GLYCINE RESISTANCE IN *AGROBACTERIUM TUMEFACIENS*

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Received for publication April 20, 1961

ABSTRACT

BEARDSLEY, ROBERT E. (Manhattan College, New York, N. Y.). Glycine resistance in *Agrobacterium tumefaciens*. J. Bacteriol. **83**:6-13. 1962.—The application of the fluctuation test of Luria and Delbrück to the distribution of glycine-resistant bacteria among cultures of *Agrobacterium tumefaciens* strain B6 indicates that resistance arises by mutation in the absence of glycine. On glycine-supplemented medium, additional resistant colonies arise during prolonged periods of incubation. Their appearance is preceded by L-form growth. In general, the number of generations over which glycine resistance is inherited in the absence of glycine is increased by serial transfers on the selection medium. In liquid medium containing glycine, sensitive bacteria form spheroplasts. Resistant bacteria continue to grow as rod forms. In the medium employed, spheroplasts are unstable.

The mechanism by which *Agrobacterium tumefaciens* (Smith and Townsend) Conn induces autonomous growth in cells of susceptible plants remains an unresolved problem (Braun and Stonier, 1958). One experimental approach to an understanding of this mechanism is through the isolation of attenuated clones from initially virulent cultures. Longley et al. (1937) developed a technique for consistently attenuating cultures of the crown gall organism by serial transfers on media containing high concentrations of glycine. Although these observations have been confirmed and extended in several reports (Van Lanen, Baldwin, and Riker, 1940, 1952a; Van Lanen, Riker, and Baldwin, 1952b; Stapp, 1951; Rack, 1953), the mechanisms involved in attenuation have not been established.

The early work of McLeod and his group (Wyon and McLeod, 1923; Gordon and McLeod, 1926; McLeod, Wheatley, and Phelon, 1927) and of Whitehead (1924) clearly established the toxicity of certain monoamino, monocarboxylic amino acids, including glycine, for bacteria.

Gordon and Gordon (1943) observed the production of large forms by glycine. Subsequently, Dienes, Weinberger, and Madoff (1950) and Dienes and Zamecnik (1952) extended this finding and reported that glycine induces L forms. McQuillen (1960) recently reviewed the evidence indicating that glycine may interfere with normal cell-wall synthesis, resulting in osmotically fragile forms of bacteria that are lysed in nonstabilizing media. Gordon, Hall, and Stickland (1951) investigated the kinetics of bacterial lysis in media containing glycine and reported the resistance to lysis of organisms previously selected in glycine-supplemented media (Gordon, Hall, and Stickland, 1949). The mutational origin of resistance to amino acid toxicity was studied by Davis and Maas (1949). The effects of the selection of mutants resistant to amino acids on virulence have been investigated in *Brucella* (Goodlow, Mika, and Braun, 1950; Goodlow et al., 1952) and *Salmonella typhimurium* (Page, Goodlow, and Braun, 1951). The present paper reports experiments designed to test the assumption that the selection of glycine-resistant mutants is involved in the attenuation of *A. tumefaciens* and to determine the fate of nonresistant bacteria in media containing high glycine concentrations.

MATERIALS AND METHODS

Bacteria. *A. tumefaciens* strain B6 (Stonier, 1960) was used throughout these experiments. Several derivatives of the B6 strain were isolated from colonies growing on media supplemented with glycine. All stock cultures were maintained at 5 C on nutrient agar slants.

Media. 1) Liquid media:—Modifications of the medium employed by Stonier (1956) were used. Minimal medium consisted of the inorganic salts and citrate in the concentrations designated by Stonier but did not contain glutamate. Glutamate medium contained Na glutamate (Matheson Co., Matheson, Coleman & Bell Division, Norwood, Ohio), 2 g/liter. Nutrient medium was prepared by adding 0.5% yeast ex-

tract (Difco) and 0.5% casein hydrolysate (Difco) to minimal medium.

2) Solid medium for plating:—In poured plates, the bottom layer of medium was solidified with 1.5% agar (Difco), the top layer with 0.7% agar. Bacteria were plated by suspending them in 2.5 ml of molten top-layer agar. This suspension was poured onto the surface of bottom-layered plates containing about 30 ml. All platings were made on nutrient medium to which supplemental glycine was added to obtain the concentrations designated. The glycine concentration in the unsupplemented medium was not determined.

3) Nutrient agar slants:—Slants were prepared with nutrient broth (Difco), 16 g/liter, plus 0.5% NaCl, and solidified with 2% agar.

4) Solutions:—Stock solutions of 1 M glycine (Eastman Organic Chemicals, Division of Eastman Kodak Co., Rochester, N. Y.) in minimal medium without citrate were sterilized by autoclaving 10-ml portions at 18 psi for 5 min.

Procedures. All cultures and plates were incubated at 26 C. Flask cultures contained 25 ml of medium and were not aerated. Tube cultures contained 5 ml and were aerated on a tissue-culture roller rotating at 1 rev/min.

The phage-sensitive strain and experimental techniques employed in phage assays were those previously reported (Beardsley, 1960).

RESULTS

Effect of glycine on survival. When growing cultures of B6 in glutamate medium were diluted and plated on nutrient medium supplemented with glycine at concentrations greater than 0.012 M, there was a marked reduction in the number of colonies formed, even when incubation periods were prolonged for 30 days in a moist chamber. When colonies were counted after 4 to 6 days of incubation, fewer than 0.1% of the plated bacteria formed visible colonies on medium supplemented with 0.013 M glycine, but 50% or more formed colonies in the presence of 0.012 M glycine (Fig. 1). Following this abrupt decrease, the surviving fraction decreased exponentially. The surviving fraction on 0.02 M glycine was less than 10^{-6} .

When bacteria isolated from colonies grown on plates supplemented with 0.02 M glycine were cultured in glycine-free glutamate medium and replated on various concentrations of glycine, the surviving fraction on 0.02 M glycine was

FIG. 1. Survival curves of *Agrobacterium tumefaciens* strain B6 on glycine-supplemented nutrient agar. Survival curve of original strain (○). Survival of initially resistant clone isolated at 0.02 M level (●) and resistant clone isolated at 0.03 M level (×). Cultures were grown in glutamate medium prior to plating.

usually greater than 0.4. Increasing concentrations above 0.02 M prevented the growth into visible colonies of a constantly decreasing fraction of the plated bacteria (Fig. 1).

When bacteria isolated from colonies formed by the 0.02 M resistant derivatives on 0.03 M glycine plates were grown in glutamate medium and again replated, the colony-forming potential on 0.03 M glycine was usually equivalent to that on control plates. As indicated in Fig. 1, these isolates were glycine-resistant at the 0.035 M level, but at higher concentrations there was an exponential decrease in the surviving fraction.

The fraction of plated bacteria, which produced macroscopically detectable colonies on concentrations of glycine greater than 0.012 M, increased with prolonged incubation. The rate of increase in the colony count on plates supplemented with 0.02 M glycine decreased rapidly after 7 days and approached zero after 25 days

TABLE 1. *Survival of Agrobacterium tumefaciens B6 on nutrient agar supplemented with 0.02 M glycine**

	Incubation period				
	4 days	7 days	11 days	20 days	25 days
Surviving fraction	3.3×10^{-7}	7.0×10^{-6}	1.4×10^{-5}	2.0×10^{-5}	2.2×10^{-5}
Mean counts (glycine plates)	0.9	18.7	36.9	53.1	58.7
Variance	1.6	21.6	35.3	144.9	193.3
Chi-square	15.3	9.9	8.1	24.5	29.4
P	0.08	0.35	0.52	0.004	<0.001

* Number of plates = 10.

of incubation. During incubation periods of 30 days, the total colony count on glycine plates increased about 50-fold above counts determined at 4 days. When several samples were plated from the same culture, the variance of the colony counts obtained on 0.02 M glycine plates after 4 days of incubation was usually approximately equal to the mean. However, the variance significantly exceeded the mean when the colonies that appeared later were included in the surviving fraction (Table 1).

Figure 2 presents, for comparison, the colonies formed by the parental strain on unsupplemented medium (2A) and on medium containing 0.02 M glycine (2B) after 5 days of incubation. The colonies formed on 0.02 M glycine during this period of incubation exhibited considerable variation in size and opacity. The first macroscopically visible colonies to appear were similar in morphology to colonies appearing on control plates. Microscopic examination of glycine-supplemented plates revealed numerous macroscopically undetectable colonies (Fig. 2C) consisting entirely of soft protoplasmic elements without definite morphology. Some of these colonies became visible to the unaided eye with further incubation. When this happened, the colony either remained small and somewhat more translucent than colonies on control plates, or eventually assumed the size and frequently the opacity of control colonies. In the latter case, microscopic examination of stained smears made from the colony revealed organisms with the morphology typical of *A. tumefaciens*. Occasionally, in addition to small gram-negative rods, larger forms were seen (Fig. 2D). These large forms frequently decolorized less readily after gram-staining than did the small rods, but none was isolated which could be described as gram-positive. Large forms were also observed

on heavily seeded glycine-supplemented plates examined under phase contrast.

All colonies that were isolated produced pure cultures of motile rods with the size and staining characteristics described for *A. tumefaciens* (Breed, Murray, and Smith, 1957) when transferred to either glutamate medium or nutrient slants. Bacteria from these cultures produced the temperate phage characteristic of strain B6 (Beardsley, 1960).

The colonies formed by 0.03 M glycine-resistant derivatives of B6 on both control plates and plates supplemented with 0.03 M glycine were similar to those formed by the parental strain on control medium. However, when an isolate was replated on 0.04 M glycine, colonies of the type shown in Fig. 2B and C were observed. The colonial morphology of first-step resistant isolates selected on 0.02 M glycine was variable. When replated on 0.02 M glycine, some clones formed colonies exclusively similar to those shown in Fig. 2A. However, after storage on stock slants, these clones frequently produced small translucent colonies when plated on 0.02 M glycine.

The level of glycine resistance exhibited by populations derived from colonies growing on glycine-supplemented plates was variable. Although Fig. 1 represents a general pattern obtained in several experiments, there were many exceptions to the survival ratios indicated. For example, one isolate obtained from a plate supplemented with 0.03 M glycine gave the survival ratios indicated in Fig. 1 up to the 0.036 M level. However, it was not possible to grow the isolate on 0.038 M or higher levels of glycine. Another isolate from the same plate gave markedly lower survival ratios than those indicated in Fig. 1 at the 0.03 to 0.035 M levels, but the pattern of resistance above 0.036 M was that indicated in

FIG. 2. Effects of glycine on the growth of *Agrobacterium tumefaciens*. Colonies formed by strain B6 on unsupplemented nutrient agar (A) and nutrient agar supplemented with 0.02 M glycine (B) after 5 days of incubation. Plates originally photographed at 2.5X on a Quebec colony counter. Microscopic colonies consisting of soft protoplasmic bodies formed on 0.02 M glycine-supplemented agar (C). Photographed originally at 100X by bright-field illumination. Long forms isolated from a colony formed by a 0.03 M glycine-resistant clone on 0.04 M glycine-supplemented agar (D). Gram-stained preparation was photographed originally at 960X.

Fig. 1. Similar variability was observed with isolates selected at the 0.02 M glycine level.

In a few experiments, replicate samples from the same culture resulted in colony counts on 0.02 M glycine plates after 4 days of incubation. These counts gave probabilities of less than 0.05 when tested for homogeneity by chi-square analysis. In each case, microscopic examinations revealed that colonies consisting of the soft protoplasmic elements described above had been included in the counts.

Distribution of resistant clones. The variance of the number of resistant colonies among samples drawn from different cultures was invariably greater than that obtained with samples from the same culture when plates were counted after 4 days of incubation. Table 2 presents the results obtained in one experiment. The increase in the number of resistant colonies obtained, when samples were plated at successive times during the growth of a culture in glutamate medium, was consistently greater than the increase in total viable count as measured on unsupplemented plates. The generation time for several initially resistant isolates was determined in aerated glutamate medium. In no case was it less than 88 min, which was the generation time obtained for the parental strain. In most cases it was longer, averaging 96 min.

Inheritance of glycine resistance. The inheritance of glycine resistance in the absence of added glycine was investigated by growing isolates on nutrient agar slants and determining the resistant fraction after various periods of growth. In general, the resistance of isolates at the 0.02 M glycine level decreased with continued growth in the absence of glycine.

In another experimental approach, a single isolate at the 0.02 M glycine level was inoculated into 5 ml of glutamate medium. After 18 hr of incubation, 0.1 ml of the resulting culture was inoculated into each of two flasks containing 25 ml of glutamate medium. Serial transfers were made by inoculating 0.1 ml from the flask cultures every 40 hr into fresh medium. In this manner, parallel cultures from a single isolate were maintained in nearly continuous logarithmic growth. Each flask in the series supported approximately 20 generations. At each transfer, platings were made on both unsupplemented and 0.02 M glycine medium. Both cultures established from the isolate manifested a decrease in glycine resistance

FIG. 3. Effect of continuous growth in glutamate medium on glycine resistance in *Agrobacterium tumefaciens* strain B6. The proportion of bacteria resistant to 0.02 M glycine in parallel cultures of a glycine-resistant isolate selected at the 0.02 M level (○ and ●). The proportion of bacteria resistant to 0.03 M glycine in parallel cultures of a resistant isolate selected at the 0.03 M level (× and +).

during growth (Fig. 3). The results of a similar experiment with an isolate at the 0.03 M level are also presented in Fig. 3. In this case, platings were made on control and 0.03 M glycine medium. In general, these isolates were not only resistant to higher levels of glycine, but also did not lose resistance in the absence of glycine as readily as the first isolates. Further, glycine resistance was inherited over a greater number of generations, without significant change in survival ratios, following repassage of 0.02 M glycine isolates on plates supplemented with 0.02 M glycine.

Observations on cultures in liquid medium. Although the nutrient medium employed in these studies was chosen to preclude the possibility of selecting against nutritionally deficient glycine-resistant bacteria, none of the resistant clones that would grow in nutrient medium failed to grow in glutamate medium. However, the first isolates at the 0.02 M level would not grow in glutamate medium supplemented with 0.02 M glycine. Those tested did grow in liquid nutrient medium supplemented with 0.02 M glycine.

When bacteria from the parental strain were inoculated into liquid nutrient medium containing 0.02 M glycine, many of the bacteria were converted to spherical forms. These grad-

TABLE 2. *Distribution of colonies formed by Agrobacterium tumefaciens B6 on nutrient agar supplemented with 0.02 M glycine**

Samples from independent cultures		Replicate samples	
Culture no.	No. of resistant bacteria	Culture no.	No. of resistant bacteria
1	95	21	31
2	241		31
3	56		32
4	156		36
5	47		36
6	55		37
7	114		39
8	22		39
9	85		39
10	75		40
11	44		40
12	58		41
13	78		42
14	60		43
15	92		43
16	41		44
17	50		46
18	70		50
19	163		51
20	113		55
Average: 85.8		Average: 40.8	
Variance: 2,680		Variance: 41.4	

* Twenty 0.5-ml cultures (no. 1 to 20) and one 5-ml culture (no. 21) were grown in glutamate medium. Samples of 0.1 ml containing 4.3×10^8 bacteria (avg) were plated.

ually swelled and eventually became indistinguishable under phase contrast. Resistant bacteria selected at the 0.03 M level of supplemental glycine grew normally in nutrient medium supplemented with 0.02 M glycine.

Occasionally, motile rods were seen when 10^6 or more bacteria of the parental strain were inoculated into nutrient medium containing 0.02 M glycine. Eventually such cultures were overgrown by these resistant bacteria. Cultures inoculated with 10^4 cells were completely sterilized within 48 hr.

Pathogenicity of glycine-selected isolates. The pathogenicity of glycine-selected isolates was tested by inoculation into the stems of susceptible plants. Either a decrease or loss of virulence for the host plants studied was observed with most of the colonies isolated from glycine-supple-

mented plates. The results of these studies will be published in detail in a subsequent publication.

DISCUSSION

The data presented here are consistent with the hypothesis that the first colonies to appear on glycine plates are derived from resistant bacteria present in the plated samples (Luria and Delbrück, 1943).

When resistant clones isolated from 0.02 M glycine medium are maintained by serial transfers on medium containing 0.02 M glycine, resistance is more stable during subculture, in the absence of glycine, than it is among clones maintained on glycine-free medium. Van Lanen et al. (1952b) observed a similar effect of continued selection during the course of their studies on the attenuation of the crown-gall organism. Although determinations on the proportion of glycine-resistant cells in populations were not reported, they found that the number of generations over which loss of virulence was inherited in the absence of glycine was a direct function of the number of transfers in glycine-supplemented medium during the attenuation process.

The level of resistance inherited within a clone is determined, in part, by the concentration of glycine in the medium on which selection took place. In addition, clones selected at the same level of glycine exhibit variability in resistance. These observations suggest that either multiple alleles or several genes are involved in the mechanism of glycine resistance.

Typical rods may appear subsequent to the growth of soft protoplasmic elements in colonies on glycine-supplemented plates. The soft protoplasmic elements which do produce rod forms are not propagated indefinitely in the medium. However, if the glycine concentration is increased, the atypical elements reappear. On the basis of the arguments presented by Lederberg and St. Clair (1958), the designations "L form" and "L colony" appear to be applicable to the atypical growth. L forms of *A. tumefaciens* that had been induced by glycine were previously described by Rubio-Huertos and Desjardins (1956).

With prolonged incubation, the colony counts, obtained when replicate samples are plated on glycine-supplemented medium, fail to follow the Poisson distribution law. This indicates that the distribution of bacteria from which the

colonies arise involves variables not under experimental control. The lack of significant deviations from the mean colony counts, among replicate samples plated on control medium, indicates that this is not due to a lack of precision in distributing bacteria among plates. Apparently, the large variance reflects a lack of precision in distributing bacteria to sites favorable to the survival and growth of bacteria as L forms. The possible factors which affect the ability of plated bacteria to initiate L colonies in glycine-supplemented medium were not studied in detail nor were the factors which determine the continuation of L-form growth to produce macroscopically visible colonies. It was observed that variations in the thickness of the agar top layer affected the number of colonies formed. In general, as the depth of the top-layer medium increased, more of the plated bacteria gave rise to visible colonies.

Apparently attenuation and glycine resistance are pleiotropic effects. Thus, elucidation of the mechanism of glycine resistance may indicate the mechanism responsible for reduced virulence. For example, the lowered pathogenicity of the resistant strains may reside in the failure of bacteria in the infecting population to respond to possible spheroplast-inducing mechanisms within wounded plant tissues.

ACKNOWLEDGMENTS

This work was supported by a research grant from the National Science Foundation and by grants from the New York City Cancer Committee of the American Cancer Society.

I am grateful to Tom Stonier of the Rockefeller Institute, who performed the pathogenicity tests referred to in this report, and to Judy Abraham for technical assistance throughout the course of these studies.

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