptically with solutions of the metabolites to be tested to bring the medium to normal concentration. When a solidified medium was desired, Bacto agar was prepared at 3.0% (unless stated otherwise), autoclaved, and mixed aseptically with an equal volume of double strength basal medium. Such procedure avoids browning of the agar, and avoids breakdown products, as well as facilitating easier visual inspection of growth (Stanier, Palleroni, and Doudoroff, 1966).

For replica plating, strains were hand inoculated in patches

with a sterile needle onto a TSY master plate following their successive subcultivation on TSY plates. Sufficient inoculum was transferred to give pin point colonies of cells even before growth (Shifrine, Phaff, and Demain, 1954). After 15-18 hours growth, the master plate was inverted and pressed against sterile velveteen fabric which was then used as an inoculum for test media. The order of replication was as follows: synthetic non nutrient plate, which usually contained only inorganic salts and served to remove excess inoculum, the blank, which was the control plate without the growth factor in question but which contained other required growth supplements, 6 or 7 plates, each supplemented with a different growth factor to be tested, and a terminal control plate on TSY which determined whether sufficient growth had been transferred to the velveteen to inoculate the entire series.

For successful replication of plates some procedures are essential to avoid false negative results or inconclusive results. The number of replications was limited to no more than 10 plates, since smearing of the colonies usually became pronounced after this number, complicating reading of the results. Also for this reason, and to avoid any possible interaction between colonies, the number of patches on the master plate was limited to either 16 or 17. To avoid early substrate exhaustion, the plates were furnished with a large volume of medium (Stanier, Palleroni, and Doudoroff, 1966). It is important that plates are well dried for successful replica plating; thus, freshly made plates were dried by incubating them for several days at 30°C or at room temperature,

and then stored for up to 3 weeks. When the agar concentration was reduced below 1.2%, plates were used within a week, for the solid media had a tendency to crack if stored longer than 8-10 days.

Each carbon compound tested as a growth substrate was added to a semi-defined medium at a concentration of 0.2% except in cases of propionate, succinate, and phenol. These compounds were added at only 0.1%, since they appeared to be inhibitory at higher concentrations. The following substrates were tested; (in the case of the acidic substances, the sodium salts were used):

a. sugars: D-ribose, D-xylose, D-arabinose, L-rhamnose, D-glucose, D-mannose, D-galactose, D-fructose, maltose, lactose, sucrose, cellobiose, trehalose, melibiose, raffinose, melezitose.

b. organic acids: acetate, propionate, butyrate, lactate, succinate, citrate, D and L-tartarate, malate, pyruvate.

c. Alcohols: ethanol, n-propanol, isopropanol, n-butanol, isobutanol.

d. polyalcohols and glycols: erythritol, mannitol, sorbitol, meso-inositol, adonitol, glycerol, ethylene glycol.

e. cyclic compounds: benzoate, phenol, mandelate.

f. nitrogenous compounds: putrescine, betaine.

g. amino acids: L-glutamate, L-asparate, L-asparagine.

The vitamins were divided into 3 major groups and nutritional requirements studied by the deletion method. Several vitamins were investigated further as potential growth factors. The

vitamins tested and their initial groupings were as follows:

	Final concentration in medium: (μg./ml.)
Group I.	
niacin	1.0
thiamine · HCl	1.0
riboflavin	0.1
Group II.	
para-aminobenzoate	0.05
calcium pantothenate	1.0
folic acid	0.01
Group III.	
biotin	0.002
vitamin B_{12}	0.5
pyridoxal · HCl	1.0

Because of the likelihood of the presence of vitamins as contaminants in agar (especially, the ubiquitous and highly potent vitamin, biotin) replica plating was used only as a tentative determination of vitamin requirements. Instead, reliance was placed on the method of serial subcultivation of liquid cultures. The initial inoculum was suspended into a basal medium to give just visible turbidity (Knight and Proom, 1950). One drop of this cell suspension was added to a series of 25 ml. flasks containing vitamin mixtures in various combinations, and aerated by shaking. Strains which showed growth were serially subcultivated into the same vitamin medium approximately every 22-26 hours for at least 3 successive days to eliminate positive growth due to a carry-over of nutrient from the previous tubes. Biotin, especially, required several subcultures before growth of biotin-requiring strains ceased in a medium deficient in biotin, an observation noted similarly by Knight and Proom (1950).

The requirements for amino acids as growth factors were

tested by adding several amino acids to the supplemented synthetic medium. These metabolites which were found to be stimulatory in preliminary work were retested as possible growth factor requirements by means of replica plating and by serial subcultivation in liquid media. The liquid medium contained the following at final concentration of 200 µg/ml: L-glutamate, L-aspartate, L-asparagine, L-glutamate + L-aspartate, L-glutamate + L-asparagine. For replica plating, each amino acid was added at 40 µg./ml., and Bacto agar was added at 0.9%.

Nitrogen compounds were tested as a source of nitrogen on the synthetic medium (minus the ammonium salts), by replica plating. The following compounds were tested individually.

	Final concentration in medium: (g./l.)
L-glutamate	1.0
L-aspartate	1.0
L-glutamate + L-aspartate	1.0 (each)
sodium nitrate	1.0
betaine	1.0
putrescine	1.0
casamino acids	0.05
yeast extract	0.05

Scoring of plates for all nutritional requirements was visual. The inoculated plates were examined and the results tabulated periodically for up to 2 weeks. The utilization of a given compound was assumed to have occurred in replica plating when growth was clearly heavier in its presence than on the synthetic or semi-defined medium alone. In some cases, single colonies appeared very late. These were probably mutants and were scored separately (see Legend to Tables 6-11 for scoring).

The efficacy of a defined medium in supporting growth of liquid cultures was determined by turbidity measurements. Readings were taken with a Bausch and Lomb Spectronic 20 colorimeter at a wave length of 660 mμ.

III. Media

a. Maintenance medium

All strains of S. ureae were maintained as slant cultures on TSY. The maintenance procedure followed that described in Chapter 2, Part I. The composition of the TSY medium was as follows: (per liter of deionized water):

25.0 g Difco tryptic soy broth
4.0 g Difco yeast extract
15.0 g Bacto agar

b. Synthetic basal medium

A basal medium was prepared at ten fold strength and stored in the cold to be used as needed. The medium was buffered with 0.1M Tris (Tris(hydroxymethyl) amino methane). Sufficient 1N HCl was added to the salts to give a pH of 8.5 before autoclaving. $CaCl_2$ was autoclaved separately and added aseptically to the autoclaved basal medium. The composition of the synthetic medium was as follows: (per liter of deionized water):

12.11	g	Tris (Sigma)
0.2	g	K_2HPO_4
0.2	g	$MgSO_4 \cdot H_2O$
1.6	g	NH_4Cl
0.02	g	$CaCl_2$
1.0	ml.	trace element solution

c. Media for nutritional determination

1. Supplemented synthetic medium for vitamin assay.

For a preliminary investigation, the synthetic liquid

medium was supplemented with 0.1% Bacto casamino acids and 0.5% filter sterilized glucose. The vitamin assay was repeated for all the strains by replica plating, and for selected strains which had given contradictory results by serial subcultivation in liquid medium. The synthetic medium was supplemented with sodium acetate at 0.5% as the carbon and energy source (in place of casamino acids), and for replica plating, 0.9% purified agar was added. The vitamin compositions added singly or in various combinations, were as follows:

	Final concentration in medium: (µg./ml.)
biotin	0.002
niacin	1.0
thiamine·HCl	1.0

2. Medium for testing utilization of carbon compounds.

Preliminary investigation indicated that one to several vitamins are required for growth by some of the strains; these were therefore included in the synthetic medium. Glutamate and asparagine were also included because preliminary investigations indicated these amino acids may be required as growth factors or are stimulatory for a number of strains. The medium finally adopted contained (in addition to the components listed in paragraph IIIb):

	Final concentration in medium: (µg./ml.)
biotin	0.002
niacin	1.0
thiamine·HCl	1.0
L-glutamate	20.0
L-asparagine	20.0

A semi synthetic medium containing 0.01% yeast extract and 0.02% casamino acids was employed for strains which would

not grow in the medium described in the preceding paragraph. This low concentration of complex organic compounds supports discernible growth of these strains in the absence of any added carbon source. Substrate utilization could be qualitatively evaluated, however, for growth was distinctly heavier in the presence of a utilizable carbon and energy source.

3. Supplemented synthetic medium for amino acid assay.

The synthetic medium was supplemented with sodium acetate at 0.5% and with niacin, biotin, and thiamine in the concentrations described for the carbon-utilization experiments.

4. Medium for testing utilization of nitrogen compounds.

The synthetic medium (minus the ammonium salts), was supplemented with 0.5% sodium acetate as the carbon and energy source, and with various nitrogenous compounds as described previously.

IV. Diagnostic tests

a. Starch hydrolysis. Plates of starch agar (Manual of Microbiological Methods, 1957), were supplemented with 0.2% yeast extract and streaked. When colonies showed appreciable growth, sectors of the plate were flooded with Lugol's iodine solution. A clear zone beneath or around areas of growth provided evidence for extracellular amylase. Results were recorded at 2, 4, 7, and 10 days.

b. Fat hydrolysis. Plates of nutrient agar, prepared with tween 80 (Sierra, 1957), were supplemented with 0.2% yeast extract. The time of appearance of turbid halo surrounding growth was very

variable; strains were arbitrarily scored after not more than 8 days.

c. Plates of nutrient agar containing 0.4% gelatin were streaked (Smith, Gordon, and Clark, 1952). Sectors of the plates were flooded with acid mercuric chloride; a clear zone underneath and around the growth in contrast to the opaque precipitate provided evidence of proteolytic activity. Plates were read at 4, 6, and 8 days.

d. Nitrate reduction. Cultures were grown in nitrate broth (Difco), supplemented with yeast extract at 0.2% (Difco Manual, 1969). After 3 days growth, a few drops of sulfanilic acid and alpha naphthylamine reagents were added to one ml. of each broth culture. A red color indicates the presence of nitrites.

e. Urease production. Cell suspensions were tested for urease activity with the aid of urease test tablets (Key Scientific Products Co., Los Angeles, California). This test measures the change in pH due to the release of ammonia from urea.

f. Indole production. Cultures were grown in a Bacto-tryptone medium (Difco Manual, 1969), which was supplemented with 0.2% yeast extract, and tested with freshly prepared Kovac's reagent at 2, 4, and 7 days.

g. Catalase production. Cultures grown for 6 days on TSY agar were flooded with 3.0% hydrogen peroxide. Copious evolution of bubbles was evidence for the presence of catalase.

h. Voges-Proskauer test. Cultures were grown in the V.P. medium (Manual of Microbiological Methods, 1957), modified by the addition of 0.2% yeast extract and the substitution of pyruvate

for glucose as the carbon and energy source. To insure favorable aerobic conditions, wide test tubes (6x5/8"), were used, each containing 5 mls. of the medium. After 7 days growth, cultures were tested for acetoin production with KOH and creatine.

i. Ammonia production. Strains were grown in wide test tubes (6x5/8") in arginine broth (Niven, Smiley, and Sherman, 1942), modified by the omission of glucose and the addition of 0.2% yeast extract; the growth medium was adjusted to pH of 8.2 with 1N NaOH. Cultures were read at 2, 4, 5, 7, and 8 days by adding several drops of Nessler's solution to spot plates, and an equal amount of the growth culture.

j. Hydrogen sulfide production. Bacto-Lead Acetate agar medium (Difco Manual, 1969), was modified with the addition of 0.2% yeast extract. Plates were examined for evidence of browning in the vicinity of growth at 2, 4, 7, and 10 days.

k. Growth in 10% urea concentration. Because it was noted during isolations that some strains differed in their tolerance to urea, cultures were tested for tolerance to this compound on TSY plates, supplemented with 10% filter-sterilized urea. Plates were examined periodically up to 10 days for evidence and extent of growth.

l. Anerobic growth. Colonies which had been grown on TSY plates for 16 hours were hand patched to fresh TSY plates containing 0.5% yeast extract, and incubated for 14 days in an anerobic jar (BBL Gas Pak). In each test where yeast extract supplemented the test medium (other than the last 2 tests described), control plates or tubes were included, which contained the yeast extract but not

the substrate.

V. Manometry

Respiration of a selected strain which had shown positive growth on acetate, ribose, and fructose by replica plating, was measured by Warburg manometry (Umbreit, Burris, and Stauffer, 1964). This organism was grown on a series of synthetic agar media; the basal medium was supplemented with niacin, biotin, and thiamine (see Methods and Media, Part II), and with fructose, ribose, or acetate, each supplied at 0.2%. After growth for 4 days, cells were transferred to respective liquid media (having the same composition as the agar media), and incubated for 28 hours. Cells were then washed twice with 0.1M Tris buffer, and sedimented by centrifugation. Oxygen uptake of the cells was measured following the addition of 50 μ moles fructose, 50 μ moles ribose, or 100 μ-moles acetate.

VI. Determination of pigments

Pigmentation was determined by growing cultures on solid medium of TSY/5 supplemented with 0.5% sodium acetate. Although pigmentation was never as heavy on this medium as on TSY, the reduced concentration of tryptic soy broth and particularly of yeast extract, provided a much lighter background for observing subtle differences in pigment color. Furthermore, diffusible yellow pigments which darken TSY plates, were not a problem at the reduced concentration of TSY.

Ten strains which showed a representative spectrum of colors on solid media, were selected for further investigation. Cells were

harvested after growth in TSY/5 + 0.5% acetate, washed with 0.02M NaCl and sedimented by centrifugation. Various solvent mixtures of methanol, petroleum ether, acetone, and water were tested for the ability to remove pigment. The most successful was 90% methanol in water. Cells were suspended in 90% methanol and stored at 4° C for 4 hours, then re-sedimented. The absorption spectrum was determined on the methanol extract.

Saponification was necessary before any pigment could be partitioned into petroleum ether. Saponification was carried out by incubating extracted pigments for 10 minutes at room temperature with methanolic KOH; sufficient amount of 25% KOH in methanol was added to the extract to provide a final concentration of the alkali of 5% (Karrer and Jucker, 1950; Goodwin, 1965). The pigments were then extracted from the methanol phase by 2 successive extractions with a small quantity of petroleum ether (b.p. 30-60° C). A small amount of residual pigment always remained in the methanol phase; its identity was not determined but might represent chemically altered pigments, or non saponifiable pigments. The petroleum ether was washed free of the alkali and dried over anhydrous $NaSO_4$. To minimize cis-trans isomerizations and oxidation of the lipid material, illumination during the extraction procedure was kept minimal and the extracted material was kept in the dark (aluminum foil covered the tubes). The absorption spectra were determined with a Beckman DU Spectrophotometer.

VII. Morphology

Colony and cellular morphology were studied with cultures

grown on both solid and liquid media of TSY. For obtaining cellular measurements, cultures were inoculated into TSY liquid medium and incubated on a rotary platform shaker for 12-15 hours.

The spore medium (MacDonald and MacDonald, 1962) was used for obtaining spores for studying spore morphology. Cells were examined after 24 hours growth, and then periodically until all strains had formed fully mature spores.

8. RESULTS

On the basis of the nutritional tests, it is possible to group the strains according to growth factor requirements (Tables 6-11). Group I is represented by those organisms that grow on a minimal acetate medium without added growth factors. Group II contains the bulk of the strains tested, and revealed a spectrum of definable growth factor requirements. All strains of this group seem to require biotin, either singly or in combination with niacin and/or thiamine, and some strains have growth factor requirements for asparate. Group III consists of strains which have complex growth factor requirements or requirements which have not been determined. It was necessary to supplement the basal medium with low concentrations of yeast extract and casamino acids when investigating their nutrition. Group IV appears to be the most nutritionally fastidious strains of all those tested. Three of these organisms grew on basal medium only when it was supplemented with casamino acids or with glutamate as the carbon and energy source and one strain showed growth only on TSY. (see Table 12).

All strains of Groups I-III could utilize glutamate and acetate (or butyrate, with one or two exceptions), for carbon and energy. Carbohydrates, in general, were not utilized. No strain could attack glucose, but some members of the first three groups gave slow but positive reactions with ribose and/or fructose as the sole carbon and energy source. Positive results with these sugars, however, were difficult to reproduce by replica plating. Strains which were re-tested as liquid cultures generally gave slow but weakly positive

TABLES 6 - 11

Nutritional properties of S. ureae

Legend

1. Utilization:
   - + = growth distinctly enhanced over control
   - ± = scanty growth but significantly more than on control plate
   - w = late positive reaction (between 9-12 days)
   - ? = inconsistent results
   - M = late growth of a few colonies in area of patch; these are presumed to be mutants.
   - (p) = presence of diffusible pigments with benzoate.
   - blank = not tested

   - B = biotin
   - N = niacin
   - T = thiamine
   - ASP = aspartate
   - COM = complex supplements required for growth with test media.

2. Growth factor requirements:
   - + = required
   - - = not required

List A: xylose, arabinose, rhamnose, glucose, mannose, galactose, maltose, lactose, sucrose, cellobiose, trehalose, melibiose, raffinose, melezitose, D-tartarate, ethanol, n-propanol, iso-propanol, n-butanol, iso-butanol, erythritol, mannitol, sorbitol, meso-inosotol, adonitol, ethylene glycol, phenol, mandelate, putrescine, betaine

List B: nitrate, putrescine, betaine, 0.005% casamino acids, 0.005% yeast extract

* strain P12 forms a ring of growth on these substrates.

** growth of these strains is generally very slow on all substrates tested. The presence of aspartate as a growth factor enhanced growth considerably, but was not shown to be an absolute requirement for these strains.

† although glutamate was not indicated as an absolute growth requirement, growth of these strains was considerably enhanced when it was added in conjunction with aspartate.

TABLE 6
Utilization of carbon sources
GROUP I

Strain	List A	ribose	fructose	acetate	butyrate	pyruvate	citrate	malate	succinate	lactate	L-tartarate	propionate	glycerol	glutamate	aspartate	asparagine	benzoate
P38	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-
P35	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-
P36	-	±	±	+	+	+	+	+	+	+	+	+	-	+	+	+	+(p)
981	-	±	+	+	+	+	-	+	±	+	+	+	-	+	+	+	-
P33	-	±	±	+	+	±	+	-	+	+	-	w	-	+	-	+	-
P15	-	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	-
P34	-	±	+	+	+	M	+	+	±	+	+	+	-	+	+	+	M
P4	-	+	±	+	+	M	+	+	+	+	-	+	+	+	+	+	-
380	-	±	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
P6-A	-	±	±	+	+	+	±	+	+	+	-	-	-	+	+	+	M
P18-A	-	±	±	+	+	+	+	+	±	+	-	-	-	+	+	+	+(p)
P16-A	-	±	-	+	+	M	+	+	+	+	-	+	?	+	-	+	-
860	-	?	-	+	+	+	w	+	±	+	-	-	-	+	±	+	-

TABLE 7
Utilization of nitrogen sources and growth factor requirements
GROUP I

Strain	nitrogen sources				growth factors	
	List B	ammonia	glutamate	aspartate	vitamins	other growth factors
P38	-	+	+	±	-	-
P35	-	+	+	w	-	-
P36	-	+	+	-	-	-
981	-	+	+	-	-	-
P33	-	+	+	w	-	-
P15	-	+	-	-	-	-
P34	-	+	-	-	-	-
P4	-	+	-	-	-	-
380	-	+	-	w	-	-
P6-A	-	+	-	w	-	-
P18-A	-	+	-	-	-	-
P16-A	-	+	-	-	-	-
860	-	+	-	-	-	-

TABLE 8
Utilization of carbon sources
GROUP II

Strain	List A	ribose	fructose	acetate	butyrate	pyruvate	citrate	malate	succinate	lactate	L-tartarate	propionate	glycerol	glutamate	aspartate	asparagine	benzoate
P8	–	±	–	+	+	+	+	+	±	+	–	+	+	+	–	+	–
P37	–	±	–	+	+	+	+	+	±	+	?	+	?	+	–	±	–
P10	–	±	±	+	+	+	–	–	±	+	–	±	?	+	M	+	–
P6-B	–	±	–	+	+	+	+	–	+	+	–	+	+	+	–	+	–
P17-A	–	–	±	+	+	+	+	+	–	+	–	+	–	w	–	+	–
P13	–	–	–	+	+	+	–	+	+	–	–	–	–	+	–	+	–
P12	–	–	±	+*	+*	+*	w	M	±	+*	–	–	–	+	±	+	–
P39	–	±	+	+	+	+	+	+	M	+	+	+	–	+	+	+	+(p)
684	–	±	±	+	+	+	+	–	–	+	–	M	–	+	–	±	+(p)
P3	–	±	±	+	+	+	+	+	+	+	–	–	+	+	–	±	–
P11	–	±	±	+	+	+	+	+	+	+	–	w	–	+	±	+	+(p)
P16-B	–	?	–	+	+	M	+	+	±	+	–	?	–	+	±	+	–
P21-A	–	±	±	+	+	+	±	+	+	+	–	+	–	+	+	+	+(p)
P21-C	–	±	?	+	+	+	+	+	+	+	–	–	–	+	+	+	–
P22	–	–	±	+	+	+	?	+	w	+	–	–	w	+	+	+	–
P14	–	?	±	+	+	+	±	±	M	+	–	–	M	+	+	+	–
P2	–	–	–	+	+	M	+	+		+	–	+	–	+	±	+	–
P20-B	–	–	–	+	+	±	–	±	w	–	–	–	+	+	–	+	–
P18-B	–	–	–	+	+	M	+	–	–	M	–	–	–	+	+	+	–
P31	–	±	?	+	+	+	–	+	+	+	–	–	+	+	±	+	–
634	–	±	±	+	+	+	+	±	+	+	–	?	–	+	+	+	+(p)
752	–	–	±	+	+	+	+	–	–	w	–	M	–	±	–	±	+(p)
P25	–	–	±	±	+	+	–	+	+	+	–	+	?	+	±	+	–
P19	–	–	±	+	+	M	+	–	±	w	–	–	±	+	–	+	–
P9**	–	±	–	+	+	–	M	–	±	–	–	+	+	+	w	w	–
P28-A**	–	?	±	+	+	–	–	–	±	+	–	–	–	+	–	M	–
P28-B**	–	±	–	+	+	–	–	M	±	+	–	–	–	+	–	+	–
204**	–	–	–	+	+	+	w	–	–	+	–	+	–	w	–	w	–
P5	–	±	–	+	+	+	+	+	±	+	–	–	+	+	–	+	–
P30	–	±	w	+	+	±	–	+	±	±	–	–	–	+	+	+	–
P32-B	–	–	±	+	+	w	–	+	+	±	–	–	+	+	+	+	–
P1	–	–	–	+	+	–	w	+	±	+	–	–	+	+	±	+	–
P21-B	–	±	–	+	+	–	+	±	±	M	–	–	w	+	–	–	–
P26-A	–	–	–	+	+	–	–	–	–	±	–	M	w	+	–	+	–
P26-B	–	–	–	+	+	–	–	–	–	±	–	M	w	+	–	+	–
P32-A	–	–	–	+	+	M	–	M	w	–	–	–	±	+	±	+	+(p)
P20-A	–	–	±	+	+	+	+	–	±	+	–	–	w	+	–	±	–

TABLE 9
Utilization of nitrogen sources and growth factor requirements
GROUP II

Strain	nitrogen sources				growth factors	
	List B	ammonia	glutamate	aspartate	vitamins	other growth factors
P8	-	+	-	-	B	-
P37	-	+	-	-	B	-
P10	-	+	-	-	B	-
P6-B	-	+	-	-	B	-
P17-A	-	+	-	-	B	-
P13	-	+	-	-	B	-
P12	-	+*	-	-	B	-
P39	-	+	-	-	N(T or B)	-
684	-	+	-	-	N(T or B)	-
P3	-	+	-	-	N(T or B)	-
P11	-	+	-	-	TB	-
P16-B	-	+	-	-	TB	-
P21-A	-	+	-	-	TB	-
P21-C	-	+	-	-	TB	-
P22	-	+	-	-	TB	-
P14	-	+	-	-	?	-
P2	-	+	-	-	TB	-
P20-B	-	+	-	-	TB	-
P18-B	-	+	-	-	TB	-
P31	-	+	-	-	TB	-
634	-	+	-	-	NTB	-
752	-	+	-	-	NTB	-
P25	-	+	-	-	NTB	-
P19	-	+	-	-	NTB	+
P9**	-	+	-	-	NTB	-
P28-A**	-	+	-	-	NTB	-
P28-B**	-	+	-	-	NTB	-
204**	-	+	-	-	NTB	-
P5	-	+	-	-	NTB	ASP
P30	-	+	-	-	NTB	ASP
P32-B	-	+	-	-	TB	ASP
P1	-	+	-	-	TB	ASP
P21-B	-	+	-	-	TB	ASP
P26-A	-	+	-	-	NTB	ASP
P26-B	-	+	-	-	NTB	ASP
P32-A†	-	+	-	-	TB	ASP
P20-A†	-	+	-	-	TB	ASP

TABLE 10
Utilization of carbon sources
GROUP III

Strain	List A	ribose	fructose	acetate	butyrate	pyruvate	citrate	malate	succinate	lactate	L-tartarate	propionate	glycerol	glutamate	aspartate	asparagine	benzoate
S1			±	+			+	-		-		-	M	++			
P17-B			w	+			M	+		-		+	-	±+			
P24			±	+			+	+		+		±	+	±			
P23			-	+			-	-		-		+	-	+			
C1			-	+			±	+±		+		-	+	+			
P7			-	+			+±	±		+		-	+	+			
P27			w	+			±	w		+		-	w	-			

TABLE 11
Utilization of nitrogen sources and growth factor requirements
GROUP III

Strain	nitrogen source				growth factor	
	List B	ammonia	glutamate	aspartate	vitamins	other growth factors
S1		+	-		NTB	COM
P17-B		+	-		no growth	COM
P24		+	-		TB	COM
P23		+	-		no growth	COM
C1		+	-		TB	COM
P7		+	-		TB	COM
P27		+	-		NTB	COM

TABLE 12

Growth of organisms of Group IV on complex media. Acetate and ammonia, when present, were added to the synthetic medium at 0.5% and at 0.1%, respectively. ++ = heavy growth. + = good growth but slow. - = no growth.

Substrate	Strains			
	858	C2	P40	P29
1. Casamino acids, Difco (0.4%), no acetate, no ammonia	++	++	++	-
2. Casamino acids (0.25%), + ammonia, no acetate	++	++	++	-
3. Casamino acids (0.1%), + acetate, no ammonia	-	++	++	-
4. Casamino acids (0.1%), + acetate + ammonia	++	++	++	-
5. Glutamate (0.4%), no acetate, no ammonia	-	-	-	-
6. Glutamate (0.2%), + ammonia, no acetate	+	+	+	-
7. Glutamate (0.2%), + acetate, no ammonia	-	-	-	-
8. Glutamate (0.2%), + acetate + ammonia	++	++	++	-

reactions. When the respiratory activity of washed cells of selected strains was measured manometrically, the results did demonstrate the complete oxidation of fructose and ribose but at a slower rate than for acetate, (Fig. 2 and 3). Furthermore, the utilization of these sugars is inducible, since cells did not oxidize ribose or fructose when grown on acetate (Fig. 2).

Utilization of other substrates as carbon and energy sources revealed no pattern or common reaction among strains. All organisms of Group I and a number of strains of Group II and Group III could utilize lactate, and a spectrum of activity was shown with intermediates of the tricarboxylic acid cycle. Most members of Group I showed strongly positive activity on all three of these compounds tested (citrate, malate, and succinate). Growth on succinate, however, was not always reproducible, and the same difficulty was experienced with propionate; these compounds inhibited growth of a number of strains, even at the lower concentration. Many strains of Group I could attack L-tartarate, but only one strain of Group II consistently gave positive results with this substrate. All strains of the first three groups showed some activity on glycerol. Benzoate was attacked by only a few strains of Group I and Group II; this compound was not tested with Group III or IV. For more test results, refer to Tables 6, 8, and 10.

Although the presence of both glutamate and aspartate (or asparagine), appeared to enhance growth, no strain indicated an absolute requirement for glutamate as a growth factor (and, in fact, growth of some organisms was inhibited in its presence).

FIGURE 2

Oxidation of fructose, ribose, and acetate by acetate-grown cells of S. ureae (strain P15).

The main vessel of the Warburg flask contained 2.0 ml cell suspension in 0.1M Tris buffer. The center well contained 0.2 ml 20% NaOH. Oxygen uptake was measured following the addition of 50 μ moles fructose or ribose, or 100 μ moles acetate from the sidearm.

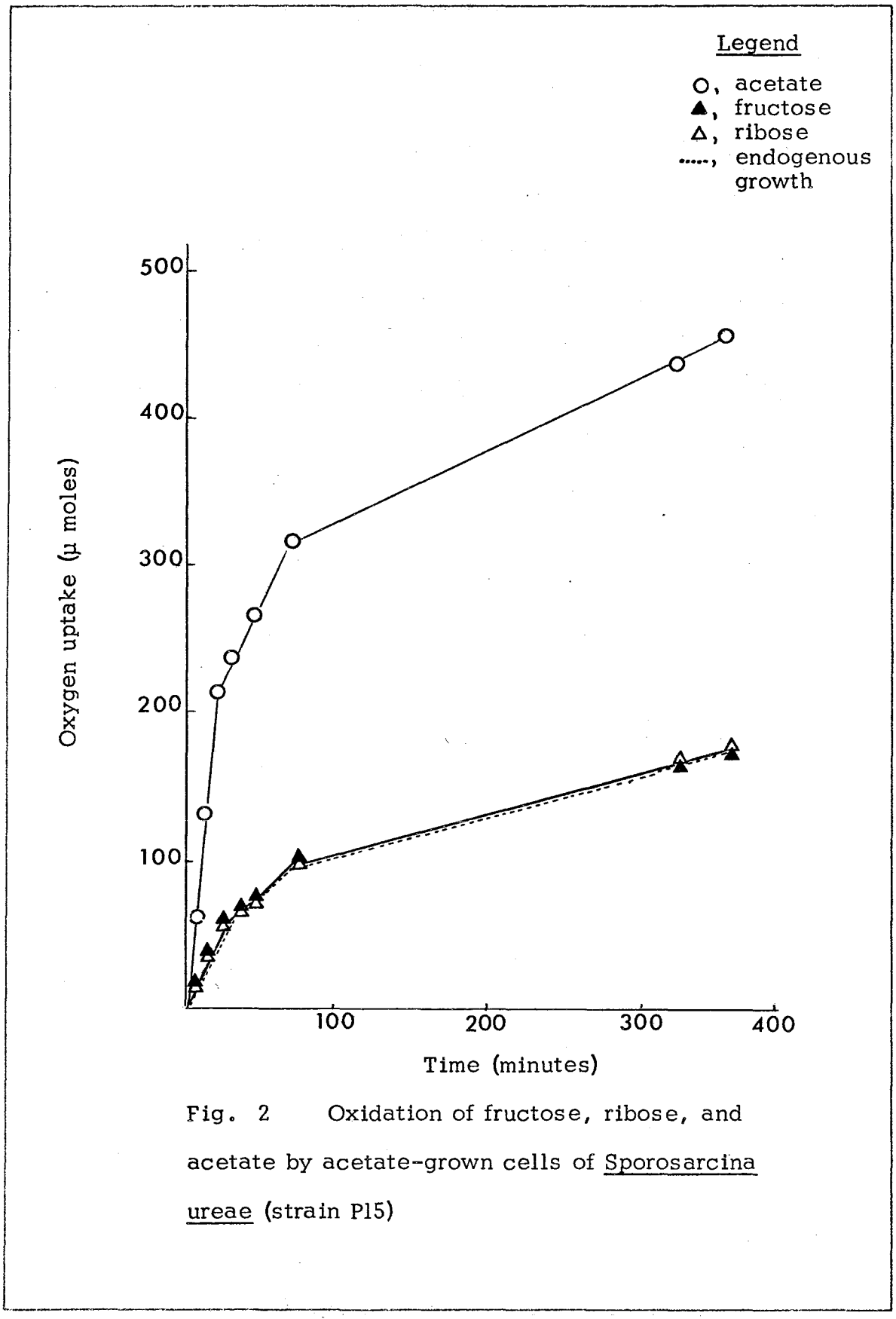

Fig. 2 Oxidation of fructose, ribose, and acetate by acetate-grown cells of Sporosarcina ureae (strain P15)

FIGURE 3

Oxidation of fructose, ribose, and acetate by fructose-grown cells of S. ureae (strain P15).

The main vessel of the Warburg flask contained 2.0 ml cell suspension in 0.1M Tris buffer. The center well contained 0.2 ml 20% NaOH. Oxygen uptake was measured following the addition of 50 μ moles fructose or ribose, or 100 μ moles acetate from the sidearm.

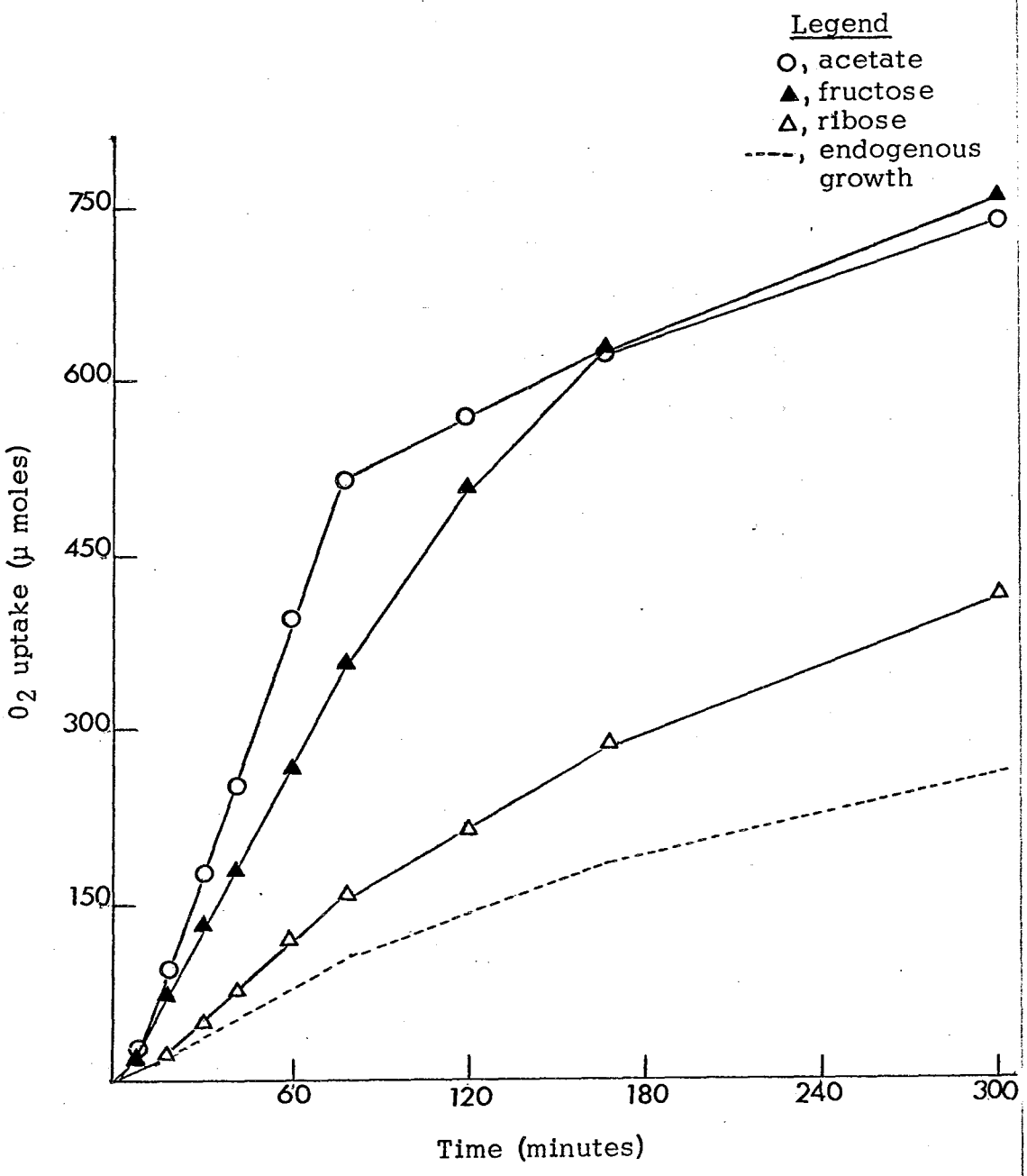

Fig. 3 Oxidation of fructose, ribose, and acetate by fructose-grown cells of S. ureae (strain P15)

Relatively few strains showed a requirement for aspartate; however, even for strains which indicated no requirement for aspartate, growth was very slow in its absence (Table 8). Growth of several organisms was considerably enhanced when both glutamate and aspartate were present (Table 8). Several other strains showed growth in the absence of aspartate only after a number of sub-cultivations in liquid media.

Ammonium salts could serve as a nitrogen source for all groups except Group IV. Three organisms of this group required both ammonia and amino acids. As noted previously, casamino acids or glutamate could replace ammonia as the nitrogen source only when supplied at substrate level. Only a few strains of Groups I and II could utilize glutamate as the sole nitrogen source. Growth on this substrate, however, was slow and generally weakly positive. Neither putrescine, betaine, nor the nitrate ion could be utilized as a nitrogen source. For detailed results, refer to Tables 7, 9, and 11.

Four strains (Group IV), were treated separately because they would not grow on normal nutritional media even when supplied with small amounts of complex organic materials. Table 12 illustrates some conditions of growth of these organisms with complex media. Although the complete requirements have not been determined, three of the four strains of this group appear to require glutamate as a carbon and energy source.

One study indicated that one of the strains of this group, 858, might have a base composition of its DNA that is somewhat

different from the average (Boháček, Kocur, and Martinec, 1968). Interpretations are difficult, however, because determinations made by different methods do not agree.

Diagnostic tests-Tables 13 and 14)

a. Starch hydrolysis. The results were negative for all but a few strains which showed weak or doubtfully positive reactions. MacDonald and MacDonald (1962), found a number of strains to be weakly positive. In my hands, all but one of the same strains gave negative results.

b. Fat hydrolysis. Kocur and Martinec (1963), found all the strains they tested to be negative. In this study, the time of appearance of a turbid halo surrounding growth was variable. A weak reaction occurring after an extended period of growth may reflect autolysis of the bacteria and liberation of intracellular esterases (Stanier, Palleroni, and Doudoroff, 1966). Although a number of strains were scored positive for this test, no strain showed the extremely dense deposits of calcium salt that were characteristic of two strains of sarcinae collected from soils; moreover, these sarcinae produced these deposits within 2 days. A few Sporosarcina produced moderately dense, spreading halos, but most of the strains were weakly positive or were negative after 8 days.

c. Gelatin Hydrolysis. MacDonald and MacDonald (1962), reported only one strain to be weakly positive; Kocur and Martinec (1963), noted no gelatin hydrolysis. In my hands, all strains gave negative results.

TABLE 13

Data on diagnostic tests

* a number of strains of Sporosarcina ureae used in this study have been examined by other workers; † indicates those strains which were examined by MacDonald and MacDonald, (1962); ‡ indicates those strains which were examined by Kocur and Martinec, (1963).

** = Voges-Proskauer

Explanatory notes

(1) Starch hydrolysis. ±, clear zone of hydrolysis beneath or around areas of growth was narrow or doubtfully positive.

(2) Tween hydrolysis. +++, extremely dense halo formed within 2 days. The following designations were scored after 8 days growth; ++, moderately dense halo completely and widely encompassing each colony; +, halo less dense but completely encompassing the colonies; ±, feathery-like halo, encompassing most of the colonies.

(4) Nitrate reduction test. +, appearance of red color after 3 days' growth.

(9) Arginine dihydrolase test. +++, deep orange color after 2 days' growth; ++, deep gold color within 5 days; +, yellow color after 8 days' growth; s, slight color change after 8 days' growth.

(10) 10% urea (TSY agar). All strains showed only punctiform growth after 8 days' incubation. ++, substantial tolerance; +, moderate tolerance; s, slight tolerance.

Other symbols: -, negative response; n.t., not tested.

TABLE 13
Data on diagnostic tests

Strain*	(1) Starch	(2) Tween 80	(3) Gelatin	(4) Nitrate	(5) Urease	(6) Indole	(7) Catalase	(8) V.P.**	(9) Arginine	(10) H_2S	(11) 10% urea
P1	-	±	-	+	+	-	+	-	+	-	+
P2	-	±	-	+	+	-	+	-	+	-	+
P3	±	±	-	+	+	-	+	-	+	-	+
P4	-	±	-	+	+	-	+	-	+	-	++
P5	-	±	-	+	+	-	+	-	+	-	-
P6-A	-	±	-	+	+	-	+	-	+	-	++
P6-B	-	±	-	+	+	-	+	-	++	-	++
P7	-	±	-	+	+	-	+	-	+	-	+
P8	±	±	-	+	+	-	+	-	+	-	-
P9	-	±	-	+	+	-	+	-	s	-	++
P10	-	++	-	+	+	-	+	-	+	-	++
P11	-	±	-	+	+	-	+	-	+	-	++
P12	-	+	-	+	+	-	+	-	++	-	++
P13	-	+	-	+	+	-	+	-	-	-	++
P14	-	±	-	+	+	-	+	-	++	-	
P15	-	-	-	+	+	-	+	-	++	-	++
P16-A	-	-	-	+	+	-	+	-	+	-	+
P16-B	-	±	-	+	+	-	+	-	+	-	++
P17-A	-	±	-	+	+	-	+	-	-	-	-
P17-B	-	±	-	+	+	-	+	-	s	-	s
P18-A	-	±	-	+	+	-	+	-	+	-	++
P18-B	-	±	-	+	+	-	+	-	+++	-	++
P19	-	±	-	+	+	-	+	-	s	-	+
P20-A	-	±	-	+	+	-	+	-	s	-	s
P20-B	-	-	-	+	+	-	+	-	+	-	-
P21-A	±	-	-	+	+	-	+	-	+	-	+
P21-B	-	-	-	+	+	-	+	-	++	-	+
P21-C	-	±	-	+	+	-	+	-	+	-	++
P22	-	-	-	+	+	-	+	-	-	-	-
P23	-	±	-	+	+	-	+	-	+++	-	-
P24	±	-	-	+	+	-	+	-	++	-	-
P25	-	-	-	+	+	-	+	-	+	-	s
P26-A	-	-	-	+	+	-	+	-	+	-	s
P26-B	±	±	-	+	+	-	+	-	s	-	-
P27	-	-	-	+	+	-	+	-	-	-	-
P28-A	-	-	-	+	+	-	+	-	+++	-	+
P28-B	-	-	-	+	+	-	+	-	++	-	+
P29	±	-	-	+	+	-	+	-	+	-	s
P30	±	-	-	+	+	-	+	-	+	-	s

TABLE 13
Data on diagnostic tests

Strain*	(1) Starch	(2) Tween 80	(3) Gelatin	(4) Nitrate	(5) Urease	(6) Indole	(7) Catalase	(8) V.P.**	(9) Arginine	(10) H_2S	(11) 10% urea
P31	±		-	+	+	-	+	-	+	-	+
P32-A	-	±	-	+	+	-	+	-	s	-	-
P32-B	±		-	+	+	-	+	-	s	-	s
P33	-	++	-	+	+	-	+	-	+	-	++
P34	-	-	-	+	+	-	+	-	+	-	++
P35		+	-	+	+	-	+	-	-	-	++
P36	-	±	-	+	+	-	+	-	s	-	++
P37	-	±	-	+	+	-	+	-	+	-	-
P38	-	++	-	+	+	-	+	-	-	-	++
P39	-	±	-	+	+	-	+	-	s	-	++
P40	-	±	-	+	+	-	+	-	++	-	-
C1†	-	+	-	+	+	-	+	-	+++	-	++
C2†	-	±	-	+	+	-	+	-	++	-	s
204†‡	-	+	-	+	+	-	+	-	+++	-	++
380†‡	-	-	-	+	+	-	+	-	+++	-	s
634†‡	-	±	-	+	+	-	+	-	++	-	++
684 ‡	±	+	-	+	+	-	+	-	++	-	++
752†‡	±	+	-	+	+	-	+	-	++	-	++
858†‡	-	+	-	+	+	-	+	-	++	-	++
860†‡	-	++	-	+	+	-	+	-	+	-	++
981†‡	-	+	-	+	+	-	+	-	++	-	++
S1	-	±	-	+	-	-	+	-	++	-	+

d. Nitrate reduction. All strains were positive. The same results were found by Gibson (1935), Wood (1946), MacDonald and MacDonald (1962), and by Kocur and Martinec (1963).

e. Urease. Gibson (1935), MacDonald and MacDonald (1962), and Kocur and Martinec (1963), reported positive results for this test. In this study, all but one strain were positive.

f. Indole. All strains were negative. The same results were found by Gibson (1935), Wood (1946), and by Kocur and Martinec (1963).

g. Catalase. All strains were positive. These results agreed with those obtained by MacDonald and MacDonald (1962) and by Kocur and Martinec (1963).

h. Voges-Proskauer (V.P.). All strains were negative. The same results were also noted by Kocur and Martinec (1963).

i. Arginine dihydrolase. Most of the strains showed some color change indicative of a positive reaction, but there were quantitative differences in reactions. It is interesting to note that all the strains which were initially obtained as pure laboratory cultures consistently showed strong positive reactions within 2-5 days. Many of the freshly isolated strains, however, gave negative or only slightly positive reactions after 8 days; only a few of these strains initially gave a reaction as strong as that shown by the laboratory cultures. Several strains on retesting after several months, however, demonstrated a more positive activity than originally noted. The same strains tested and found to be positive by both MacDonald and MacDonald (1962), and by Kocur and Martinec (1963), also

gave positive reactions in this study.

j. Hydrogen sulfide. Gibson (1935), reported negative results; Kocur and Martinec (1963), noted some positive results for some strains. In my hands, all the strains were negative.

k. 10% urea. Strains varied in their tolerance to this medium from no growth to substantial growth.

l. Anerobic growth (Table 14). No strain could grow anerobically. The same results were also reported by Gibson (1935), MacDonald (1960), and Kocur and Martinec (1963).

Pigmentation

Although strains showed a range of colors on solid media, (Table 14), pigments extracted from various strains had the same absorption spectrum (Fig. 4). The shape and position of the peak indicates that the extracted pigments are carotenoids which are present in the orange-pigmented organisms. The variations in color from one strain to another is a consistent characteristic but these variations apparently reflect a quantitative difference in pigment concentration, not a qualitative difference. Several organisms which appeared white or cream colored on the medium used for determining pigment color, exhibited a slight yellowish color when grown on TSY. Upon analysis, however, several such strains did not reveal a detectable amount of carotenoids by our methods. It is apparent that those strains which did not produce pigments on the pigment medium also did not do so on the rich TSY medium. The slight color exhibited by some strains on TSY is presumed to be due to background color of the medium.

TABLE 14
Selected morphological and physiological properties of S. ureae

	Colony Color	anerobic growth	motility	mean diameter cell (µm)	spore production	mean diameter spore (µm)
P1	brt. orange	-	+	1.7	+	1.1
P2	cream	-	+	1.8	+	1.0
P3	orange	-	+	1.5	+	1.1
P4	cream	-	+	1.5	+	1.0
P5	lt. orange	-	+	1.4	+	1.2
P6-A	cream	-	+	1.9	+	1.2
P6-B	orange	-	+	1.7	+	1.1
P7	lt. orange	-	+	1.7	+	1.0
P8	orange	-	+	2.0	+	1.1
P9	cream	-	+	1.7	+	1.1
P10	cream	-	+	1.8	+	0.9
P11	cream	-	+	1.5	+	1.0
P12	cream	-	+	1.3	+	1.0
P13	cream	-	+	1.3	+	1.0
P14	lt, orange	-	+	1.4	+	0.9
P15	cream	-	+	1.4	+	1.0
P16-A	orange	-	+	1.6	+	1.1
P16-B	cream	-	+	2.0	+	1.2
P17-A	dark cream	-	+	1.4	+	1.1
P17-B	orange	-	+	1.6	+	1.1
P18-A	greyish-cream	-	+	1.4	+	1.0
P18-B	brt. orange	-	+	1.7	+	1.2
P19	lt. cream	-	+	1.6	+	1.0
P20-A	lt. cream	-	+	1.6	+	1.0
P20-B	orange	-	+	1.7	+	1.1
P21-A	brt. orange	-	+	1.5	+	0.9
P21-B	lt. orange	-	+	1.8	+	1.0
P21-C	greyish-cream	-	+	1.7	+	1.0
P22	orange	-	+	2.2	+	1.0
P23	cream	-	+	1.6	+	1.0
P24	lt. orange	-	+	1.8	+	1.0
P25	orange	-	+	2.0	+	1.0
P26-A	lt. orange	-	+	2.1	+	1.3
P26-B	orange	-	+	1.8	+	1.3
P27	cream	-	+	1.8	+	1.0
P28-A	lt. orange	-	+	1.7	+	1.0
P28-B	orange	-	+	1.5	+	1.1
P29	lt. orange	-	+	2.0	+	1.0
P30	lt. orange	-	+	1.7	+	1.0

TABLE 14

Selected morphological and physiological properties of S. ureae

	Colony Color	anerobic growth	motility	mean diameter cell (µm)	spore production	mean diameter spore (µm)
P31	intensely brt.orange	-	+	2.0	+	1.0
P32-A	brt. orange	-	+	1.8	+	1.0
P32-B	orange	-	+	1.6	+	1.1
P33	lt. cream	-	+	2.1	+	1.2
P34	cream	-	+	2.1	+	1.1
P35	lt. cream	-	+	1.8	+	1.1
P36	lt. cream	-	+	1.9	+	1.0
P37	lt. cream	-	+	2.0	+	1.1
P38	lt. cream	-	+	2.0	+	1.1
P39	greyish-cream	-	+	2.1	+	1.0
P40	cream	-	+	2.2	+	1.0
C1	orange	-	+	2.3	+	1.0
C2	cream	-	+	1.7	+	1.0
204	cream	-	+	1.3	+	-
380	lt. orange	-	+	2.3	+	-
634	lt. cream	-	+	1.6	+	-
684	dark cream	-	+	1.5	+	-
752	cream	-	+	2.0	+	1.0
858	cream	-	+	1.5	+	-
860	cream	-	+	2.1	+	1.2
981	greyish-cream	-	+	2.2	+	1.1
S1	cream	-	+	1.2	+	1.0

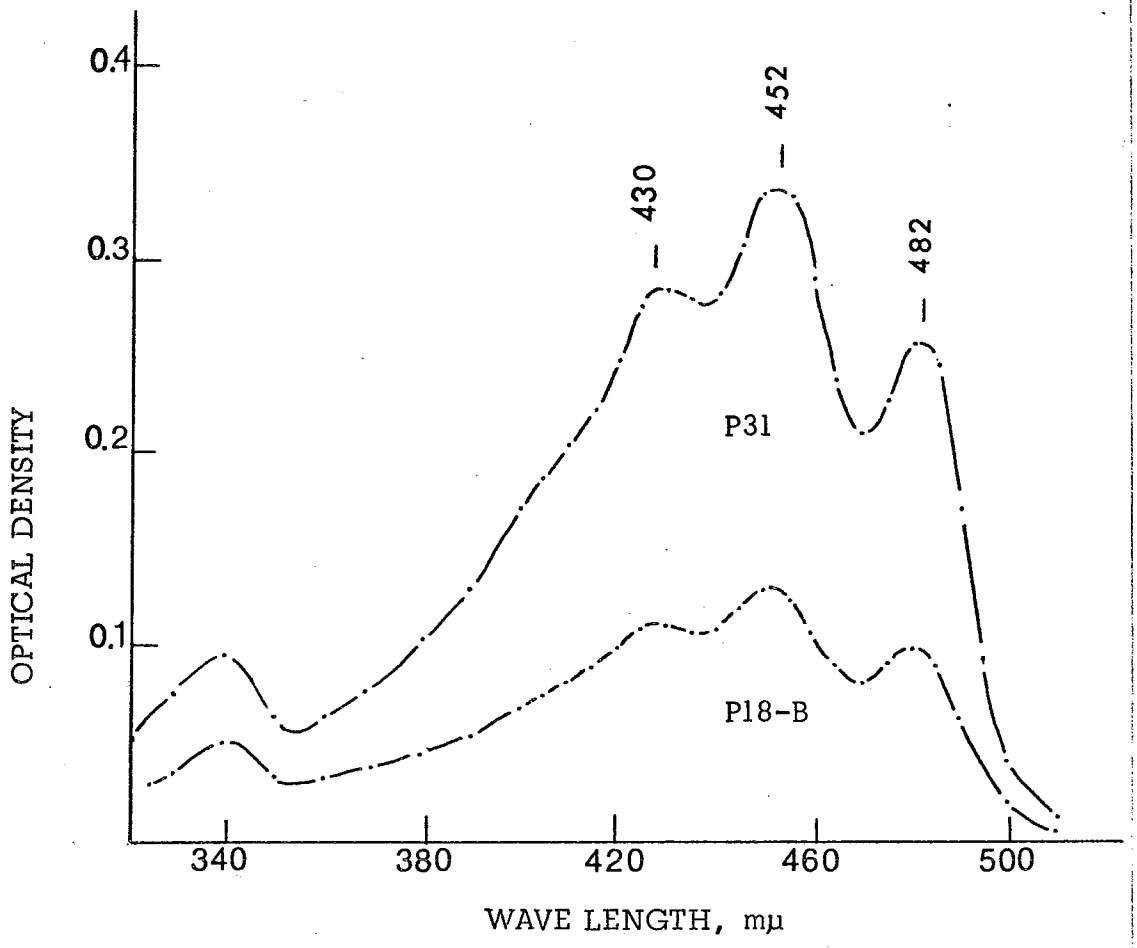

FIG. 4 Absorption spectrum of pigments of two strains of Sporosarcina ureae in petroleum ether. Methanol extract was saponified and partitioned in petroleum ether. Equal quantities of cells of P18-B and P31 were extracted and partitioned as described.

Some extracts showed a relatively small peak at 400 mμ after lengthy extraction with methanol. The presence of this material is not correlated with the presence of carotenoids and identification was not attempted.

The partition test resulted in the extraction of virtually all the yellow pigments into the petroleum ether fraction (epiphase). This behavior is consistent with the presence of non-hydroxylated or monohydroxylated carotenoids. Some methanolic fractions (hypophasic), were slightly pigmented but the absorption data did not reveal the presence of any di- or polyhydroxylated carotenoids, which one would expect to find in this layer. No attempt was made to characterize further the extracted carotenoids.

Pigmentation was produced with a number of substrates, but pigment colors were noticeably more intense in the presence of lactate. The addition of glutamate at low concentration, generally resulted in inhibition of pigmentation, which may be due to the general inhibitory effect this metabolite exerts on growing cultures; similarly, both succinate and propionate inhibited pigmentation of strains which otherwise showed good growth on these substrates.

An intensely yellowish, water soluble pigment was produced by a few organisms in Groups I and II when grown on sodium benzoate. This pigment was not identified.

Morphology

The formation of packets of cells is a consistent feature of all the strains examined when plated on a variety of media. The most common packet formation is the tetrad, but single cells as well as

doubles and packets of 8 are commonly seen. Cell size ranged from 1.2 to 2.2 μm. These measurements are in general agreement with those obtained by other workers; they found the range to be 1.0-2.5 μm. Young vegetative cells showing typical packet formation are illustrated in Figure 5 in photomicrographs taken in phase-contrast.

A number of aberrant forms were noted in young and in older cultures, and of single cells as well as of cells within packets, or of packets themselves. Both young and older cultures grown on both solid and liquid media of TSY or in supplemented synthetic media, regularly showed abnormally large cells contained within packets or found singly. Old cultures from TSY plates commonly showed irregular conformation of the packet into short chains or into small clustering of cells, which may be the result of asynchronous cell division. Such forms, however, were not seen in young cultures.

Motility

Flagellar insertion was not examined. Flagella have been described by numerous workers as being present usually singly or occasionally several to a cell, exceedingly long and arranged peritrichously (Beijerinck, 1901; Ellis, 1902; van Niel, 1923; Hucker and Thatcher, 1929; Pijper, Crocker, and Savage, 1955; Hale and Bisset, 1958; and Leifson, 1960). Motility was observed with all young cultures (ca 12-15 hours), both on TSY plates and in TSY liquid medium. Strains grown on solid media showed motility for a much longer period of time than when grown in liquid media, an observation noted also by Gibson (1935). Kocur and Martinec

(1963), reported that strains were motile when grown in nutrient broth or on agar only when 1.0% urea was added. In our experience, colonies on nutrient agar do not require the presence of urea for motility. Growth is generally poor on this medium, however, and motility was not as vigorous as under alkaline conditions.

Although motility generally ceases by the time each cell of a packet produces a highly refractile spore, on several occasions, vigorous motility has been observed with pairs or tetrad of cells containing a "full house."

Cultural characteristics

Colony morphology has been described by a number of workers (Beijerinck, 1901; Ellis, 1902; Gibson, 1935; Kocur and Martinec, 1963). A tentative description proposed by Gibson* for the 8th edition of Bergey's Manual, describes the colonies as being "circular, gray, microscopically coarsely granular, becoming opaque and yellowish, brown, or orange in different strains or on different media." On TSY, freshly isolated strains produce smooth, round colonies which are slightly convex, glistening, and semitransparent. Pigmentation, when present, is not evident for the first day or two, but then becomes increasingly apparent so that by the 4th or 5th day, the colonies may range in color from pale yellow or cream-colored to bright orange. Pigmentation generally is visible first at the center of the colonies, but occasionally the edges show pigmentation first. Some strains produce concentric rings of pigmentation. As noted previously, when cells are grown on TSY, which contains rich concentrations of yeast extract and tryptic

* personal communication.

soy broth, a considerable amount of yellow diffusible pigment is produced after several days, especially by the pigmented strains; most of the organisms which are presumed to lack carotenoid pigments did show a small amount of diffusible pigment on this medium. On synthetic media containing acetate as the carbon and energy source or on TSY/5 + acetate, these water soluble pigments are produced minimally or are absent from all the strains.

Some strains occasionally showed variations in surface characteristics, forming rough, dried, and crusty surfaces. Surface granularity is a characteristic found in all strains studied. This feature becomes increasingly apparent after 24 hours, and is often accompanied by a loss of surface glossiness. The edges of colonies are entire but may appear rough due to this granularity, especially after 3-4 days. A number of strains produce bubbles near the center of colonies, this surface effect becoming more pronounced with time. The consistency of colonies ranges from soft to firm and dry growth. A number of organisms showed slight to moderate viscosity and several organisms produce rubbery growth so that small colonies could sometimes be detached whole with a needle. All cultures show a uniformly smooth opacity when viewed at 8-25 x magnification. This characteristic appears to be related to the specific cellular morphology. Some Sarcina species and bacilli which produce short rods, are often mistaken for Sporosarcina ureae because their colony morphology also reveals smoothly opaque interiors. Those colonies which show non-uniform opacity, that is, clumpy or web-like interiors, almost invariably contain long rods.

After 48 hours growth on TSY, the colonies generally range in size from 2-4 mm. in diameter; after 7 days, they range from approximately 6-10 mm. in diameter.

Growth in liquid media

In general, liquid cultures were not examined as closely as cultures grown on solid media. It has been observed that cells sediment very rapidly on standing and may show a high degree of agglutination. Some cultures grown for 12-15 hours on TSY, or for one to two days on supplemented synthetic media, may form cell masses which can be drawn out with a loop. These characteristics have not been studied further, but there seems to be no direct relationship between the degree of agglutination and sporulation, a relationship that has often been noticed among the bacilli.

Sporulation

Many investigators have studied this organism because of the interest in spore production. (Beijerinck, 1901; Ellis, 1902; Gibson, 1935; Wood, 1946; MacDonald and MacDonald, 1962; Kocur and Martinec, 1963; Mazanec, Kocur, and Martinec, 1965). Our observations are in keeping with their descriptions. The spores are spherical (with phase contrast microscopy); they occupy a central position in the cell and take up most of its interior (see photomicrograph, Fig. 6). They range in size from approximately 0.8 to 1.3 μm. There may be considerable variation in size, however, within strains and under differing cultivation conditions.

Crowded conditions on plates seem to stimulate spore production. Experience has shown that the most likely place to find

FIGURE 5

Vegetative cells of a 12 hour culture of S. ureae, strain P6-A, grown on TSY agar at 30°C (phase contrast microscopy).

FIGURE 6

Sporulating cells of a 28 day culture of S. ureae, strain P6-A grown on TSY agar at 30°C (phase contrast microscopy).

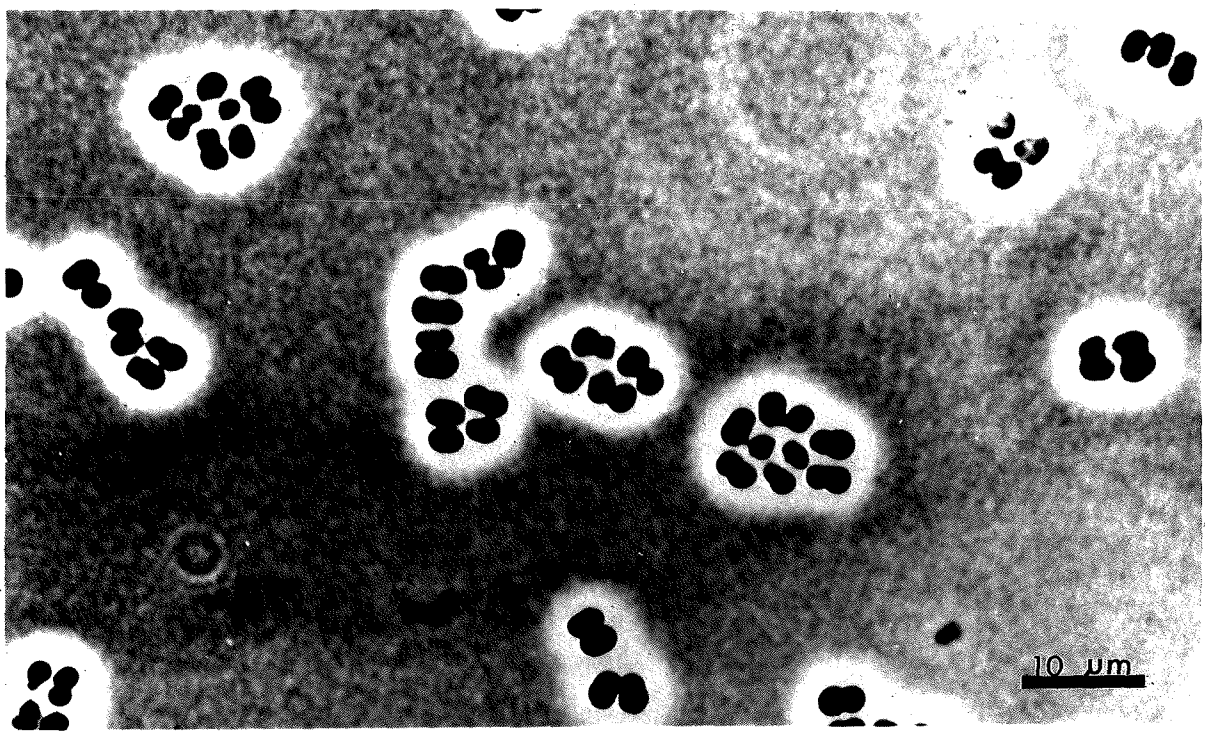

FIGURE 5

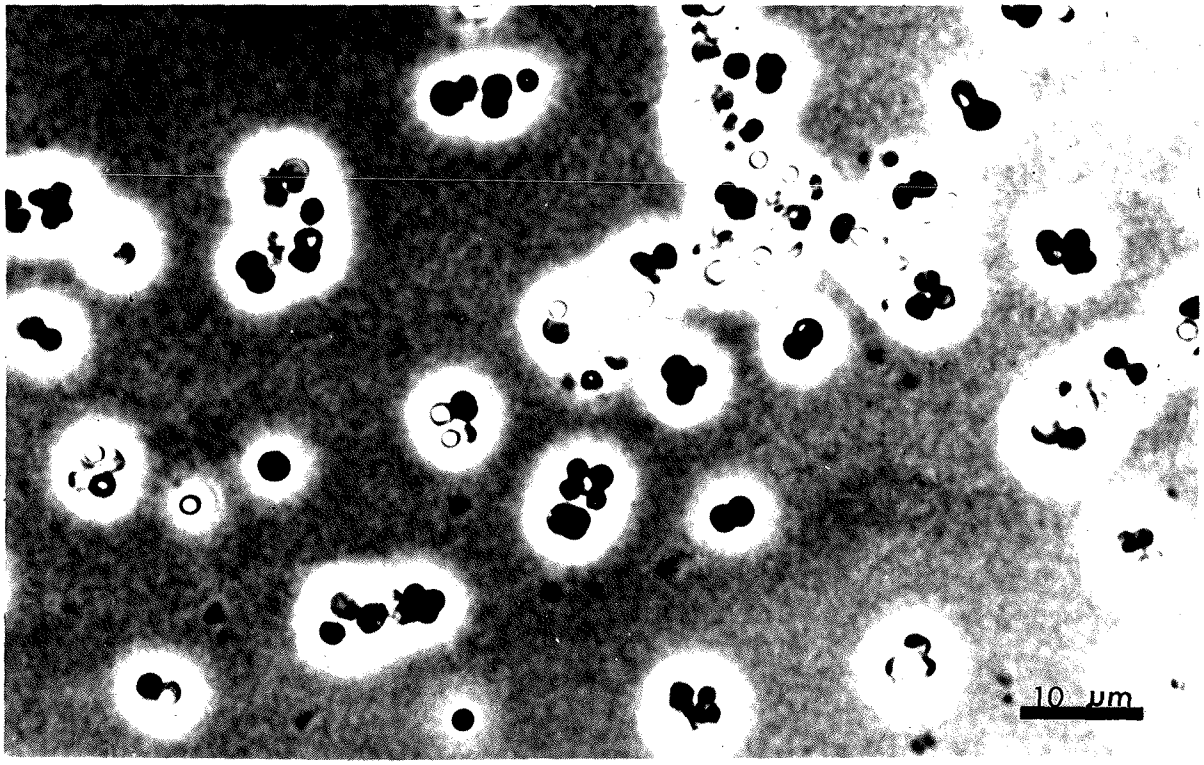

FIGURE 6

spores is in densely populated areas where plates are heavily streaked. The single, well isolated colony, however, may show little spore production, or if present, spores are usually not as plentiful as in areas of heavy growth. Environmental conditions along the margins of colonies seem to favor the production of spores.

Opacity appears to be associated with sporulation since spores were never noted from transparent (or nearly transparent) variants which were occasionally produced by some cultures.

Each isolate showed production of spores on MacDonald's spore medium but there was considerable variation in the onset and extent of sporulation. Some strains produced only a few spores after 4-5 weeks of growth and other strains produced spores within 24 hours, and an abundance of spores within 3-4 days, but results were very often not reproducible. Several organisms appeared to sporulate as readily (or as poorly), on TSY as on the spore medium.

Several variations of the spore medium were tried. Salts were filter-sterilized and added separately to the autoclaved complex medium, the pH was varied between 7 and 9 (before autoclaving), the agar concentration was varied between 1.5% and 3.0%, and casamino acids (at 0.3%) were substituted for peptone. A notable improvement in sporulation was evidence when the medium initially was made quite alkaline. Because the pH of the spore medium drops considerably with time, an initial pH of 8.8-9.0 (before autoclaving), resulted in more satisfactory sporulation of most of the cultures than at lower pH, especially if strains were streaked within a few days. Increasing the agar concentration to 3.0%

also resulted in some improvement, an observation also noted by MacDonald and MacDonald (1962). Several strains produced spores more readily when casamino acids replaced peptone. Five isolates, however, showed little production of spores on any of the various media and five pure cultures from other laboratories showed no sporulation, despite evidence from other workers of their sporulating capacities (MacDonald and MacDonald, 1962; Kocur and Martinec, 1963). It is possible that these laboratory strains, which were received as lyophillized cultures, were not subcultured sufficiently in this study for them to regain their capacity for spore production.

Among the different strains, no correlation was noted between their nutritional and their sporulating capacities, although three of the four members of Group IV (858, P 40, and P 29), showed little or not sporulation on any of the spore media tested. However, one member of this group, C 2, generally showed 60%-80% spore production within a few days, on a variety of spore media. The fastidious mutants of nutritional Group III exhibited a range of sporulating capacities.

9. DISCUSSION

As detailed in Chapter 7, the nutrition of this organism was investigated using two procedures--serial subcultivation of liquid cultures and replica plating. Various investigators have found this latter procedure to be an efficient method for comparing the nutrition of relatively large numbers of organisms. As examples, Stove (1963), used this method for studying the biology of Caulobacter, and Stanier, Palleroni, and Doudoroff (1966), for a taxonomic study of pseudomonads. Replica plating is a particularly useful technique for testing the utilization of potential carbon or nitrogen sources. However, it is less useful for determining growth factor requirements which are needed at such low concentrations that they may be supplied as impurities in the agar or transferred with the organism as endogenous reserves. Hence, to determine growth factor requirements greater reliance was placed on the method of serial subcultivation of liquid cultures. Such a procedure effectively dilutes carryover on even small quantities of unwanted nutrients.

In this study, the technique of replica plating was not as satisfactory as had been hoped. In the examination of Group I, the results obtained by replica plating were reproducible and unambiguous, and agreed with the results obtained by serial subcultivation in liquid cultures. However, when replica plating was employed with other groups, the results were inconsistent. Furthermore, these results generally did not agree with those

obtained by liquid subcultivations. One difficulty encountered, and noted by other workers, is that relatively little total growth on a plate suffices to give a marginal but positive reading. Distinguishing positive responses was also hampered by the normal turbidity of the agar gel; the use of purified agar at a low concentration (0.9%) did provide a clear background for reading results, but still did not give reproducible results for a number of strains. Another difficulty encountered with replica plating is that some organisms, such as P 35, P 39, and 981, are highly efficient utilizers of even the reduced concentrations from chemical contaminants or residual material carried over from previous cultivations. These organisms consistently gave early positive results by replica plating with substrates which were not utilized when subsequently retested by serial subcultivation of liquid cultures.

Aside from growth factor requirements, the strains tested show a relative constancy of metabolic characteristics. For example, the strains require only ammonium salts for a nitrogen source in general, sugars are not utilized by these strains or are utilized very poorly (glucose can not be oxidized by any strain tested), while two substrates, acetate and glutamate, are apparently universal substrates for this organism. For the most part, the strains also show a constancy in the commonly tested biochemical properties (Table 13), being generally inactive, in agreement with the results of most other workers. They do not produce hydrogen sulfide, indole, or acetylmethylcarbinol, nor do they hydrolyze

gelatin or starch although they are positive for catalase and urease and reduce nitrates to nitrites. Conflicting results reported for the presence of arginine dihydrolase may be due to different criteria used for evaluating the results. This test is essentially a quantitative measure of the activity of this enzyme, since the vast majority of strains evinced some degree of positive activity, providing the incubation period extended for at least 8 days. The relative homogeneity of this organism in biochemical properties could, of course, be more apparent than real since only a limited number of biochemical reactions were tested.

Whatever variations in biochemical properties were observed were not regular enough to suggest categorizations at the species or subspecies level. The only feature which permitted subdivision among the strains was the growth factor requirements which ranged from no requirements at all to the requirement for definable growth supplements, to complex and undefined requirements. Of the 4 catagories used, only Group I showed a general internal uniformity; most members of this group were efficient utilizers of substrates, giving early and positive response to tests. One member of this group, P 38, was notable in its ability to utilize residual material on plates and was scored as positive on substrates, which on subsequent examination turned out not to be utilized by this organism. It was necessary to re-test growth of this strain on these substrates by cultivation in liquid media or by subcultivation on plates. Two other members of Group I, P 35 and 981, showed a similar behavior but to a lesser degree. Both Group II and Group III are

characterized by an irregular pattern of vitamin requirements. In general, however, those strains which required only a single vitamin, biotin, did not require aspartate, and those organisms that required this amino acid usually required both thiamine and biotin or niacin as well. Because of the great potency of biotin, clear cut results with this vitamin were never easy to obtain. The most reliable approach for assay of biotin was successive subculturing in biotin-free liquid media. This method, however, enhanced the possibility of the selection of variants which could readily grow in the absence of biotin. With a number of strains, the results were unequivocal, but with others, it is possible that both biotin in conjunction with niacin and/or thiamine are required. Several organisms gave puzzling results in that they required niacin and either thiamine or biotin (Table 9). It is not clear how either biotin or thiamine could supplant one another, unless, of course, neither is actually required but some contaminant common to both is present. No growth of these organisms was ever found on niacin alone. This test was repeated by serial subcultivation using acetate as the carbon and energy source instead of casamino acids, with the same results. Growth was noticeably slow when cultures were subcultured in the casamino acid basal medium used to test for vitamins; presumably this was due to the limited concentration of substrates. Glucose had been provided in these initial experiments, but as was learned later, it was not utilized. When the vitamin assay was repeated with selected strains using acetate as the carbon and energy source, conflicting results were sometimes

obtained. Several organisms which earlier seemed to require vitamins in the casamino acid medium grew without them later when acetate was the substrate. One organism which previously had shown a need for thiamine and biotin later showed a requirement for niacin as well. In general, the strains exhibited a heterogeneous array of growth factor requirements with no clear cut evidence of any well defined biotypes. It is entirely possible that the failure to define nutritional subgroups may be due to inadequate representation of organisms. Had more strains been examined, internally homogeneous groups may have been found. There is some evidence of such groups, particularly with respect to vitamin requirements. More work needs to be done, however, to characterize some of the strains whose vitamin requirements are ambiguous. On the other hand, it may be that the internal heterogeneity of this group of organisms tested is a natural feature characterizing this organism as a whole, and that the differences noted are an insignificant fraction of the total.

The organisms placed in Group IV seem to exhibit nutritional requirements greatly different from all other organisms and it might be possible on this basis to assign them to a different species. However, such differences might be caused by a simple metabolic defect in an unknown but critical enzyme system, in which case the organisms would simply be defective strains of the same species. Until the exact nutritional requirements can be worked out, it will be impossible to decide between these choices. Determination of the GC content of their DNA might be helpful in deciding this

question, although it would only give a definitive answer if the base composition differed from the remainder of strains.

It might be argued that nutritional characters of laboratory cultures are too subject to selective pressures to be used for this purpose. However, strains maintained for years in the laboratory indicated the same general nutritional and biochemical pattern as newly isolated strains. Thus, there is ample evidence to suggest that these characteristics do not change and that laboratory strains may be maintained without fear of selecting for a particular characteristic. Knight and Proom (1950) commented on the similarity in vitamin requirements of old laboratory cultures and fresh isolates of Bacillus species, and similar observations have been made with Proteus species (Proom and Woiwood, 1951). There is cumulative evidence suggesting that the basal vitamin requirements of a bacterial species are among one of its most constant characteristics (Proom, 1955). It was necessary in this study to subculture these strains a number of times before the characteristic nutritional activity was restored, but when this was done the cultures reverted to their previously described characteristics. One slight discrepancy noted was that most cultures obtained from other laboratories gave markedly positive reactions for arginine dihydrolase compared to initial isolates from this study. Preliminary investigations indicated that subculturing may lead to stronger reactions. Another exception noted in a number of laboratory cultures was their failure to regain ability to sporulate despite evidence from other workers of their sporulating abilities (MacDonald and MacDonald, 1962; Kocur and

Martinec, 1963). However, it should be pointed out that considerable difficulty was also encountered in obtaining reproducible results on various spore media with initial isolates from this study, and substantial differences were noted among all strains tested in sporulating capacities. Future investigations might focus on comparative studies of sporulation of S. ureae and on the sporulating process itself. To my knowledge, these areas have not been explored with this organism.

As pointed out earlier, S. ureae is closely allied with the genus Bacillus on the basis of spore production, similar GC content in DNA and similar biochemical reactions. Results from the nutritional study suggest further correspondence with Bacillus species. The requirement for biotin and thiamine and the ability to utilize ammonium salts as sources of nitrogen are nutritional characteristics often encountered among the aerobic sporeforming rods (Knight and Proom, 1950; Proom and Knight, 1955).

The Bacillus species which is most often compared with S. ureae is Bacillus pasteuri because this species, like S. ureae, is ureolytic and has a similar GC content (MacDonald and MacDonald, 1962; Auletta and Kennedy, 1966; Venner, 1967; Boháček, Kocur, and Martinec, 1968). This organism shows some nutritional properties which are not generally encountered with other Bacillus species, but which it shares with Sporosarcina. From a nutritional study of ten strains of B. pasteuri, Knight and Proom (1950), reported that the nutritional requirements of this organism were more heterogeneous than with most of the other species of Bacillus they studied. Some

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strains of B. pasteuri showed an additional need for niacin in addition to thiamine and biotin, notable since the requirement for niacin is rare among Bacillus species (Knight and Proom, 1950). Furthermore, both S. ureae and B. pasteuri share a distinguishing physiological characteristic, namely the inability to utilize glucose. Only two other Bacillus species show this same metabolic limitation. Unlike S. ureae, B. pasteuri requires the amino acids of casein hydrolysate either with or without ammonia for growth and either urea or an alkaline medium. One might speculate that perhaps some members of Group IV might have similar requirements for growth. Several clues point to such a possibility (refer to results of nutritional groups).

It would appear that selective pressures due to growth in a common environment have resulted in evolutionary convergence of Sporosarcina ureae and Bacillus pasteuri with respect to their physiological characteristics or divergence from a common prototype. Their possession of a relatively unique constellation of mutual properties and indications of a general restrictive type of metabolism suggests this convergence.

In this study, an attempt was made to correlate physiological and nutritional properties with habitats from which organisms were isolated. Stanier, Palleroni, and Doudoroff (1966), noted with pseudomonads, for example, an almost perfect correlation between the property of liquefaction of gelatin and the source of isolation; isolates from soils were non-liquefiers, those obtained from water sources were liquefiers. These workers also described differential

activities on certain substrates between these two ecological groups of organisms. Unfortunately all of the water samples tested in this work were negative, and the only isolate from water (Wood, 1946), was unavailable.

A comparison of isolates between soils from different areas failed to give consistent differences. Physiological and morphological variability within samples collected from the same site was essentially the same as samples collected from widely separated geographical sites (e.g., those areas differing particularly in climate and/or in soil type). In other words, there appeared to be no marked similarities between isolates from a particular site which might separate them from other isolates. For example, although both P16-A and P16-B isolated from the same soil sample exhibited a similar nutritional and biochemical pattern of activity (Tables 6-9, 12-13), P16-A showed no absolute requirement for growth factors, whereas P16-B indicated a requirement for both thiamine and biotin. These strains, in fact, exhibited properties more in common with members of other environments than with each other. Similarly, among three organisms isolated from a single soil sample from Berkeley, California, P21-A, P21-B, and P21-C, one (P21-B), showed far more complex requirements than the other two. This one organism appeared to be as closely allied in nutritional activity with several other organisms from different environments (Group III) as with the other two strains from the same soil sample.

On the other hand, two organisms, P35 and P38, isolated from

widely different geographical areas and from different types of soils, exhibited remarkably similar nutritional activity and behavior. Both, for example, were extremely efficient utilizers of small amounts of substrates, required no growth factors and showed strong positive growth on almost all utilizable substrates. Close examination of Tables 6-11 reveals other examples of lack of correlation of strains isolated from the same samples.

In general, no overall differences in nutritional behavior were observed with strains isolated from different collection sites with one possible exception. Within areas of Japan, there is a suggestion of such a correlation. Isolates from this area were generally among the slowest growing organisms and included some of the most nutritionally fastidious isolates collected. Only one strain of the fifteen collected from Japan belonged to Group I needing no growth factors. Six of the Japanese strains grew poorly or not at all unless both vitamins and amino acid supplements were included, and two strains required low concentrations of complex materials for growth. Moreover, one strain, P29, was unique among all the strains studied in its inability to grow on any medium except TSY (See Table 12).

It is evident that organisms showing a spectrum of nutritional fastidiousness were isolated from the soil. It is not likely, however, that such isolates were inadvertently selected for a given set of nutritional requirements, since the medium was nutritionally rich and complex, and thus would not likely have favored the selection of good synthesizers of essential metabolites. Knight and Proom

(1950), made the observation that a disproportional number of nutritional intermediates were encountered by them when strains of Bacillus species were isolated from soils rather than from other sources.

Organisms belonging to the genus Sporosarcina are readily identifiable because they share some striking characteristics which permits unambiguous recognition. Only one species has been recognized in this genus. This species has been defined on the basis of the criteria studied--by its morphology, spore production, DNA base composition, and biochemical reactions. In general, these strains show a homogeneity of these characters and, unlike a number of other bacterial species, do not reveal a constellation of differential characters which might permit recognition of additional species. In this study it was hoped that a nutritional pattern might emerge which could be also correlated, perhaps, with corresponding differences in morphology, physiology, or ecology of this organism. The results of this study did not, however, reveal any consistent differences in morphology, physiology, or in the ecology of Sporosarcina that could be justifiably correlated with nutritional differences. This study did find, although the data collected is very incomplete, that the leading criterion, that is, the character that appears to have the greatest significance in species identification and classification, may be the nutritional differences shown by this organism. It remains to be seen whether this heterogeneity is a reflection of imperfect differentiation because too few strains were examined or is a natural feature

characterizing this group as a whole.

In view of the specific nutritional requirements indicated by some strains it would be worthwhile investigating this organism at the biochemical level. It should not be difficult to locate the specific metabolic lesions by determining the enzymatic constitution of certain enzyme systems.

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