DWARF COLONY MUTANTS OF SALMONELLAE

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Bacterial variants which differ from corresponding normal strains by forming much smaller colonies on solid media are not uncommon. They can be isolated from natural sources or be produced in the laboratory from many bacterial species (Clowes and Rowley, 1955). Among the salmonellae, a dwarf colony variant of *Salmonella typhosa* was isolated by Jacobsen (1910) during a typhoid epidemic. Later, Kristensen and Kauffmann (1935) isolated a similar variant of *Salmonella eastbourne* from a normal strain and found it to be also anaerogenic. More recently, Morris *et al.* (1943) reported an out-break of typhoid fever due to a small colony form of *S. typhosa*.

Several other species of the genus *Salmonella* including *S. pullorum*, *S. typhi-suis*, and *S. abortus-ovis* also grow slowly on conventional media and form small colonies (Stokes and Bayne, 1957). This creates difficulties in isolation because these slowly growing strains may be overgrown in liquid enrichment media by competitors and, also, they may not be detected on agar media.

In continuation of our previous attempts to determine the cause of slow growth, efforts were made to isolate slowly growing mutants from normal, rapidly growing Salmonella strains. It was thought that a detailed comparison of the properties of parent strains and mutants might reveal the reasons for slow growth. Such mutants have been obtained from a number of *Salmonella* species and are described in the present paper.

MATERIALS AND METHODS

Growth of salmonellae on solid media was determined by measuring the diameter of colonies on trypticase soy agar, nutrient agar, and other media. The colonies were obtained by streaking plates from 4 or 5 hr trypticase soy broth cultures. Usually 4 to 8 well isolated colonies on a plate were measured under a wide-field microscope equipped with a calibrated ocular micrometer, at magnifications of 12, 24, or 36 and the results were averaged. Growth in liquid media was measured in a Klett-Summerson photometer equipped with a red filter. All cultures were incubated at 35 C.

The salmonellae strains investigated were *S. newport*, *S. derby*, *S. typhimurium*, *S. oranienburg*, *S. worthington*, *S. bareilly*, *S. senftenberg*, *S. anatum*, *S. montevideo*, and *S. meleagridis*. Additional details of technique will be given later as needed.

RESULTS

Isolation of dwarf mutants. The 10 serotypes of salmonellae used were normal strains which grew rapidly on agar and in liquid media (Stokes and Bayne, 1957). To obtain slowly growing mutants, the parent strains were inoculated into tubes of nutrient broth which contained 1 per cent of either lithium sulfate or sodium selenite as possible mutagenic agents. The cultures were inoculated at 35 C for several weeks and during this time they were periodically streaked on trypticase soy agar plates to detect the presence of dwarf colonies.

Usually, after one or two weeks, dwarf colonies were obtained from the lithium sulfate cultures and occasionally from the selenite tubes but never from plain nutrient broth cultures. The original dwarf colonies when restreaked usually gave rise to a mixture of large and small colonies in which the large or parent type colony frequently predominated. Stable dwarf strains were obtained by repeated reisolations from small colonies. The only exception was the dwarf mutant of *S. oranienburg* which did not revert to the large colony form at any time. Dwarf mutants were isolated from all of the 10 *Salmonella* species.

On trypticase soy agar, the parent strains gave rise to colonies that were 2 mm or more in diameter within 18 hr, whereas the colonies of the dwarf strains were about 0.7 mm in diameter or

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about one-third that of the parent strains (table 1). On nutrient agar, growth of both parent and dwarf strains is slower than on trypticase soy agar and the dwarf colonies may be as little as 0.1 mm or less in diameter in 18 hr. The difference in colony size of parent and dwarf strains is more striking when the colonies themselves are viewed (figure 1).

On incubation beyond one day, the colonies of both parent and dwarf strains continue to increase in size but the difference between the two remains fairly constant. For example, in the case of *S. oranienburg*, the colony diameters of the parent strain at 24, 48, and 72 hr were 2.9, 5.1, and 5.6 mm, respectively, whereas those for the dwarf strain were 0.9, 1.2, and 1.4 mm.

TABLE 1

Size of colonies of parent and dwarf strains of salmonellae

Species	Colony Diameter*	
	Parent	Dwarf
	mm	*mm*
Salmonella newport	2.1	0.7
Salmonella derby	2.2	0.7
Salmonella typhimurium	2.1	0.8
Salmonella oranienburg	2.3	0.6
Salmonella montevideo	2.3	0.7

* After 18 hr on trypticase soy agar.

The best way of measuring the difference in growth rates of colonies of parent and dwarf strains is to determine the number of cells in each type of colony. This has been done for several strains by plating representative colonies. Data for *S. typhimurium* and *S. oranienburg* are given in table 2. Although the diameters of dwarf colonies are $\frac{1}{3}$ to $\frac{1}{4}$ those of the parent strains, the number of cells in dwarf colonies is only $\frac{1}{50}$ that in parent strain colonies. The difference between the two types is therefore quite large.

These marked differences in growth rates of parent and dwarf strains are also evident in broth cultures. As shown in figure 2, the parent strain of *S. oranienburg* grew rapidly and abundantly in trypticase soy broth whereas the dwarf strain derived from it grew slowly and sparsely.

Properties of parent and dwarf strains. There were no differences in the morphology or reaction to the Gram stain of parent and dwarf cultures. Both consisted of short, gram-negative rods. Also, the dwarf strains agglutinated readily with the same group specific O-sera as the corresponding parent strains. A more detailed serological analysis might disclose antigenic differences between the two types of strains but such an analysis has not been made.

A significant difference, however, was noted in the ability of parent and dwarf strains to ferment carbohydrates. The formation of acid and gas in

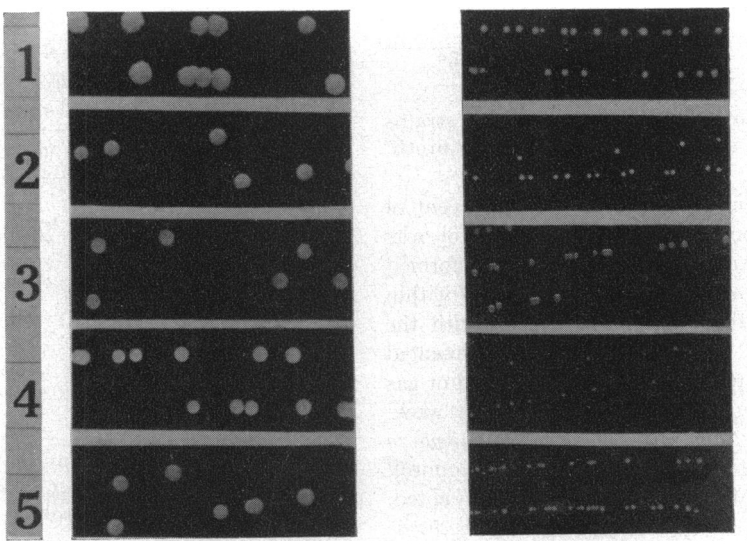

Figure 1. Colonies of salmonellae and dwarf mutants derived from them. Natural size on trypticase soy agar after 18 hr incubation. *1. Salmonella bareilly, 2. Salmonella montevideo, 3. Salmonella anatum, 4. Salmonella newport,* and *5. Salmonella oranienburg.*

TABLE 2

Relation of colony size to numbers of cells in colonies of parent and dwarf strains of salmonellae

	Colony Size	Bacteria	Ratios	
			Colonies	Bacteria
	mm	×10⁶		
Salmonella typhimurium				
Parent	2.0	164	3.3:1	53:1
Dwarf	0.6	3.1		
Salmonella oranienburg				
Parent	2.2	178	3.7:1	49:1
Dwarf	0.6	3.6		

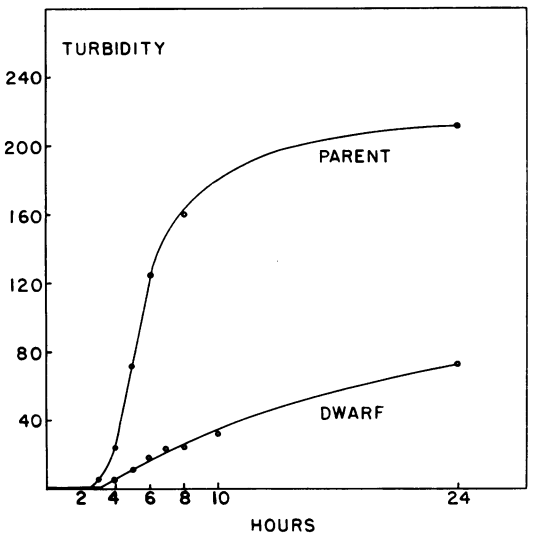

Figure 2. Growth of parent and dwarf strains of *Salmonella oranienburg* in trypticase soy broth.

tubes of nutrient broth containing 1 per cent of glucose, arabinose, dulcitol, and mannitol was investigated. All of the parent strains formed acid and gas from the four carbohydrates within one day. The dwarf strains, however, with the exception of *S. derby* and *S. anatum*, fermented the carbohydrates much more slowly. Acid or gas or both sometimes did not appear for a week. Moreover, the dwarf strain of *S. oranienburg* failed entirely to ferment dulcitol and mannitol. Loss of fermentative capacity has been noted, also with small colony variants of *Staphylococcus aureus* (Swingle, 1935) and *Escherichia coli* (Colwell, 1946) in addition to that of *Salmonella eastbourne* mentioned previously.

A limited study was made of the oxidation of amino acids by several of the parent and dwarf cultures. The usual Warburg manometric techniques were employed. The parent strains rapidly oxidized alanine, serine, threonine, proline, aspartic acid, and glutamic acid. Two dwarf strains were equally good oxidizers but the dwarf of *S. oranienburg* failed to oxidize these amino acids or attacked them slowly. In contrast, this dwarf strain oxidized glucose almost as rapidly as its parent strain.

Growth factor requirements. All of the parent strains grew well in a simple, chemically defined medium composed of the following ingredients and their percentages: glucose, 1.0; $(NH_4)_2SO_4$, 0.1; Na citrate·$2H_2O$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.05; phosphate buffer, pH 7.0, M/20 and distilled water.

When the dwarf strains were inoculated into this medium, four of them failed to grow. The possibility existed, therefore, that these four dwarf strains were biochemically deficient mutants which required vitamins, amino acids, or other growth factors for development. Further investigation proved this to be the case as shown by the data in table 3. Three of the dwarf strains grew when the above basal medium was enriched with amino acids in the form of casein hydrolyzate. The fourth mutant, that of *S. meleagridis*, grew when a mixture of B-vitamins was added in addition to the amino acids.

TABLE 3

Growth factor requirements of dwarf mutants of salmonellae

Dwarf Mutant	Basal Medium Plus			
	Nil	B-vitamins*	Amino acids†	B-vitamins + Amino acids
Salmonella oranienburg	−	−	+	+
Salmonella anatum	−	−	+	+
Salmonella bareilly	−	−	+	+
Salmonella meleagridis	−	−	−	+

* Mixture of thiamin, pyridoxin, riboflavin, biotin, and pantothenic, nicotinic, folic and *p*-aminobenzoic acids (Stokes and Gunness, 1945).

† 0.3 Per cent vitamin-free casein hydrolyzate plus 25 μg of L-cystine and 100 μg of DL-tryptophan per ml of medium.

The casein hydrolyzate could be replaced by a mixture of 20 amino acids (Stokes and Gunness, 1945). By eliminating one amino acid at a time from the mixture and observing the effect of such elimination on growth, it was possible to determine the specific amino acids required by the nutritionally deficient dwarf mutants. The vitamin requirement of *S. meleagridis* was also determined in a similar manner. The specific growth factor requirements were: *S. oranienburg*, cystine; *S. anatum*, cystine; *S. meleagridis*, cystine and pyridoxin; and *S. bareilly*, cystine, methionine, tryptophan, tyrosine, and phenylalanine. The outstanding requirement is for cystine. All four dwarf mutants need it for growth and for three of them it is the only amino acid required.

Effect of cystine. These results suggested that slow growth of the mutants on trypticase soy agar might be due to a shortage of cystine in that medium. Cystine combines with reducing sugars and other aldehydes even in neutral solutions at room temperature (Schubert, 1939). Such complexes are formed also in microbiological media on autoclaving and, in addition, there may be outright destruction of the cystine. Some cystine-requiring bacteria can use the complex fully as well as cystine itself, whereas for other bacteria it is only partially effective (Riesen *et al.*, 1947; Lankford *et al.*, 1957).

Trypticase soy agar contains considerable amounts of carbohydrate which is supplied by the phytone component (Baltimore Biological Laboratory, 1956). It is possible, therefore, that cystine inactivation as well as destruction may occur during the commercial preparation of the dehydrated medium and during autoclaving. If the medium indeed lacks adequate amounts of cystine and if this is the factor which limits growth of the dwarf mutants, then addition of sufficient cystine to the medium should permit the mutants to grow rapidly and produce colonies of the same size as the parent strains.

As shown in figure 3, addition of 25 μg of L-cystine per ml of trypticase soy agar, greatly stimulated the cystine-requiring dwarf strains of *S. anatum* and *S. oranienburg*. The growth rates of the two mutants were equivalent to those of the corresponding parent strains and like the latter, the colonies formed in 18 hr were 2 mm or more in diameter.

Quantitative experiments indicated that 5 μg of cystine must be added per ml of medium for maximum growth stimulation. Cysteine can fully replace cystine, but glutathione, methionine, tetrathionate, and sulfite exhibit little or no activity.

The *S. bareilly* mutant, which needs several amino acids for growth, was not stimulated by addition of cystine to trypticase soy agar but was greatly stimulated by addition of a mixture of amino acids in the form of 0.3 per cent casein hydrolyzate enriched with cystine and tryptophan. Cystine, apparently, is not the only amino

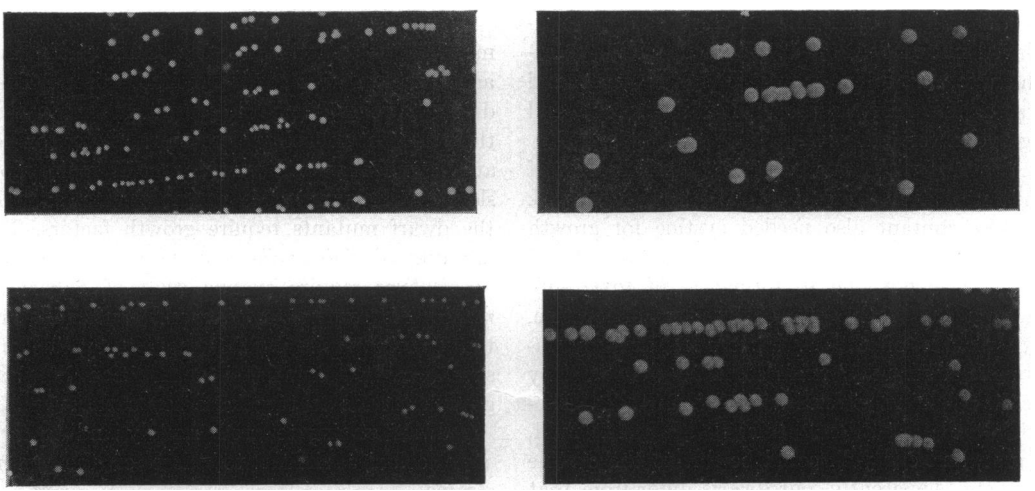

Figure 3. Stimulation of salmonellae dwarf colonies by cystine (25 μg/ml). Natural size after 18 hr incubation on trypticase soy agar. (*Upper set*) *S. anatum*, without and with cystine; (*Lower set*) *S. oranienburg*, without and with cystine.

acid present in suboptimum amounts in the medium.

The dwarf strain of *S. meleagridis* did not respond to addition of cystine and pyridoxin to trypticase soy agar although these were the only two growth factors required. Substitution for pyridoxin, however, of the coenzyme form, namely, pyridoxal-5-phosphate (1 μg/ml), led to more rapid growth of this dwarf mutant.

Unidentified growth factors appear to be required by some of our Salmonella dwarf mutants. These are the 6 strains which grew in the chemically defined medium without any vitamins or amino acids and yet they grew slowly on trypticase soy agar. These mutants apparently need unknown stimulatory factors rather than completely indispensable ones in order to grow rapidly. The possibility that slow growth may be due to toxic substances in the media has not been entirely eliminated although the data do not seem to lead in that direction. Dwarf colony variants of *Alcaligenes faecalis*, *Shigella sonnei*, and other bacteria which require vitamin B_1, vitamin B_{12}, or other unidentified growth factors have been described by Weinberg (1950).

DISCUSSION

Jacobsen (1910) concluded that the slow growth of his dwarf mutant of *S. typhosa* on nutrient agar and other media was due to inhibitory substances formed by heat during preparation and sterilization of the media. Moreover, he noted that the dwarf mutant produced large colonies even on repeatedly autoclaved nutrient agar if 0.1 per cent sulfite, tetrathionate, or other inorganic sulfur compound was added. Also, ascitic fluid, blood serum, and egg yolk were effective.

Results with our cystine requiring dwarf mutants strongly suggest that Jacobsen's *S. typhosa* mutant also needed cystine for growth and that retardation of growth in autoclaved media was not due to formation of toxic substances but rather to destruction of cystine. Stimulation of the *S. typhosa* mutant by inorganic sulfur compounds could be due to ability of the organism to form cystine from them. Likewise, the stimulation obtained with ascitic fluid, serum, and egg yolk could be due to the cystine in them. Apparently, our dwarfs differ from that of Jacobsen in that they cannot convert inorganic sulfur to cystine.

Cystine may play still another role in the growth of salmonellae under special conditions. North and Bartram (1953) found that addition of small amounts of cystine to selenite enrichment broth stimulated the growth of salmonellae. They used apparently normal strains of salmonellae, mainly *S. typhimurium*, in their experiments. Such strains do not usually require cystine or any other exogenous growth factor. Their need for cystine may be related to the fact that the selenite in selenite broth not only retards the growth of *E. coli* and other salmonellae competitors but also, to some extent, that of the salmonellae themselves (Stokes and Osborne, 1955). It is known that selenium can interfere with normal sulfur metabolism by substituting for sulfur in the synthesis of amino acids to form toxic selenium analogues of these amino acids (Shrift, 1954 *a*, *b*; Cowie and Cohen, 1957). The stimulatory effect of cystine on growth of salmonellae in selenite broth may be due, therefore, to neutralization of the toxic action of selenium by cystine.

SUMMARY

Dwarf colony mutants have been isolated from 10 normal salmonellae strains by growing the latter in nutrient broth containing lithium sulfate or sodium selenite. The dwarf mutants produce colonies on trypticase soy agar which are ⅛ or less in diameter than those of the corresponding parent strains and contain only 1/50 as many cells.

The mutants resemble the parent strains in morphology, reaction to the Gram stain and agglutination with group-specific antisera. They differ, however, from the parent strains in that they ferment carbohydrates more slowly, or not at all, and may oxidize amino acids much more slowly. Also, unlike the parent strains, some of the dwarf mutants require growth factors. The mutants of *Salmonella anatum* and *Salmonella oranienburg* require cystine, that of *Salmonella meleagridis* requires cystine and pyridoxin, and that of *Salmonella bareilly* requires cystine and several other amino acids. Addition of these growth factors to trypticase soy agar permitted he dwarf strains to grow rapidly and to form arge colonies similar to those of the parent strains.

The data suggest that dwarf colony strains of salmonellae are growth-factor-dependent mu-

tants which grow slowly on conventional media because the latter do not contain adequate amounts of the required essential or stimulatory growth factors.

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