GROWTH RATES OF SALMONELLA COLONIES

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Most species of Salmonella grow rapidly and luxuriantly on ordinary agar media. In contrast, Salmonella pullorum and a few other species or types such as Salmonella abortus-ovis, Salmonella sendai, and Salmonella typhi-suis develop slowly and sparsely (Kaufmann, 1954). The reasons for the poor growth of these strains are not known. Also, their sparse growth makes isolation more difficult. In the present investigation, the rates of growth of a large number of Salmonella on solid media, and to a limited extent in liquid media, have been determined under a variety of environmental conditions. Particular attention has been given to the slow growth of S. pullorum and to attempts to increase its rate of development.

MATERIALS AND METHODS

The rate and extent of growth of Salmonella on solid media were determined by measuring the diameter of the colonies on trypticase soy agar, nutrient agar, and other media. The colonies were obtained by streaking from 4 or 5 hour trypticase soy broth cultures. An important advantage of this method is that most colonies will arise from single cells and therefore the size of inoculum for all of the strains in all experiments was essentially identical and constant. Provided well isolated colonies are measured, agreement between replicate colonies on a plate and between replicate plates is close. Moreover, colony size is not significantly affected by the depth of agar, total amount of growth on the plate, and age of inoculum, in the range of 4 to 24 hr. Usually 4 to 8 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 the Klett-Summerson photometer equipped with a red filter. All cultures were incubated at 35°C unless otherwise indicated.

The Salmonella species and strains investigated included S. paratyphi B (2 strains), S. derby, S. senftenberg (4 strains), S. meleagridis (2 strains), S. anatum (2 strains), S. bareilly, S. panama, S. montevideo (2 strains), S. typhimurium, S. worthington, S. newport, S. oranienburg (3 strains), S. muenchen, S. gallinarum (12 strains), and S. pullorum (10 strains). Towards the end of our investigations several additional species and strains were examined briefly. These included S. typhi, S. sendai (2 strains), S. paratyphi A (3 strains), S. abortus-ovis (2 strains), S. typhi-suis (2 strains), and S. fulica.

RESULTS

Growth rates. The extent to which Salmonella strains grow in 17 hr at 35°C on trypticase soy, nutrient, and brilliant green agar is shown in table 1. The results are representative for all of the 44 strains originally examined. On trypticase soy agar, all species except S. gallinarum and S. pullorum give rise to colonies which are approximately 2 mm in diameter. In contrast, colonies of all strains of S. gallinarum are about 1 mm in diameter and those of S. pullorum roughly 0.5 mm. On this basis, therefore, growth of S. gallinarum is only one-half and S. pullorum one-fourth of the other Salmonella species. These differences are increased by a factor of two if the areas rather than the diameters of the colonies are compared and even more if volumes are considered. These striking differences in colony size of the 3 groups of Salmonella are shown in the photographs in figure 1.

Similar colony diameter ratios were obtained on nutrient and brilliant green agar. Growth, however, of all Salmonella strains was less on these media than on trypticase soy agar. The poor growth of S. pullorum on brilliant green agar is especially significant because this medium is frequently used to isolate S. pullorum from enrichment cultures. Such small colonies may be missed if the plates are not carefully examined.

Growth curves can be obtained by following changes in colony diameter with time of incubation as shown in figure 2. S. gallinarum and especially S. pullorum exhibit a marked lag
TABLE 1

Differences in amount of growth of Salmonella on solid media

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number</th>
<th>Colony Diameter, mm*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tp-1</td>
<td>Trypticase soy agar</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>2.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Salmonella oranienburg</td>
<td>2.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Salmonella worthington</td>
<td>2.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Salmonella gallinarum</td>
<td>371</td>
<td>1.4</td>
</tr>
<tr>
<td>Salmonella gallinarum M</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Salmonella gallinarum 9</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Salmonella pullorum</td>
<td>3470</td>
<td>0.7</td>
</tr>
<tr>
<td>Salmonella pullorum</td>
<td>3340</td>
<td>0.5</td>
</tr>
<tr>
<td>Salmonella pullorum</td>
<td>3083</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* After incubation for 17 hr.

Most of the rapidly growing Salmonella, however, develop best at 40 C, whereas 35 C is the optimum for the gallinarum-pullorum groups. At 46 C, only an occasional strain of the rapidly growing Salmonella develops and the growth is slight.

The Salmonella strains will grow fully at the lower temperatures if the incubation period is prolonged beyond 1 day. At 20 C essentially full growth of all strains is obtained within 3 days. The same is true at 15 C after 7 days except that S. pullorum strains show no more than a trace of growth. At 10 C, however, there is virtually no growth of any of the Salmonella except for slight growth of a few fast growing species after 2 to 4 weeks of incubation. Not the slightest growth of any of the Salmonella was obtained in 1 month at 5 C and 1 C. The minimum temperature for the growth of Salmonella is, therefore, approximately 10 C. Likewise, the results at 46 C mentioned above plus additional data obtained by use of temperatures between 40 C and 46 C and by prolonged incubation at the latter temperature indicate that 46 C is about the maximum temperature for the growth of Salmonella. Similar results were obtained when trypticase soy broth rather than agar was used.

In general, however, S. pullorum and to a lesser extent, S. gallinarum, do not tolerate the extremes of the indicated temperature range as well as other Salmonella species. They also tend to develop best at 35 C compared to 40 C for most of the latter. It is clear that the slow growth of S. pullorum in the previous experiments cannot be ascribed to the use of an unfavorable incubation temperature.

Effect of pH. The media used in the previous experiments were at pH 7.0 to 7.2. It was of interest to determine to what extent hydrogen ion concentration influences the growth of Salmonella, especially S. pullorum. Trypticase soy agar was adjusted to pH 5, 6, 7 and 8 by addition of appropriate mixtures of 1 M phosphate buffer. The buffers were sterilized separately and added to the melted sterile trypticase soy agar in amounts sufficient to give a final concentration of 0.05 M. In addition, a small amount of H3PO4 was used to reduce the medium to pH 5. The indicated pH values of the media are those determined by direct measurement.

None of the Salmonella strains grow at pH 5. S. derby, representative of the rapidly growing strains, grew well at pH 6 whereas S. gallinarum and especially S. pullorum grew poorly at this
Figure 1. Comparative size of colonies of different Salmonella species grown on trypticase soy agar for 18 hr; natural size. (a) Salmonella worthington (b) Salmonella gallinarum (c) Salmonella pullorum.
pH level. The optimum for all strains is pH 7 and many but not all strains grow equally well at pH 8.

In another series of experiments in which the agar medium was adjusted with acid or alkali to pH 5, 6, 7, 8, 9, and 10, results similar to those with phosphate buffer were obtained. Moreover, all strains grew considerably at pH 9 and 10. At the latter pH level, the gallinarum-pullorum groups were more inhibited than the other Salmonella strains.

In trypticase soy broth, S. oranienburg, S. worthington, S. senftenberg and S. typhimurium, did not develop at pH 5 but made some growth at pH 5.6 whereas four strains of S. pullorum and two strains of S. gallinarum grew only when the pH was raised to 6.0.

In general, therefore, S. pullorum and S. gallinarum do not grow as well as other Salmonella species at the extremes of the pH range used. In this respect, the situation parallels that in the temperature experiments. Also, the growth rate of S. pullorum cannot be increased by shifting the pH level of the medium from pH 7; such shifts usually lead to slower growth.

Stimulation of S. pullorum by yeast extract. Most of our attempts to increase the rate of growth of S. pullorum were unsuccessful. It was not increased by the addition of the following materials to trypticase soy agar: (a) glucose or other sugars, (b) supernatant or aqueous extracts of rapidly growing S. typhimurium, (c) a large variety of plant and animal extracts, (d) CO₂ and compounds of the tricarboxylic acid cycle, (e) B-vitamins including pyridoxal phosphate, coenzyme A and lipoic acid and also purines and pyrimidines. Moreover, substitution of a variety of complete dehydrated media for trypticase soy agar did not lead to more rapid growth of S. pullorum.

The only substance which markedly and consistently increased the growth rate of S. pullorum on trypticase soy agar was yeast extract either in the form of the dehydrated product (Difco) or as yeast autolysate or the Basaminobact of Anheuser-Busch. The extent of stimulation is shown in table 2. The diameter of S. pullorum colonies increased by approx 40 to 70 per cent and approached or exceeded the diameter of S. gallinarum colonies on trypticase soy agar alone. The rapidly growing Salmonella and S. gallinarum

Figure 1c.
yeast extract has been noted also by Gilfillan et al. (1955).

The data in table 3 show that the addition of 2 per cent yeast extract to the selective media, brilliant green, SS, and bismuth sulfite agar (commonly used for the isolation of Salmonella) also increases the growth of S. pullorum. These results and others that we have obtained indicate that, in general, strains of S. pullorum do not grow as well on selective agars as on trypticase soy agar and that some strains grow very poorly on such agars. Bismuth sulfite medium is the

also are stimulated by yeast extract so that the effect is not a preferential one. Five tenths per cent yeast extract is as effective as 2 per cent and greater amounts may be inhibitory. There is very little stimulation with 0.1 per cent yeast extract. The responsible factors in yeast extract are not known but they are not inorganic, since the ash is not stimulatory. Yeast extract also increases growth in trypticase soy broth. Interestingly, it does not appreciably reduce the lag period of S. pullorum but rather increases its rate of growth during the logarithmic phase and also the total cell crop. Growth stimulation of S. pullorum by

**TABLE 2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number</th>
<th>Colony Diameter, mm*</th>
<th>Without 2 per cent yeast extract</th>
<th>With 2 per cent yeast extract</th>
<th>Per Cent Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pullorum</em></td>
<td>3179</td>
<td>0.54</td>
<td>0.74</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td><em>S. pullorum</em></td>
<td>3470</td>
<td>0.52</td>
<td>0.87</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td><em>S. pullorum</em></td>
<td>1431</td>
<td>0.71</td>
<td>1.1</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td><em>S. pullorum</em></td>
<td>222</td>
<td>1.0</td>
<td>1.2</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td><em>S. pullorum</em></td>
<td>4411</td>
<td>1.0</td>
<td>1.3</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td><em>S. pullorum</em></td>
<td>3253</td>
<td>2.0</td>
<td>2.3</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td><em>S. pullorum</em></td>
<td>6266</td>
<td>2.1</td>
<td>2.8</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

* After incubation for 18 hr.
most inhibitory and brilliant green the least. Inhibition of Salmonella by bismuth sulfite agar when streaked from selenite enrichment cultures has been reported by Williams Smith (1952).

The addition of yeast extract to selective agar media would be helpful in the isolation of S. pullorum provided the selective properties of such media are retained in the presence of yeast extract. Experiments with Proteus vulgaris, Escherichia coli, Aerobacter cloacae and Alcaligenes faecalis indicate that the selective activity of brilliant green is largely nullified by yeast extract as might be expected from previously published data (Stokes and Osborne, 1955). However, the inhibition of the above mentioned bacteria was equally as effective on SS and bismuth sulfite agar with yeast extract as without it.

Additional slow growing Salmonella. After completion of most of the experimental work, additional cultures of Salmonella which have been reported to grow slowly on ordinary agar media were obtained from various sources and examined. These included S. typhi, S. paratyphi A, S. sendai, S. pullorum, S. abortus-ovis and S. typhi-suis. On both tryptcaseoy and nutrient agar, S. typhi, 2 strains of S. paratyphi A and 1 strain of S. sendai grew as well as other rapidly developing Salmonella. One strain each of S. paratyphi A, S. sendai and S. abortus-ovis and also S. pullorum exhibited the rate characteristic of S. gallinarum. The remaining S. abortus-ovis strain grew as slowly as S. pullorum. The latter results are in accord with those of Lovell (1931) who reported an average diameter of 0.7 mm for 6 strains of S. abortus-ovis when grown on agar for 18 hr at 37 C. The 2 cultures of S. typhi-suis developed extremely slow on both media; readily visible colonies were present only after 2 days of incubation. This organism, therefore, grows even more slowly than S. pullorum. As in the case of other Salmonella strains, the 6 species were stimulated by the addition of 2 per cent yeast extract to tryptcase soy agar.

The extremely slow growth of S. fulica, S. abortus-ovis and S. typhi-suis on brilliant green agar is particularly striking and significant. The colonies were 0.1 mm or less in diameter after 17 hr incubation. These organisms could easily be missed during isolation from natural sources unless extended incubation periods were used and the plates carefully examined. Such small colonies do not redden the medium surrounding them. S. fulica frequently failed to grow on brilliant green agar and only occasionally formed a few small colonies.

**DISCUSSION**

The results indicate that most species of Salmonella grow rapidly on solid culture media but that strains of S. pullorum develop much more slowly while those of S. gallinarum occupy an intermediate position. The differences in growth rates between the 3 groups are large and constant and may offer still another diagnostic means for identifying and differentiating S. pullorum and S. gallinarum (Hinshaw, 1941).

The slow development of S. pullorum compared to other Salmonella is due to a combination of a longer lag period and a lower rate of multiplication during the exponential phase. The more limited pH and temperature tolerance of S. pullorum may be due to this lower growth rate and may disappear when rapid growth is achieved. The slow growth of S. pullorum differs from that of the dwarf colonies of S. eastbourne and S. typhi (Kaufmann, 1954) in that the latter do not increase in size on continued incubation. Also, the dwarf colony variants give rise to normal sized colonies when grown in the presence of small amounts of thiosulfate or sulfite, whereas these compounds have no effect on the growth of S. pullorum.

The difficulty encountered in preferentially increasing the growth rate of S. pullorum by modification of pH, temperature, nutrients and growth factors suggests that inadequate permeability of the cells to nutrients may be a controlling factor in its slow growth. In this connection it may be significant that S. pullorum strains require exogenous supplies of amino
acids for growth (Johnson and Rettger, 1943). If permeability to essential amino acids were indeed limiting, this could account for the slow growth of *Salmonella pullorum*. Interestingly, many strains of *S. gallinarum*, *S. sendai*, *S. paratyphi A* and all of the other slow growing Salmonella also require either growth factors or amino acids or both, since they will not grow in a medium of glucose, ammonium nitrogen, and inorganic salts. In contrast virtually all other Salmonella strains—and these grow rapidly—can develop in this or similar simple media (Kauffmann, 1954; Lederberg, 1947). Nutritional data on our strains support these conclusions. The rapidly growing Salmonella developed well in a medium composed of glucose, citrate, ammonium nitrogen and mineral salts, whereas the slowly growing strains required amino acids for growth and in some instances also B-vitamins. Recently Clowes and Rowley (1955) suggested that permeability may be the limiting factor in the growth of small colony variants of *Escherichia coli*. We have experiments now under way to determine to what extent the metabolism of amino acids and permeability influence the growth rates of *S. pullorum* and other Salmonella.

ACKNOWLEDGMENTS

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SUMMARY

The growth rates of a large number of *Salmonella* strains on agar media, and to a more limited extent in liquid media, have been determined under a variety of cultural conditions. Most species grow rapidly on nutrient and trypticase soy agar and form colonies that are 1.5 to 2.5 mm or more in diameter in 18 hr at 35 C. In contrast, all strains of *Salmonella pullorum* develop slowly and the colonies are therefore small, 0.3 to 0.8 mm, or approximately one-fourth the diameter of the rapidly growing Salmonella. Strains of *Salmonella gallinarum* exhibit growth patterns that are intermediate between the above two groups. The slower development of *S. pullorum* is due to a combination of a longer lag period and a lower rate of multiplication during the exponential phase.

The minimum, optimum and maximum growth temperatures for most Salmonella species are 10 C, 40 C, and 46 C, respectively. The pullorum-gallinarum groups grow best at 35 C and do not tolerate the extremes of the temperature range as well as other Salmonella. They also do not develop as readily as the rapidly growing Salmonella at pH 5 to 6 and above pH 8.

The rate of growth of *S. pullorum* on trypticase soy agar could not be increased by a variety of changes in physiological and nutritional conditions. Only the addition of yeast extract markedly increased the growth rate. This occurred also with the other Salmonella species. The data indicate that the addition of yeast extract to selective agars such as SS and bismuth sulfite may be helpful in the isolation of slow growing Salmonella.

REFERENCES


Hinshaw, W. R. 1941 Cysteine and related compounds for differentiating members of the genus *Salmonella*. Hilgardia, 13, 583-621.


