OCCURRENCE OF CATALASE IN PLEUROPNEUMONIA-LIKE ORGANISMS AND BACTERIAL L FORMS

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ABSTRACT

WEIBULL, C. (Central Bacteriological Laboratory of Stockholm City, Stockholm, Sweden) AND KERSTIN HAMMARBERG. Occurrence of catalase in pleuropneumonia-like organisms and bacterial L forms. J. Bacteriol. **84:**520–525. 1962.—The catalase activity of six pleuropneumonia-like organisms (PPLO), four stable Proteus L forms, and four normal strains of *Proteus mirabilis* was determined quantitatively. The PPLO (strains of the species *Mycoplasma agalactiae, hominis, laidlawii, mycoides,* and strains isolated from tissue cultures) exhibited no measurable catalase activity. The L forms decomposed H_2O_2 approximately ten times as rapidly as the normal bacteria from which they were derived.

Reports on the catalase activity of pleuropneumonia-like organisms (PPLO) are conflicting. Lecce and Morton (1954) stated that this enzyme is present in the PPLO strain named Campo, and Rodwell and Rodwell (1954) reported its presence in the organism of bovine pleuropneumonia. Freundt (1958) was unable to confirm these observations. Kandler and Kandler (1955) found no catalase in seven saprophytic PPLO strains and one strain isolated from mice.

With the exception of the study by Rodwell and Rodwell, the investigations mentioned above were performed by means of qualitative tests. To obtain more precise information on the catalase activity of PPLO, the present authors determined the catalase activity of several strains quantitatively. The catalase of four stable Proteus L forms was measured to provide a comparison. The activity of the normal bacteria from which these L forms were derived was also determined.

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MATERIALS AND METHODS

Organisms. (1) *Mycoplasma agalactiae*, a strain isolated from a goat ill with agalactia. (2) *M. hominis*, strain Campo (Smith, Peoples, and Morton, 1957). (3) *M. laidlawii*, strain A (Laidlaw and Elford, 1936). (4) *M. mycoides* var. *mycoides*, strain Institut Pasteur. (5 and 6) Tissue culture strains AE and 8490 T of PPLO. (7) A stable Proteus L form, strain L 9, derived from *Proteus mirabilis* strain 9 (Klieneberger-Nobel, 1956; Weibull and Lundin, 1961). (8) A stable Proteus L form, isolated from *P. mirabilis* strain D52 (Dienes, 1949; Taubeneck, 1956). (9) A stable Proteus L form, derived from *P. mirabilis* strain VI (Taubeneck, Böhme, and Schuhmann, 1958). (10) A stable Proteus L form, isolated from *P. mirabilis* strain 18 (Tulasne, 1949). Tests performed in our laboratory have shown that this *Proteus* strain exhibits the fermentation properties characteristic of *P. mirabilis.* (11–14) The normal *Proteus* strains from which the L forms listed above were derived. (15) *Lactobacillus arabinosus* ATCC 8014.

Organisms 1, 2, 5, and 7 were obtained from E. Klieneberger-Nobel, Lister Institute of Preventive Medicine, London, England. Organisms 3, 4, and 6 were obtained from E. A. Freundt, State Serum Institute, Copenhagen, Denmark. Organisms 8 and 9 were obtained from U. Taubeneck, Institut für Mikrobiologie und Experimentelle Therapie, Jena, Germany. Organism 10 was obtained from M. Sensenbrenner, Institut de Chimie Biologique, Strasbourg, France. In the following, the L forms will be designated as Proteus L VI, L 9, L 18, and L D52.

Growth conditions. M. agalactiae and *M. mycoides* were grown at 37 C in meat broth supplemented with 10% (inactivated) horse serum.

The pH of the medium was 7.8. *M. laidlawii* was grown at 30 C in meat broth (pH 7.8) supplemented with 1% horse serum. The other PPLO strains were grown in an aqueous solution containing 2% Tryptose (Difco), 0.4% Yeast Extract (Difco), 0.001% deoxyribonucleic acid, and 10% (inactivated) human serum. The pH of the medium was adjusted to 7.8.

All L forms and the normal strains of *Proteus* were grown at 30 C in Abrams (1955) liquid serum-free medium. However, no penicillin was included in this medium. *L. arabinosus* was grown at 37 C in an aqueous solution containing 2% Yeast Extract, 1% glucose, 0.5% peptone (Difco), 0.1% KH_2PO_4, and 0.1% K_2HPO_4. The pH of this medium was adjusted to 6.8.

All organisms except *L. arabinosus* were grown in 200-ml Erlenmeyer flasks, each containing 50 ml of medium. The cultures of the L forms and of *M. laidlawii* were incubated on a rotary shaker (100 rev/min). *L. arabinosus* was grown in 50-ml bottles completely filled with medium.

The cultures were ordinarily incubated for 18 to 24 hr before being used for the catalase determinations. After incubation, the PPLO cultures were concentrated about 100-fold by centrifugation for 20 min at 78,000 $\times$ *g* and resuspension of the organisms in a small volume of the supernatant liquid. The *L. arabinosus* cultures were concentrated about tenfold by centrifugation. The cultures of the L forms and the normal bacteria were not concentrated before being used for the enzyme assays.

Determination of catalase activity. The method of Bonnichsen, Chance, and Theorell (1947) was used. When this method was applied, the material to be assayed was added to a 0.01 M solution of H_2O_2 in phosphate buffer (pH 7.0). The decomposition of H_2O_2 was measured by titrating the remaining substrate with permanganate after stopping the enzymatic reaction with sulfuric acid. Each assay mixture consisted of 1 ml of 0.25 M H_2O_2, 25 ml of 0.01 M phosphate buffer, and 0.1 to 1.0 ml of a suspension of bacteria in growth medium. The decomposition of H_2O_2 was allowed to proceed for 5 to 60 min at 25.0 C. At least four samples (2.0 ml each) were taken from each assay mixture at suitable time intervals and titrated for remaining H_2O_2.

The assays were generally carried out in duplicate. To obtain enzyme blanks, the amounts of permanganate consumed by the bacterial samples in the absence of H_2O_2 were determined separately.

Analysis of variance was used to indicate activity data which showed a 95% probability of being significantly different; 95% confidence limits were calculated for these data. In several assays, it was not directly evident from the experimental data whether or not a small amount of H_2O_2 was split during the incubation of the reaction mixtures. In these cases, covariance analysis was used to reveal any significant correlation between reaction time and content of H_2O_2 in the assay mixtures.

Dry weight determinations. The dry weight of normal bacteria was determined after washing cells with distilled water and drying them at 100 C for 24 hr. To determine the dry weight of PPLO, a sample of the culture in question was centrifuged at 78,000 $\times$ *g* for 20 min, and the pellet obtained was suspended in distilled water. The suspension was transferred to a beaker, dried at 100 C, and its dry weight (I) determined. The dry weight (II) of the medium included in the centrifugal pellet was calculated according to Weibull and Beckman (1960). Furthermore, since the yield of PPLO per ml of growth medium was rather low (one-tenth or less of an average crop of normal bacteria), it was found necessary to take into account the dry weight of material of nonbacterial nature sedimented with the PPLO. This was done by centrifuging a sample of the uninoculated growth medium concurrently with the bacterial culture. The dry weight (III) of the pellet obtained was determined. The dry weight of the PPLO was obtained by subtracting the sum of weights II and III from weight I. The dry weight of bacterial L forms was determined as described by Weibull and Beckman (1960).

RESULTS

Precision of the enzyme assays. The precision of the permanganate titrations was reduced to some extent by the presence of large amounts of growth media and bacterial material in the assay mixtures. Therefore, a maximum of 1 ml of medium, containing 10 mg (dry wt) of bacterial material was added to each reaction mixture. Under these conditions, the end point of one

titration could be determined with a precision of 0.05 ml.

Kinetics of the enzymatic reactions. The decomposition of H_2O_2 by the bacteria studied followed the kinetics of a monomolecular reaction. For one and the same crop of bacteria, the velocity constant was found to be proportional to the amount of bacterial material added to the assay mixtures. No inactivation of the bacterial catalase was noted when the organisms were incubated with H_2O_2 for 60 min (Virtanen and Karström, 1925; Bonnichsen et al., 1947; Molland, 1947). The standard deviation of individual assays of the catalase activity of one and the same culture of *Proteus* bacteria was $\pm 7\%$.

Catalase activity of PPLO, normal Proteus bacteria, and Proteus L forms. Control experiments showed that none of the growth media caused any measurable decomposition of H_2O_2 when added to the dilute H_2O_2 solution used for the enzyme assays. Other experiments showed that these media had no or only a slight inhibitory effect on the decomposition of H_2O_2 by *Proteus* bacteria. Therefore, the organisms studied by us were not washed before being added to the assay mixtures. The omission of washing procedures prevented any extensive lysis of the PPLO and L forms before they were assayed for enzymatic activity.

TABLE 1. *Catalase activities of PPLO, Lactobacillus arabinosus, normal Proteus bacteria, and Proteus L forms*

Organism	Activity*
Mycoplasma agalactiae	<0.012
M. laidlawii	<0.012
M. mycoides	<0.012
PPLO, strain Campo	<0.012
PPLO, strain AE	<0.012
PPLO, strain 8490 T	<0.012
L. arabinosus	<0.028
P. mirabilis, strain 9	25.2 ± 3.4
P. mirabilis, strain VI	25.2 ± 3.4
P. mirabilis, strain 18	25.2 ± 3.4
P. mirabilis, strain D52	25.2 ± 3.4
Proteus L form, strain L 9	166 ± 35
Proteus L form, strain L VI	264 ± 38
Proteus L form, strain L 18	264 ± 38
Proteus L form, strain L D52	264 ± 38

* The activities are expressed as μmoles of H_2O_2 decomposed per mg (dry wt) of bacterial material per min. The ± sign indicates 95% confidence limits of the mean values given in the table.

To evaluate the influence of age of the bacterial cultures on catalase activity, samples of one and the same culture of normal *Proteus* bacteria and of a Proteus L form were withdrawn within 10 to 36 hr after the inoculation of the cultures. The variations in the activities of these samples were not found to be more significant than those of samples taken from cultures grown independently for 18 to 20 hr.

The values obtained for the catalase activities of the PPLO, the normal *Proteus* bacteria, and the Proteus L forms are given in Table 1. A figure obtained from the catalase-negative bacterium *L. arabinosus* is also included. Three independently grown crops of each of the normal *Proteus* bacteria and the Proteus L forms, and two crops of *L. arabinosus* and the PPLO, were assayed for catalase.

When the PPLO were tested for catalase, the H_2O_2 content of the assay mixtures remained practically constant during the entire reaction time. Thus, these organisms exhibited either a very low catalase activity or none at all. If the former were true, the rate of H_2O_2 decomposition during the assays could be assumed to be practically constant. This implies that there would exist a linear regression of the H_2O_2 content of an assay mixture on the reaction time. Therefore, the available titration data were subjected to covariance analysis to reveal whether or not such a regression existed in one or several of the assays. A regression coefficient significantly lower than zero would indicate that the PPLO studied possessed a weak but definite catalase activity.

The covariance analysis revealed that the mean square deviations from regression in the individual assays varied homogeneously. The regression coefficients of these assays did not differ significantly. Thus, a regression coefficient, b, common to all assays, could be calculated. The value of this coefficient was $+ 0.00113 \pm 0.00232$ μmoles of H_2O_2 decomposed per mg (dry wt) of bacterial material per min. The ± sign indicates sample standard deviation (Snedecor, 1956) of b. Thus, b did not differ significantly from zero; i.e., the content of H_2O_2 in the assay mixtures did not decrease significantly with time.

To obtain the maximal amount of H_2O_2

decomposed by a particular PPLO strain, the square of the standard deviation of *b* was multiplied by the total degrees of freedom (67) available for the calculation of *b* and divided by the degrees of freedom available from the assays carried out on this PPLO strain (on an average, 11.1). The square root of the figure obtained was multiplied by 2.20 ($t_{0.05}$ for 11 degrees of freedom). It was thus found that the maximal amount of H_2O_2 decomposed by any of the PPLO studied was 0.012 μmoles per min per mg of bacterial material ($P = 0.05$). This figure is given in Table 1.

The value for the upper limit of the catalase activity of *L. arabinosus* given in Table 1 is higher than that of the PPLO. This is due to the fact that 7.5 mg (mean value) of PPLO were used in each catalase assay, whereas the corresponding figure for *L. arabinosus* was 3.2 mg.

It can be seen from Table 1 that all the *Proteus* bacteria tested were catalase-positive. The catalase activities of the normal *Proteus* strains did not differ significantly from each other. On the other hand, one L form, L 9, exhibited a significantly lower activity than the other three. The catalase activities of the L forms were six to ten times higher than those of the normal *Proteus* strains.

DISCUSSION

According to our data, the catalase activity of all of the six PPLO studied by us is too low to be estimated by the sensitive method of Bonnichsen et al. (1947). The following calculations will show that, in all probability, none of these organisms contains any catalase at all.

Pure catalase has a turnover number of about 10^7 molecules per min per molecule of catalase (Sumner and Somers, 1947; Herbert and Pinsent, 1948). However, catalase molecules inside intact cells seem to split H_2O_2 about ten times slower (Euler and Blix, 1919; Herbert and Pinsent, 1948), and are thus characterized by a turnover number of about 10^6. The average diameter of PPLO cells is approximately 0.4 μ (Bartmann and Höpken, 1955; Weibull and Lundin, 1962). If we assume that these cells are spherical and contain 75% water, the dry weight of each PPLO would be approximately 10^{-11} mg.

Table 1 shows that less than 0.01 μmole, i.e., less than 10^{-8} moles of H_2O_2, is split per min per mg (dry wt) of PPLO. Consequently, each PPLO cell contains on an average less than

$$\frac{10^{-8} \times 6 \times 10^{23} \times 10^{-11}}{10^6},$$

i.e., less than 0.06 molecules of catalase. Thus, unless the PPLO populations studied are enzymatically highly heterogeneous, the calculations indicate that the PPLO cells do not contain any catalase molecules.

Similar calculations, based on the data for normal *Proteus* cells given in Table 1, indicate that each of these cells contains on an average approximately 2,500 catalase molecules. This value agrees satisfactorily with the calculated number of catalase molecules, 4,000 or more, in one cell of *Micrococcus lysodeikticus* (Lamanna and Mallette, 1959). The calculations of these workers were based on chemical data.

Our results are in accord with those of Kandler and Kandler (1955) and Freundt (1958). On the other hand, they do not agree with the reports of Lecce and Morton (1954) and Rodwell and Rodwell (1954). Lecce and Morton tested the catalase activity of human PPLO qualitatively by adding H_2O_2 to suspensions of organisms or cellular extracts. Rodwell and Rodwell measured the H_2O_2 decomposition of *M. mycoides* strain V5 by the Warburg technique. A Q_{O_2} value of 800, calculated from the rate over the first 5 min after tipping in substrate, was found at 25 C. However, neither Lecce and Morton nor Rodwell gave any information bout the controls included in their experiments or the time course of oxygen evolution. Thus, it seems justified to state that the evidence available favors the view that the *Mycoplasma* spp. tested so far are catalase-negative.

The similarities and dissimilarities between PPLO and bacterial L forms have been discussed repeatedly (Tulasne, 1955; Kandler, 1960; Klieneberger-Nobel, 1962). In this connection, it is of interest to note that, in contrast to the PPLO, all the Proteus L forms studied by us were catalase-positive. Our results agree with other investigations demonstrating the qualitative similarities between the enzymatic and metabolic properties of *Proteus* bacteria and their L forms (Kandler and Kandler, 1955; Kandler, Zehender, and Müller, 1956*a*, *b*; Mandel and Terranova, 1960; Weibull and Beckman, 1960).

The fact that the Proteus L forms studied by

us exhibited a stronger catalase activity than the normal *Proteus* from which they were derived does not necessarily imply that the L forms contain more of this enzyme per mg dry weight. The L forms lyse to a large extent when transferred to dilute buffers, whereas normal bacteria are highly resistant to osmotic forces. This could explain the higher catalase activity of the L forms, since it is known that a degradation of the structure of microbial cells enhances their catalase activity (Euler and Blix, 1919; Herbert and Pinsent, 1948; Preiss, 1959). The low catalase activity of one of the L forms, L 9, may also be explained by lytic effects. This strain is known to contain considerable amounts of cell-wall constituents (Weibull, 1958; Weibull and Beckman, 1960), whereas the other three strains studied by us contain less of such compounds (Morrison and Weibull, 1962). It seems reasonable to assume that the strain containing more of the cell-wall constituents should be less susceptible to lytic effects, and should therefore display a lower catalase activity than the other L forms.

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