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Release of Alkaline Phosphatase from Cells of *Escherichia coli* upon Lysozyme Spheroplast Formation*

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It has been shown that the alkaline phosphatase of *Escherichia coli* is quantitatively released into the medium when cells containing this enzyme are converted to spheroplasts by the action of ethylenediaminetetracetic acid and lysozyme. Other enzymes, such as glutamic acid dehydrogenase, glucose-6-phosphate dehydrogenase, and β-galactosidase, are retained by the spheroplasts. Release of alkaline phosphatase requires dissolution of the cell wall, since it is not observed with spheroplasts prepared by treatment with penicillin or cycloserine. However, the enzyme is not bound to existing cell-wall structure and is released in soluble form when penicillin or cycloserine spheroplasts are lysed by treatment with distilled water. It is suggested that the enzyme lies in a compartment between the cell wall and cell membrane.

In a study of the utilization of phosphate from an organic ester by resting, phosphatase-containing cells of *Escherichia coli* (Malamy, 1963) evidence was obtained for hydrolysis prior to the entry of phosphate into the cell. It was also observed, however, that the phosphate liberated when the ester was hydrolyzed failed to equilibrate completely with inorganic phosphate in the medium, suggesting that the hydrolytic enzyme must be located at or outside of the cell membrane.

In order to test this hypothesis, methods for removing the cell wall from *E. coli* cells without producing significant lysis were explored. In a preliminary report (Malamy and Horecker, 1961) we described the quantitative release of alkaline phosphatase activity into the supernatant solution when spheroplasts were produced with lysozyme and EDTA. We have now investigated spheroplast formation by the action of penicillin or D-cycloserine, and find that such spheroplasts retain most of this enzyme activity.

METHODS

Bacterial Strains.—*E. coli* K12 was obtained from Dr. Werner K. Maas of this department; *E. coli* ML308, which synthesized β-galactosidase constitutively, was provided by Dr. Jacques Monod of the Institut Pasteur. A strain constitutive for alkaline phosphatase synthesis was isolated from *E. coli* K12 by the procedure of Torriani and Rothman (1961).

Media.—"Minimal medium-TEA" was a modification of medium 63 (Cohen and Rickenberg, 1956) in which phosphate buffer was replaced by 0.05 M triethanolamine buffer. The composition per liter was: 2.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7\,H_2O$, 0.0005 g of $FeSO_4 \cdot 7H_2O$, 0.075 g of KCl, 7.5 g of triethanolamine. The medium was adjusted with HCl to a final *p*H of 7.4. The carbon source was autoclaved separately as a 20% solution and added at the time of inoculation. Phosphate in growth-limiting amounts (*ca.* 10^{-4} M) was added (Echols *et al.*, 1961). "Enriched medium" was prepared by mixing equal volumes of Difco-neopeptone broth with fresh beef infusion.

Materials.—Egg white lysozyme was a product of Nutritional Biochemicals Corp.; glucose-6-phosphate, penicillin, nucleotides, and coenzymes were commercial products; thiomethyl-β-D-galactoside and *o*-nitrophenyl-β-D-galactoside were obtained from Mann Research Corp.; *p*-nitrophenylphosphate was purchased from the Sigma Biochemical Corp. as Sigma 104; Tris was purchased from Sigma Biochemical Corp. as Sigma 121. D-Cycloserine was a gift of Dr. E. Simon of this institution.

Lysozyme Spheroplast Formation.—Cultures were grown overnight at 37° in triethanolamine medium supplemented with a carbon source and 5×10^{-5} M phosphate. The cells were harvested by centrifugation, washed once with the original volume of 0.01 M Tris buffer, *p*H 7.4, and resuspended in a solution of 20% sucrose containing 0.033 M Tris buffer, *p*H 8.0. The suspension was kept at 0° and was treated successively with 0.01 volume of 0.1 M EDTA, *p*H 8.0, and 1 μl of lysozyme suspension (5 mg/ml) per ml of suspension (Repaske, 1958). The suspension was stirred gently, and osmotic fragility was determined at intervals by diluting 0.1-ml aliquots to 1 ml with distilled water and determining the turbidity at either 490 or 600 mμ in the Beckman DU spectrophotometer. When spheroplast formation was complete, as indicated by the absence of further decrease in the turbidity of the diluted sample, the suspension was centrifuged for 15 minutes at 4° in the high-speed attachment of the International PR-2 centrifuge. The supernatant solution was carefully removed and the pellet was washed once with the sucrose-Tris buffer solution. The washed pellet was lysed by adding distilled water.

Penicillin or D-*Cycloserine Spheroplast Formation.*—

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TABLE I
RELEASE OF ALKALINE-PHOSPHATASE ACTIVITY ON LYSOZYME-SPHEROPLAST FORMATION IN *E. coli* K12

Experiment	Enzyme Assayed	Enzyme Units in Intact Cells	Enzyme Units in Toluenized Cells (units/10 ml cell suspension)	Enzyme Units in Spheroplasts	Enzyme Units Liberated
	Alkaline phosphatase			300	4100
1[a]	Glucose-6-P dehydrogenase			36	11
	Glutamic dehydrogenase			29	6
2[b]	Alkaline phosphatase	900	3300	60	4200
	β-Galactosidase	2.5	60	84	4

[a] In experiment 1 a washed culture of *E. coli* K12 (10^{10} cells/ml), grown on limiting phosphate medium containing 0.2% glucose, was treated with EDTA and lysozyme, as described in the text. Before treatment, an aliquot (10 ml) was removed for assay of enzymes in the whole cells. Spheroplasts derived from 30 ml of washed cell suspension were lysed by the addition of 10 ml of distilled water, and the lysate was assayed as described in the text. Enzyme units are reported as the total units per 10 ml of original cell suspension. [b] In experiment 2 the cells were grown as for experiment 1, except that the carbon source was 0.5% glycerol and the medium contained 8×10^{-5} M thiomethyl-β-D-galactoside to induce β-galactosidase. The cell concentration was 5×10^9/ml.

Cells of *E. coli* K12 C4B, which synthesizes phosphatase constitutively, were grown overnight on neopeptone broth. The culture was diluted 25-fold with fresh medium and incubated at 37° in a water-bath shaker for 2 hours. Sucrose and Mg^{2+} were added to final concentrations of 20 and 0.2%, respectively. Incubation was continued for 1 hour, at which time penicillin was added to a final concentration of 1000 units/ml. Alternatively, D-cycloserine was added to a final concentration of 50 μg/ml. Incubation was continued at 37° with very gentle shaking. Aliquots of the treated cultures were removed at intervals and centrifuged at high speed. The residue was resuspended in distilled water. Other aliquots were taken for determination of viable count on nutrient agar and on nutrient agar supplemented with 20% sucrose. Phosphatase assays were performed on all fractions.

Enzyme Assays.—Alkaline phosphatase was determined by a modification of published procedures (Torriani, 1960; Garen and Levinthal, 1960; Echols *et al.*, 1961). The reaction mixture, 1 ml, contained 0.1 mg of *p*-nitrophenylphosphate in 0.5 M Tris buffer, *p*H 8, and enzyme solution or cell suspension as indicated. The reaction was followed at room temperature in the Beckman DU spectrophotometer at 420 mμ. An absorbancy unit (*A* unit) of enzyme was defined as the quantity required to produce a change in absorbancy at 1.0/hour.

For the determination of β-galactosidase (Lederberg, 1950) the reaction mixture, 1 ml, contained 0.5 μmole of *o*-nitrophenyl-β-D-galactoside in 0.04 M phosphate buffer, *p*H 7.5, and enzyme solution or cell suspension as indicated. Liberation of *o*-nitrophenol was followed directly at 420 mμ. A unit of enzyme was defined as the quantity required to produce a change in absorbancy at 1.0/hour.

Glucose-6-phosphate dehydrogenase was assayed in a solution, 1 ml, containing 0.05 M Tris buffer, *p*H 7.65, 0.01 M, $MgCl_2$, 1.0 μmole of glucose-6-phosphate, 0.4 μmole of TPN, and enzyme solution. TPN reduction at 25° was followed at 340 mμ. A unit of enzyme was defined as equivalent to the quantity required to produce a reduction of 1.0 μmole of TPN/hour.

For the assay of glutamic dehydrogenase the conditions were as described for glucose-6-phosphate dehydrogenase, except that 1.0 μmole of glutamic acid was substituted for glucose-6-phosphate. The unit of enzyme was defined in the same manner.

RESULTS

Lysozyme Spheroplasts.—More than 93% of the total alkaline phosphatase activity was liberated into the medium when the cells were converted to spheroplasts with lysozyme and EDTA (Table I). In contrast, glucose-6-phosphate dehydrogenase and glutamic-dehydrogenase activities remained almost completely associated with the spheroplasts. The activity of the latter enzymes in the spheroplast supernatant fraction serves as an index of the extent of cell lysis during spheroplast formation, which apparently amounted to 15–20% in experiment 1.

In a second experiment with cells induced with thiomethyl-β-D-galactoside to a high level of β-galactosidase activity, this enzyme was retained almost quantitatively by the spheroplasts, while 99% of the alkaline-phosphatase activity was released into the medium (Table I, expt 2).

It was observed that alkaline-phosphatase activity recovered from the medium after spheroplast formation exceeded that originally detected in intact or toluene-treated cells. Such crypticity (Cohen and Monod, 1957) has previously been demonstrated for β-galactosidase, but not for alkaline phosphatase. On the contrary, it has been reported that all of the alkaline-phosphatase activity of *E. coli* W (Torriani, 1960) and *E. coli* B (Horiuchi, 1959) is expressed with intact-cell suspensions. The differences between our results and previous findings may result from the use of different strains.

To determine whether the release of alkaline phosphatase after lysozyme-spheroplast formation was confined to K12 strains of *E. coli*, experiments were carried out with an unrelated strain, ML308, which is constitutive for β-galactosidase synthesis. We found this strain to produce alkaline phosphatase when inorganic phosphate was depleted from the medium. As in K12, the synthesis of the enzyme was repressed in the presence of inorganic phosphate. On treatment with lysozyme, 79% of the phosphatase activity was released into the medium, which contained less than 3% of the β-galactosidase activity (Table II). In this strain, crypticity for alkaline phosphatase was less marked when compared with the K12 strain. There appears to have been a small loss of activity upon toluene treatment, but the total number of units recovered after spheroplast formation exceeded the amount detected in the intact cells.

Spheroplast Formation through Metabolic Disturbance.—In the studies described, spheroplast formation was accomplished by the destruction of pre-existing cell wall of resting cells. Spheroplasts can also be produced by agents such as penicillin and D-cycloserine, which interfere with the synthesis of new cell wall. A derivative of the K12 strain (C4B), which is derepressed for alkaline phosphatase, was employed in order that the experiments might be carried out in enriched media.

Following spheroplast formation due to the action of

TABLE II
RELEASE OF ALKALINE PHOSPHATASE ON SPHEROPLAST FORMATION IN E. coli ML308

Enzyme Assayed[a]	Enzyme Units in Intact Cells	Enzyme Units in Toluenized Cells (units/ml cell suspension)[b]	Enzyme Units in Spheroplasts	Enzyme Units Liberated
Alkaline phosphatase	99	67	27	94
β-Galactosidase		108	148	4

[a] Cells of *E. coli* ML308 were grown in triethanolamine medium with 0.5% glycerol and 10^{-4} M phosphate. The cells were harvested and converted to spheroplasts as described in the text. The period of contact with lysozyme before complete conversion to spheroplasts was 30 minutes, which was considerably longer than was necessary for strain K12. [b] Enzyme units, as defined under Methods, are expressed as total units per ml of the original cell suspension before spheroplast formation. The cell concentration before lysozyme addition was 7×10^9/ml.

TABLE III
RETENTION OF ALKALINE PHOSPHATASE BY PENICILLIN SPHEROPLASTS OF E. coli K12 C4B

Time (hr)	Cells as Spheroplasts[a] (%)	Phosphatase in Medium[b]	Phosphatase in Spheroplasts (units/ml culture medium)	Total Phosphatase
0	0	0	54.2	54.2
1		10.6	129.0	139.6
2	99.9	22.8	117.6	140.4
3	*ca.* 100	47.0	102.2	149.2
4	*ca.* 100	66.6	97.0	163.6

[a] Cells of *E. coli* K12 C4B for this experiment were grown overnight in neopeptone broth and converted to penicillin spheroplasts as described in the text. Aliquots of the culture were removed at zero time and at hourly intervals after addition of penicillin. One ml was centrifuged at high speed and the supernatant fluid was saved. The residue was resuspended in 1 ml of distilled water. Viable cell counts were performed by diluting aliquots in 20% sucrose and plating on sucrose agar. Samples were also diluted in distilled water and plated on nutrient agar. The cell concentration at zero time was 1.5×10^9 cells/ml. [b] Phosphatase units are reported as the total per ml of original culture.

penicillin (Table III), only a small portion of the alkaline-phosphatase activity was liberated into the medium. When more than 99% of the cells were present as osmotically sensitive forms, only 15% of the phosphatase activity was found in the medium. The total number of enzyme units increased during the course of the experiment, indicating again that some activity is masked in intact cells. This apparent increase in total activity occurred in the first hour of incubation; thereafter there was a slow leakage of enzyme from the spheroplasts into the medium. When the viable count was followed not only by plating on nutrient agar, but also with agar supplemented with 20% sucrose, it was found that a large number of spheroplasts produced colonies. At 2 hours, when less than 0.1% of the spheroplasts produced colonies on normal agar, more than 30% of the original cell population was recovered on sucrose-agar. This finding is further evidence for the integrity of the spheroplasts in the suspension. The level of viability compares quite favorably with the results of Lederberg (1956), who showed 10–50% of penicillin spheroplasts to be capable of forming colonies on sucrose agar.

Although the bulk of the alkaline phosphatase activity was associated with penicillin spheroplasts, it was not firmly attached to the residual cell wall under these conditions. When these forms were lysed with distilled water, the enzyme activity was found to be completely soluble (Table IV). Other evidence has been obtained which supports the conclusion that the alkaline phosphatase is not associated with the cell wall per se. When cell walls were prepared by shaking intact cells with glass beads in the Micle shaker, according to the procedure of Salton and Horne (1951), the enzyme activity became completely soluble and high-speed centrifugation failed to sediment any appreciable quantity of the enzyme.

When spheroplasts were formed by the action of D-cycloserine, essentially the same result was obtained. At a point when most of the cells had been converted to spheroplasts (2 hours), only 18% of the phosphatase activity was released into the medium (Table V).

TABLE IV
RELEASE OF ALKALINE PHOSPHATASE UPON LYSIS OF PENICILLIN SPHEROPLASTS

Fraction	Total Phosphatase (units per 50 ml)[a]
Intact spheroplasts[b]	800
Lysed spheroplasts,[c] 18,000 *g* supernatant	939
Lysed spheroplasts, 18,000 *g* pellet	91

[a] The quantity of enzyme is expressed as total units in the original 50 ml of spheroplast suspension. [b] Penicillin spheroplasts were prepared as in Table III. Fifty ml of culture was treated, and after incubation for 2 hours in the presence of penicillin the suspension was divided into two parts and centrifuged. One half was resuspended in sucrose and the other half was lysed by the addition of distilled water. [c] The lysed spheroplasts were recentrifuged at 18,000 *g* for 30 minutes. The supernatant solution was removed and the residue was resuspended in Tris buffer.

DISCUSSION

The experiments presented in this report establish that the alkaline phosphatase of *E. coli* is a surface-bound enzyme situated outside of the main permeability barrier of the cell. The localization satisfies the following criteria of Pollock (1962) for surface-bound enzymes:

(1) All alkaline-phosphatase activity sediments with the cells during centrifugation. The enzyme is not found in any substantial amounts in the culture medium.

(2) The enzyme is quantitatively released from the cell into the surrounding medium when lysozyme spheroplasts are formed in a medium of high osmotic pressure. (That there is no substantial lysis of the cell population during spheroplast formation is proven by the quantitative association of known intracellular

TABLE V
RETENTION OF ALKALINE-PHOSPHATASE ACTIVITY BY D-CYCLOSERINE SPHEROPLASTS OF *E. coli* K12 C4B

Time (hr)	Units of Phosphatase in Medium	Units of Phosphatase in Spheroplasts (units/ml culture medium)[a]	Total Units
0	0.6	12.8	13.4
1	3.0	31.8	34.8
2	7.6	36.0	43.6

[a] Cells for this experiment were grown in neopeptone broth and converted to spheroplasts with cycloserine as described in the text. The culture was followed microscopically with phase-contrast optics. At 2 hours the entire culture was composed of spheroplasts. At earlier times, the usual morphological progression from "rabbit-ears" to spheres was observed.

enzymes with the spheroplasts. Other workers have firmly demonstrated the biological integrity of lysozyme spheroplasts of *E. coli*. In certain media lysozyme spheroplasts derived from cells infected with bacteriophages of the T series can complete an entire growth cycle and liberate substantial quantities of mature phage (Mahler and Fraser, 1956; Zinder and Arndt, 1956). In addition, it has been possible to infect lysozyme spheroplasts of *E. coli* with purified DNA from bacteriophage λ (Meyer *et al.*, 1961) and bacteriophage φX 174 (Guthrie and Sinsheimer, 1960), and obtain replication and the liberation of mature phage.)

(3) Experiments with phosphatase-containing cells have shown that substrates such as glucose-6-phosphate are rapidly hydrolyzed by intact cells, with release of most of the phosphate into the medium (Malamy, 1963). Under conditions where inorganic phosphate was not taken up in any substantial amounts by the cell, this ion was still able to cause a marked inhibition of the activity of the phosphatase. These results demonstrate that alkaline phosphatase is located outside the spheroplast membrane; however, on the basis of the following evidence, it does not appear to be present merely in loose physical association with the surface structure.

The enzyme is not an integral component of the cell wall. When cell wall preparations are made, no phosphatase activity is found associated with the particulate fraction; it is all completely solubilized. The release of the enzyme from the cell surface into the external medium does, however, depend on the destruction of existing cell-wall material. The enzyme is released by the action of lysozyme and EDTA, which cause the dissolution of the cell wall. When spheroplasts are prepared by methods which prevent the synthesis of new cell wall, but do not destroy existing wall structure, the phosphatase is retained by the spheroplasts. When these preparations are caused to lyse by diluting the spheroplasts into water, the enzyme is completely solubilized; no activity is found in the particulate fraction[1].

It would appear either that the enzyme is trapped by the mucopeptide layer, or else is bound in a very labile manner to a structure underlying this layer. In intact cells it behaves as though it were contained in a compartment separated from the external medium and not in complete equilibrium with it. The experiments with phosphatase-containing cells have demonstrated that inorganic phosphate liberated in the hydrolysis of phosphate esters is not in complete equilibrium with inorganic phosphate in the medium. The limiting factor appears to be the diffusion of inorganic phosphate in and out of the compartment containing the phosphatase.

There is now additional chemical and cytological evidence for the existence of layers in the Gram-negative cell wall–cell membrane complex. The work of Weidel and Primosigh (1958) on bacteriophage-receptor sites has indicated that there are at least two layers in the wall of *E. coli*. This work has suggested a specific relationship of the layers to each other; the lipoprotein layer seems to be the outermost layer of the cell wall overlying the lipopolysaccharide layer. In a study of a marine *Pseudomonas*, Brown and coworkers (1962) have shown that this organism is surrounded by a double layered, 75 A unit membrane. Analytical results show that the diaminopimelic acid of the cell envelope is mostly associated with the internal membrane. This suggests that the internal membrane corresponds to the mucopeptide layer as described by Weidel and Primosigh (1958) and Work (1961).

Similar evidence for the location of enzymes between the cell wall and cell membrane has been obtained with other cells. In the case of *Saccharomyces cerivisiae*, evidence from a number of laboratories suggests that invertase and acid phosphatase are located near the surface of the cell and are liberated when protoplasts are formed (Demis *et al.*, 1954; Preiss, 1958; Friis and Ottolenghi, 1959; Burger *et al.*, 1961; Sutton and Lampen, 1962; Islam and Lampen, 1962; McLellan and Lampen, 1963; Heredia *et al.*, 1963; Schmidt *et al.*, 1963). H. Eagle and B. L. Horecker (unpublished observations) have demonstrated that intact HeLa cells and other mammalian-tissue-culture-cell lines are able to hydrolyze external phosphate esters such as glucose-6-phosphate. In this case, again, kinetic experiments suggest that the phosphatase activity is at the surface of the cell.

The function of alkaline phosphatase would appear to be to make available the phosphate of phosphomonoesters when the inorganic phosphate of the medium is exhausted. It seems reasonable that the cell might contain other enzymes located outside of the cytoplasmic membrane which would have related functions, such as the selective transport of substrates into the cell and the synthesis of cell-wall components.

[1] H. C. Neu and L. A. Heppel (personal communication) have found that osmotically sensitive cells, prepared by shaking cells with Dowex-50 followed by treatment with lysozyme. do not release alkaline phosphatase.

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Purification and Crystallization of the Alkaline Phosphatase of *Escherichia coli**

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A new procedure is described for the purification and crystallization of alkaline phosphatase of *Escherichia coli*. The starting material for this procedure is the supernatant solution obtained when cells are converted to spheroplasts. The crystalline enzyme preparations are stable, contain little or no diesterase activity, and are homogeneous in the analytical ultracentrifuge. Three protein bands, all with phosphatase activity, are obtained in agar-gel electrophoresis. The purified protein shows a sedimentation coefficient of 6.15 and appears to break up in acid solution to yield smaller fragments with a sedimentation coefficient of 2.6. The enzyme is sensitive to inhibition by thiol-carboxyl compounds such as cysteine and thioglycolic acids. This inhibition is reversed by the addition of zinc ions.

Although procedures for the purification of alkaline phosphatase from *Escherichia coli* have been described (Garen and Levinthal, 1960; Schwartz and Lipmann, 1961), the enzyme has never been crystallized nor have purified preparations been demonstrated to be completely free of other contaminating proteins. Contaminating phosphodiesterase activity hampers the application of the enzyme to the study of nucleotide sequences in nucleic acids (Harkness and Hilmoe, 1962). Previous purification procedures have used as starting material either broken-cell preparations obtained by treatment in the French press (Garen and Levinthal, 1960; Garen and Echols, 1962; Schwartz and Lipmann, 1961), or extracts of acetone powders prepared from whole cells (D. R. Harkness and R. J. Hilmoe;[1] Schwartz and Lipmann, 1961; Plocke *et al.*, 1962). These extracts contain much of the protein and nucleic acid of the cell and extensive purification, involving heat treatment as well as addition of RNAase and DNAase, is required. The use of such drastic steps introduces certain complications that may possibly alter the chemical and physical properties of the protein and confuse the interpretation of multiple electrophoretic bands seen in vertical starch-gel electrophoresis (Bach *et al.*, 1961). Treatment with RNAase and DNAase introduces contaminating proteins which are difficult to remove completely in subsequent steps.

These difficulties can be avoided by using as starting material the supernatant fraction obtained during spheroplast formation with lysozyme and EDTA in an osmotically protective medium (Malamy and Horecker, 1961, 1964). By this procedure alkaline-phosphatase activity is completely liberated from the cells, while the bulk of the other proteins is retained. Few purification steps are required to yield a crystalline preparation which possesses high specific activity and which is free of detectable phosphodiesterase. A single sedimenting species is seen in the ultracentrifuge.

MATERIALS AND METHODS

Bacterial Strains.—A strain of *E. coli* K12, designated C_4F_1, was obtained from Dr. A. M. Torriani of the Massachusetts Institute of Technology. This strain produces a constitutive alkaline phosphatase. It was tested by streaking the cells on nutrient agar and examining the resulting colonies for alkaline phosphatase according to the method of Echols *et al.* (1961). Colonies which turned yellow quickly after the addition of a drop of nitrophenylphosphate solution were considered to be constitutive and were used as the initial inoculum for growth.

Media.—The peptone-glucose-salts medium of Levinthal *et al.* (1962) was utilized. The composition of this medium was: 0.12 M Tris, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 0.003 M Na_2SO_4, 0.001 M $MgCl_2$, 0.0002 M $CaCl_2$, 2×10^{-6} M $ZnCl_2$, 0.5% glucose, and 0.5% Difco peptone. The medium was adjusted with HCl to a final *p*H of 7.4. The glucose solution was prepared separately as a 20% sterile solution and added to the sterile medium at the time of inoculation.

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