Growth Characteristics of Microorganisms Occurring in Penicillin-Treated *Brucella abortus* Cultures (37142)

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The occurrence of cell wall-defective variants in penicillin-treated cultures of *Brucella abortus*, 3183, has been demonstrated by electron microscopy (1). Many of the altered microorganisms structurally resembled unstable L-phase variants of several bacterial species described by others (2). Results of experiments dealing with viability, filterability, and osmotic stability of this strain of *B. abortus* indicated that viable micro-organisms with increased plasticity and altered growth rates were present in those cultures containing penicillin (3). On the other hand, osmotic stability of the majority of microorganisms occurring in penicillin-treated broth cultures was not significantly less at most incubation time intervals than when grown in broth medium without penicillin. As the duration of exposure to penicillin increased, the number of bacterial colonies recovered in antibiotic-free agar pour plates decreased, and a delay in (or lack of) resumption of normal bacterial growth rates was observed upon removal of penicillin from broth cultures. The latter results suggested that larger numbers of degenerating forms were present after prolonged incubation in the presence of penicillin, and/or that microorganisms incapable of reverting to bacterial forms were being induced.

The present study was initiated in an attempt to further define the growth characteristics of microorganisms occurring in penicillin-treated broth cultures of *B. abortus*, 3183; particularly with regard to their capacity for L-type and bacterial colony production. Since it appeared theoretically possible that some bacteria might survive in a dormant state under adverse experimental conditions, the extent of bacterial survival in the presence of inhibitory concentrations of penicillin was also tested. Data shown in this paper were compiled from a single set of experiments and are representative of results obtained in several similar experiments on separate occasions.

Materials and Methods. Microorganisms. Stock cultures of a virulent strain of *B. abortus*, 3183, were grown on trypticase soy agar (TSA) slants then maintained at 4° and transferred at 2 to 3 month intervals. Upon removal from the cold, stock cultures were subcultured to trypticase soy broth (TSB) and allowed to grow for 2–3 days prior to inoculation of those cultures to be used in this study.

Cultural procedures. Four-day TSB cultures were centrifuged and the turbidity adjusted by addition of either fresh antibiotic-free broth or broth containing 20.0 μg/ml of penicillin so that each initially (0 hr) contained approximately 10^{10} bacteria/ml. A minimal concentration of 20.0 μg/ml of penicillin was maintained in treated broth cultures by adding antibiotic to the cultures at 2-day intervals throughout the incubation period. After 0 and 6 hr, and 1, 2, 3, 4, and 7 days incubation, 0.01 ml samples of undiluted and diluted (10-fold dilutions) cultures were pipetted onto an approximately 1-cm diameter area of an agar plate. The plates contained either antibiotic-free TSA or TSA containing 20.0 μg/ml of penicillin. The number of microorganisms capable of producing bacterial and L-type colonies/ml of broth culture was determined from colony counts made of appropriate dilutions in each experiment

after 7 days incubation. The identity of L-colonies was confirmed by applying Dienes stain to suspected colonies on the surface of the plates and by May–Grunwald–Giemsa stains of representative agar block preparations. The relationship of bacterial isolates and revertant bacteria to the original cultures was demonstrated by agglutination of bacteria from several typical colonies isolated in each experiment with commercially prepared *B. abortus* antiserum.

Survival of bacteria was determined by pipetting antibiotic-free broth suspensions of 3-day TSA slant grown bacterial cultures onto 2.5-cm diameter Millipore filters (average pore size 0.22 μ) supported on the surface of TSA plates containing 20.0 μg/ml of penicillin. Filters inoculated with undiluted and diluted suspensions were removed at 0 and 6 hr, and at 1, 2, 3, 4, and 7 days from the original plates and placed on the surface of antibiotic-free plates, or onto fresh TSA plates containing 20.0 μg/ml of penicillin. Methods for plating, counting, and identifying L-colonies and bacteria were the same as indicated above. Inoculation of control cultures onto filters and directly onto the agar surface showed no significant differences in the number of bacterial colonies recovered.

Sensitivity tests on revertant bacterial isolates were done on TSA plates containing 2.5, 5.0, 10.0, 20.0, and 50.0 μg/ml of penicillin. All cultures were incubated at 37° under increased CO_2 tension.

Results and Discussion. Survival of bacteria on agar plates. No growth developed on filters during 7 days initial exposure of bacterial suspensions to penicillin on the surface of agar plates, but bacterial colonies grew on all filters after transfer to antibiotic-free TSA from the original plates after various periods of incubation (Table I). The results indicate that while decreased survival of bacteria began shortly after exposure to an inhibitory concentration of penicillin, the decline in surviving organisms was gradual during the first two days. When inoculated filters remained on the antibiotic-containing agar for 3 days or longer, a marked decrease in the number of surviving organisms was observed but complete elimination of bacteria was not attained during 7 days exposure to penicillin. Numbers of bacteria surviving under these conditions were proportional to the number of bacterial colonies previously recovered in antibiotic-free pour plates from penicillin-treated broth cultures after the same incubation periods (3). Persistence of bacteria over a period of several days in the presence of inhibitory concentrations of antibiotics has been demonstrated in *Hemophilus influenzae,* but survival of cell wall-defective variants was short-lived under inhibitory

TABLE I. Survival of Bacteria on the Surface of Agar Plates Containing Penicillin.

Incubation time on TSA containing penicillin[a]	Growth on filters after transfer to:[b]	
	Antibiotic-free TSA Bacterial colonies	TSA containing penicillin L-colonies
0 hr	2.0×10^{12}	—[c]
6 hr	1.3×10^{10}	—
1 day	3.1×10^{9}	—
2 days	1.0×10^{9}	—
3 days	3.1×10^{5}	1.0×10^{2}
4 days	1.0×10^{4}	1.0×10^{2}
7 days	6.0×10^{3}	4.0×10^{2}

[a] Incubation time of Millipore filters inoculated with broth suspensions of bacterial organisms on trypticase soy agar (TSA) plates containing 20.0 μg/ml of penicillin before transfer to antibiotic-free TSA or to fresh TSA plates containing 20.0 μg/ml of penicillin.

[b] Recorded as the number of microorganisms capable of producing bacterial or L-type colonies/1 ml of broth culture.

[c] No L-colonies observed on filters inoculated with 0.01 ml of an undiluted suspension of bacteria.

TABLE II. Recovery of Bacterial and L-colonies from Penicillin-Treated Broth Cultures on Agar Plates.

Incubation time in TSB containing penicillin[a]	Growth after inoculation onto:[b]		
	Antibiotic-free TSA	TSA containing penicillin	
	Bacterial colonies	Bacterial colonies	L-colonies
0 hr	1.3×10^{10}	5.0×10^{2}	1.0×10^{2}
6 hr	1.2×10^{10}	2.2×10^{4}	6.0×10^{9}
1 day	1.0×10^{10}	1.4×10^{4}	3.5×10^{9}
2 days	1.9×10^{10}	7.7×10^{3}	5.2×10^{9}
3 days	1.8×10^{10}	1.8×10^{4}	5.8×10^{9}
4 days	1.5×10^{9}	2.5×10^{3}	3.5×10^{8}
7 days	6.2×10^{9}	6.0×10^{2}	2.0×10^{8}

[a] Incubation time in trypticase soy broth (TSB) containing 20.0 μg/ml of penicillin before inoculation of 0.01 ml samples of diluted (10-fold dilutions) and undiluted cultures onto the surface of antibiotic-free trypticase soy agar (TSA) plates or onto TSA plates containing 20.0 μg/ml of penicillin.

[b] Recorded as the number of microorganisms capable of producing bacterial or L-type colonies/1 ml of broth culture.

conditions (4). Development of only a few L-colonies after transfer of some filters to TSA containing penicillin indicates that limited induction of cell wall-defective variants capable of producing L-colonies occurred after inhibition of bacterial growth by penicillin.

Recovery of bacterial and L-colonies from broth cultures. Similar decreases were not observed in the number of bacterial colonies recovered from penicillin-treated broth cultures when subcultures were made to antibiotic-free agar plates (Table II). L-colonies were also isolated with regularity from penicillin-treated broth cultures when subcultured to agar plates containing penicillin. The number of bacterial colonies isolated on antibiotic-free medium usually approximated the number of L-colonies isolated on antibiotic-containing medium suggesting that most, if not all, L-phase variants induced by penicillin treatment of broth cultures were capable of reversion to bacteria under favorable conditions. As can be seen from results of this particular experiment, smaller numbers of bacterial colonies sometimes developed on agar plates containing pencillin when inoculated with diluted broth cultures which had been treated with penicillin. Spontaneous induction of numerous microorganisms capable of producing L-colonies or resistant bacterial colonies was not obtained, however, by inoculation of untreated broth cultures after various periods of incubation to agar containing penicillin (Table III). L-colonies

TABLE III. Recovery of Bacterial and L-colonies from Untreated Broth Cultures Inoculated onto the Surface of Agar Medium Containing Penicillin.

Incubation time in TSB[a]	Growth on TSA containing penicillin[b]	
	Bacterial colonies	L-colonies
0 hr	—[c]	—[c]
6 hr	—	—
1 day	—	—
2 days	—	—
3 days	—	1×10^{2}
4 days	1×10^{2}	1.5×10^{3}
7 days	4×10^{2}	2×10^{2}

[a] Incubation time in antibiotic-free trypticase soy broth (TSB) before inoculation of 0.01 ml samples of diluted (10-fold dilutions) and undiluted cultures onto the surface of trypticase soy agar (TSA) plates containing 20.0 μg/ml of penicillin.

[b] Recorded as the number of microorganisms capable of producing bacterial or L-type colonies/1 ml of broth culture.

[c] No colonies observed on plates inoculated with 0.01 ml of undiluted broth culture.

were not detected on antibiotic-free plates under any circumstances which corroborates results discussed above suggesting that cell wall-defective variants producing L-colonies were also capable of reversion to bacteria. Failure to show an increase in the total number of colony-forming microorganisms present in penicillin-treated broth cultures as the incubation time was extended suggests that a steady state prevailed in these cultures. Similar conditions have been observed by others in penicillin-, or glycine-treated *B. suis* cultures (5).

Identical experiments using media containing 0.3 *M* sucrose, as previously reported (3), did not alter the results. Likewise, dilution of broth cultures in sterile distilled water prior to plating did not reveal significant differences in the number of osmotically sensitive forms occurring in penicillin-treated and untreated cultures. Spheroplasts induced by penicillin treatment of *B. suis* cultures were also found by Hines, *et al.* (5) to be osmotically stable although those induced by glycine or a combination of glycine and penicillin were osmotically sensitive.

Limitations in the quantitative procedures utilized in these experiments probably account for a certain amount of variation in results such as those observed under similar cultural conditions, but should not be responsible for greater differences obtained under other conditions. Thus, large differences in the numbers of L-colonies developing when bacteria were initially exposed to penicillin incorporated into solid or liquid media are most likely due to different efficiencies in L-form induction under these environmental conditions. The influence of cultural conditions upon induction and revision of L-forms has been commented upon by numerous authors.

Sensitivity tests. Recovery of bacterial colonies from penicillin-treated broth cultures on agar medium containing penicillin indicated that some bacteria had developed resistance to the concentration of penicillin (20.0 μg/ml) routinely used in these experiments. A significant degree of penicillin resistance was not formerly noted in this strain of *B. abortus*, either before or after exposure to antibiotics (6), although penicillin resistance is common in *Brucella* species (7). Increased resistance to Carbenicillin, a semisynthetic penicillin, has been demonstrated in *Pseudomonas aeruginosa* after reversion from a cell wall-defective state (8). The exact

TABLE IV. Penicillin Sensitivity of Revertant Bacterial Isolates of *B. abortus* 3183.

Revertant bacterial isolate	Time of incubation in TSB containing penicillin[a]	Maximum concentrations of penicillin (μg/ml) allowing growth of:[b]	
		Bacterial colonies	L-colonies
1	0 hr	20	≧50
2	6 hr	≧50	—[c]
3	1 day	≧50	—
4	2 days	20	≧50
5	3 days	10	—
6	4 days	≧50	—
7	7 days	≧50	—
8	7 days	10	—
—[d]	No exposure to penicillin	5	—

[a] Incubation time in trypticase soy broth (TSB) cultures containing penicillin (20.0 μg/ml) from which L-colonies giving rise to the revertant bacteria isolates were recovered.

[b] Penicillin added to trypticase soy agar (TSA) plates in final concentrations of 2.5, 5.0, 10.0, 20.0, and 50.0 μg/ml.

[c] No L-colonies present.

[d] Subcultured from original stock strain of *B. abortus* 3183.

mechanism by which the observed resistance had developed was not defined, but no evidence for induction of penicillinase or selective induction of resistant bacteria were found, and the acquired resistance persisted after many passages. Sensitivity tests were, therefore, carried out on bacterial isolates randomly picked after reversion of *B. abortus* L-phase variants induced in broth cultures by various periods of treatment with penicillin (Table IV). An increase in resistance to penicillin over that of the original stock culture was demonstrated in all eight isolates with the degree of resistance being greatly increased in four of these ($\geqq$ 50.0 μg/ml). In addition, two of the selected isolates produced L-colonies on plates containing 50.0 μg/ml of penicillin. Factors involved in resistance of revertant *B. abortus* 3183 can only be speculated upon at the present time. The presence or absence of penicillinase, and the stability of the acquired resistance upon repeated passage or after acriflavine treatment have not been determined. Unpublished data have been accumulated which show quantitative and qualitative differences between the soluble components obtained from bacteria and L-forms of this *B. abortus* strain. If soluble components derived from revertant bacteria are found to retain characteristics of L-form components, it would seem probable that resistance had arisen through structural alteration of the cell walls.

Reproduction of L-phase variants. Attempts to stabilize L-type growth by transfer of L-colonies on media containing 20.0 or 50.0 μg/ml of penicillin resulted either in failure to obtain growth of L-colonies or in development of bacterial colonies after one or two subcultures. Similar transfers made on media containing higher concentrations of penicillin yielded no growth. Microorganisms induced by penicillin-treatment of TSB cultures of *B. abortus,* 3183, thus resemble spheroplasts occurring in penicillin-treated cultures of *B. suis* which were osmotically stable but unable to reproduce themselves to any great extent under the experimental conditions provided (5). The results apparently differ with respect to reverting ability of the cell wall-defective variants induced since only a few of the *B. suis* spheroplasts were capable of reverting to bacteria. This discrepancy might be explained by different cultural procedures used in the two studies. Whether the *B. suis* spheroplasts were capable of producing L-colonies is not known.

Summary and Conclusions. Microorganisms capable of producing L-colonies were detected in penicillin-treated broth cultures of *B. abortus,* 3183, throughout a 7-day incubation period by subcultures made to agar plates containing penicillin. Isolation of comparable numbers of bacterial colonies from the same cultures when subcultures were made to antibiotic-free agar plates, and absence of L-colonies on the latter plates, indicates that most cell wall-defective forms capable of producing L-colonies were also capable of reverting to bacteria. Bacteria with increased resistance to penicillin were isolated from some treated broth cultures and after reversion of *B. abortus* L-phase variants. Failure to show an increase in the number of colony-forming organisms during 7 days of incubation in penicillin-treated broth cultures suggests that a steady state existed in these cultures. Persistence of *B. abortus* on the surface of agar plates containing penicillin points up the fact that survival of bacteria under inhibitory conditions may not necessarily require induction of cell wall-defective forms, or development of resistance, in the intervening period of time. Finally, results presented in this paper substantiate earlier evidence suggesting that microorganisms occurring in penicillin-treated cultures of *B. abortus,* 3183, cannot reproduce themselves in a cell wall-defective state for a prolonged period of time, and that most are unstable L-phase variants.

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