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Ideal Target Organism for Quantitative Bactericidal Assays

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We have developed a target organism which permits quantitative bactericidal assays. The organism is an *Escherichia coli* mutant which cannot grow at the temperature of the assay (37°C), but retains full colony-forming potential for subsequent quantitation at 25°C. We show that quantitative data on the bactericidal capacity of polymorphonuclear leukocytes and alveolar macrophages can be obtained when this mutant is used as a target. The procedure used to generate the strain is described in detail and should be applicable to many bacterial species. Characterization of the properties of the mutant indicates that it has a strong potential for use in other in vivo and in vitro investigations of host responses to microbial invasion.

For many years bactericidal assays have been used, both experimentally and clinically, to determine the capacity of phagocytic cells to ingest and kill microorganisms. Problems exist in interpreting the results of these assays because of multiplication of the bacteria during the course of the assay. Attempts to improve the quantitation of the assay by inhibiting bacterial growth with sublethal concentrations of antibiotics (3, 4, 6, 9, 12) have only complicated the problem. The complication arises from the accumulation of lethal levels of antibiotics within the phagolysosomes (1, 2). Under such conditions the killing of bacteria within the phagolysosomes by the antimicrobial mechanisms of the cells cannot be quantitated because it cannot be separated from the killing due to the antibiotic. A second approach to the minimization of background replication has been to reduce the incubation times of the assays. This approach makes it difficult to detect phagocytic or bactericidal activities of neonatal leukocytes and other slow or marginally competent cells. Other attempts to eliminate this problem have met with limited success (7, 11).

We describe here a novel solution to the problem. We have isolated a mutant strain of *Escherichia coli* which cannot replicate at the temperature of the bactericidal assay (37°C) but remains completely viable during the course of the incubation and can be quantitated by subsequent plating at 25°C. The temperature-sensitive lesion in the mutant is not corrected by nutritional factors, and the strain retains full colony-forming potential even when maintained for days at 37°C. These properties make the mutant an ideal target organism for in vitro bactericidal assays and potentially useful for in vivo experimental work.

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MATERIALS AND METHODS

Bacteria and growth media. *E. coli* strain Easter (10) was obtained from John B. Robbins (Bureau of Biologics, Bethesda, Md.). All incubations were in R broth, a complex medium containing yeast extract and tryptone (5). All platings were on G agar (M. P. Oeschger, J. Bacteriol., in press), except when otherwise indicated.

Mutagenesis and mutant isolation. Mutagenesis was performed by the method reported by Oeschger and Berlyn (8). A freshly prepared solution of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1 mg/ml in acetone) was added to a log-phase culture of *E. coli* to a final concentration of 10 μg/ml. The cells were incubated for 10 min at 37°C without aeration and then were washed twice with and resuspended in R broth. The resuspended cells were diluted to 20 times the initial volume and incubated overnight at 22°C to allow segregation of the induced mutations.

The enrichments were performed as shown in Fig. 1. The segregated cultures were diluted with R broth and incubated at 22°C. Once logarithmic growth was established, the cultures were transferred to 35°C and penicillin was immediately added to a final concentration of 5,000 U/ml. After 3 h of incubation, the penicillin was inactivated by adding penicillinase (final concentration, 10,000 U/ml) for 30 min at 35°C. The cultures were then washed, resuspended in fresh R broth, and incubated overnight at 22°C. D-Cycloserine at a final concentration of 2 mM was used for the second enrichment. The procedure was similar to the penicillin enrichment except that the antibiotic was removed by two cycles of washing. Portions of the cultures were then plated at 24°C. Colonies which

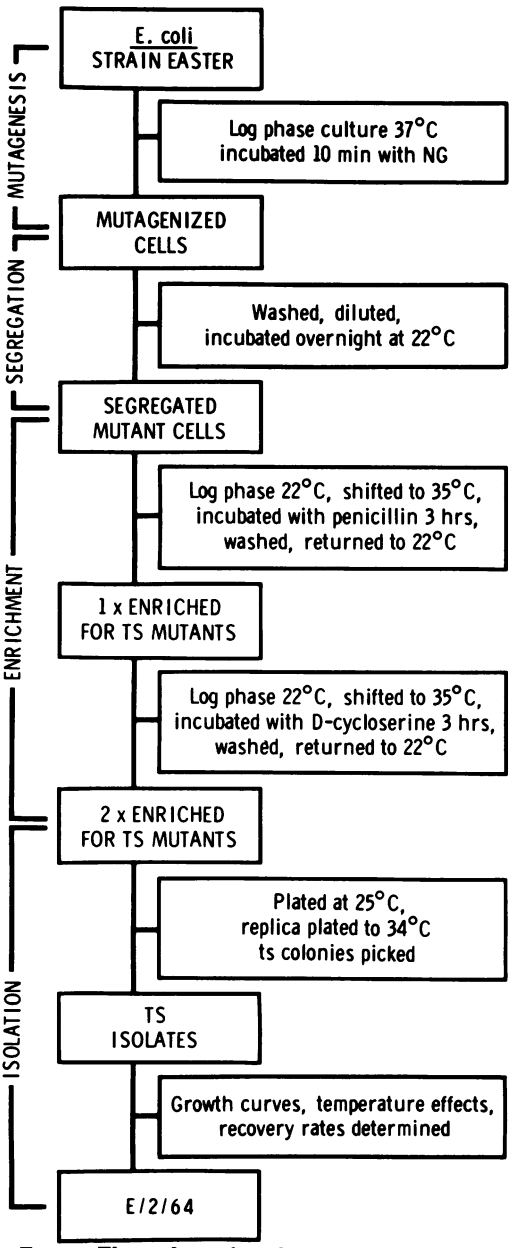

FIG. 1. *Flow sheet for the isolation of strain E/2/64. Details of the procedures are described in the text. NG, N-methyl-N'-nitro-N-nitrosoguanidine; TS or ts, temperature sensitive.*

developed on the 24°C plates were transferred to plates incubated at 34°C. Those isolates which did not grow at 34°C but formed colonies when reincubated at 24°C were tested in liquid medium. The inhibition of growth after transfer to 35°C was monitored by recording absorbance at 600 nm, and retention of colony-forming potential was determined by plating at 25°C.

Surface antigenicity. *E. coli* strain Easter possesses a characteristic set of surface antigens. To determine whether the major cross-reacting antigen had been altered as a result of mutagenesis, the mutant and its parent were grown on agar containing antiserum to that antigen (10). Using the monolayer method described by Zeligs et al. (13), we compared susceptibility of the mutant to phagocytosis with that of its parent.

Bactericidal assays. Human polymorphonuclear leukocytes (PMN) and rabbit alveolar macrophages were used for bactericidal assays. Purified suspensions of PMN were obtained by dextran sedimentation of heparinized venous blood. Contaminating erythrocytes were removed by hypotonic lysis, and the leukocytes were washed and suspended in modified Krebs-Ringers phosphate buffer (14). The target bacteria, which consisted of suspensions of either mutant or parent strains, were prepared from overnight broth cultures grown at 25°C (mutant) or 37°C (parent). The cells were collected by centrifugation at 4°C and resuspended at the desired density in Krebs-Ringers phosphate buffer containing 8% pooled fresh human serum. The parent strain was held at 4°C while the mutant was incubated for 1 h at 37°C to ensure complete arrest of bacterial growth. Equal volumes of the PMN and bacterial suspensions were mixed to a final cell ratio of 1:4 in 1 ml. Control incubations were carried out using the bacterial suspensions alone. The assays were performed in triplicate at 37°C in sterile, siliconized, polypropylene tubes (Falcon Plastics, Oxnard, Calif.) on a Fisher Roto-Rack (10 rpm). Portions were removed at 0, 1, and 2 h, the phagocytes were lysed with distilled water, and serial dilutions of the lysates were plated and incubated at 25°C (mutant) or 37°C (parent) to determine the viable bacterial cell count. To demonstrate the value of using strain E/2/64 in a situation where the phagocytic cell has less bactericidal capacity than the PMN, both mutant and parent strains were compared in an assay with alveolar macrophages. Macrophages obtained from adult New Zealand white rabbits by tracheobronchial lavage were prepared as previously described (14). The micro-bactericidal assay used has been described in detail by Zeligs et al. (13).

Chemicals and reagents. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from Aldrich, Milwaukee, Wis.; penicillin (as benzylpenicillin) and D-cycloserine were from Sigma Chemical Co., St. Louis, Mo.; penicillinase was from Difco Laboratories, Detroit, Mich.; [^{3}H]uridine (specific activity, 36.7 Ci/mmol) was from New England Nuclear Corp., Boston, Mass.; and [^{14}C]tyrosine (uniformly labeled, approximately 500 mCi/mmol) was from Amersham/Searle, Arlington Heights, Ill. Fetal bovine serum was obtained from Flow Laboratories, Rockville, Md., and heat inactivated before use. The antiserum specific for the cross-reacting antigen of *E. coli* strain Easter was the kind gift of John B. Robbins, Bureau of Biologics. All other chemicals were of reagent grade.

RESULTS

Isolation and primary characterization of temperature-sensitive mutant E/2/64.

Since bactericidal assays are performed in a nutritionally rich environment (in the presence of serum), it was important to isolate a mutant whose lesion could not be corrected by nutritional factors. For this reason, all incubations during mutagenesis, enrichment, and selection were carried out in a rich medium, R broth. The isolation scheme is shown in Fig. 1. E/2/64 was chosen from the many isolates recovered after mutagenesis and enrichment because of both the rapidity with which it ceased to grow when the temperature was shifted to 35°C and its quick recovery after the temperature was returned to 25°C. A typical growth inhibition experiment with the mutant and its parent is shown in Fig. 2. Reversion analysis showed that only one cell in 10^8 was capable of forming colonies at 37°C. To confirm that the temperature sensitivity of the mutant could not be altered by supplying nutritional factors, we tested the ability of the mutant to grow at 37°C on blood or chocolate agar and in the presence of serum. No growth was observed, even with heavy inocula.

Surface antigenicity. After growth on agar containing antiserum to the cross-reacting capsule antigen of *E. coli* strain Easter, both the mutant and its parent were surrounded by precipitin rings of comparable size, suggesting the clinically relevant antigen had not been lost as a result of mutagenesis.

Phagocytosis. The results shown in Table 1

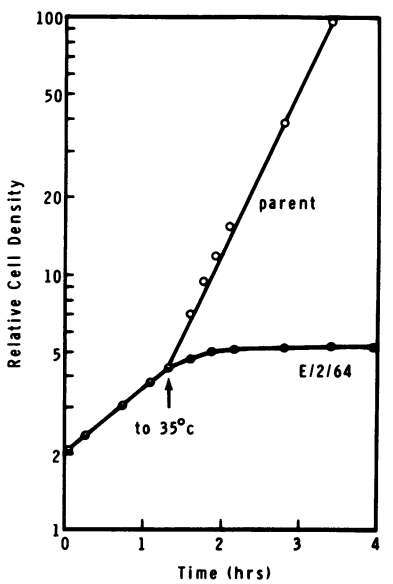

FIG. 2. *Growth of E. coli strains Easter and E/2/64. Cultures of strains Easter and E/2/64 growing in R broth at 26°C were transferred to 35°C at the time indicated by the arrow.*

TABLE 1. *Susceptibility of strain E/2/64 to phagocytosis by human PMN*

Strain	PMN containing bacteria (%)[a]	No. of bacteria per PMN[a]
E/2/64	89.0 ± 1.4[b]	5.6 ± 0.9
Easter	91.5 ± 5.1	5.8 ± 1.7

[a] Assays were performed as described in the text.
[b] Mean of four separate experiments ± standard deviation.

indicate that the mutant and its parent are equally susceptible to phagocytosis by human PMN.

Evaluation of E/2/64 as a target in bactericidal assays. Two different systems were employed to determine the value of E/2/64 as a target organism in bactericidal assays. Phagocytic cells of characteristically high and low bactericidal potential were utilized. Figure 3 compares the wild type and the mutant in an assay with human PMN, cells that normally show high bactericidal activity. Figure 3a shows that the parent strain, after dilution from overnight culture, goes through a typical lag phase before beginning exponential growth. The bactericidal activity of PMN can be measured during this lag period. However, once the cells begin logarithmic growth, quantitation becomes increasingly difficult. When the mutant is used as a target, bacterial replication is restricted and bactericidal activity is readily quantitated even in extended incubations (Fig. 3b).

Figure 4 clearly shows the value of E/2/64 as a target in a bactericidal assay system employing phagocytes whose bactericidal capacity is relatively low compared with that of PMN. The parent cannot be used as a target in this system (Fig. 4a). Figure 4b, however, shows that the quantitation of alveolar macrophage bactericidal activity can easily be made when E/2/64 is the target organism.

Further characterization of the mutant. The effect of the temperature-sensitive mutation on protein and ribonucleic acid synthesis was investigated. Table 2 shows that protein synthesis is rapidly inhibited in the mutant strain, with up to 98% inhibition within 3 min of transfer to 37°C. The inhibition of ribonucleic acid synthesis was only 70 to 80% after 3 min and 85 to 90% after 20 min. These results indicate that the direct effect of the mutation is on protein synthesis.

We then determined the ability of the mutant to survive prolonged incubations at the restrictive temperature. Figure 5 shows that the mutant can survive up to 48 h of aerobic incubation at 37°C in Krebs-Ringers phosphate buffer containing 5% heat-inactivated calf serum without significant loss of colony-forming potential. Sim-

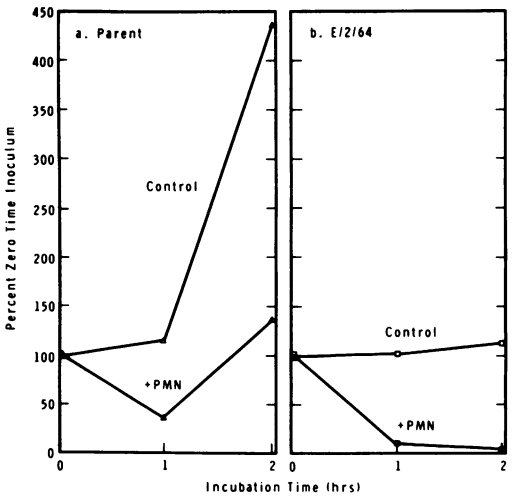

FIG. 3. *Bactericidal assays with human PMN as effector cells. (a) Strain Easter as the target organism; (b) strain E/2/64 as the target organism. Details of the assay are given in the text.*

ilar results were obtained with R broth (data not shown). When the cells were maintained on the surface of G agar plates, they remained fully viable for at least 6 days.

DISCUSSION

Choice of parental strain. In an ideal bactericidal assay, the target organism should not multiply during incubation with the phagocytes but should retain full colony-forming potential for subsequent quantitation. It is important that this be achieved without adulterating the assay medium. We decided to exploit the temperature of the assay and generate a strain whose growth would be restricted by that temperature. To produce such a target, we needed a parental strain that grew rapidly over a wide range of temperatures, had no special nutritional requirements, and was amenable to standard mutagenic and negative selection techniques. *E. coli* was a logical candidate, and strain Easter was chosen because of its clinically relevant cross-reacting capsular antigen (10).

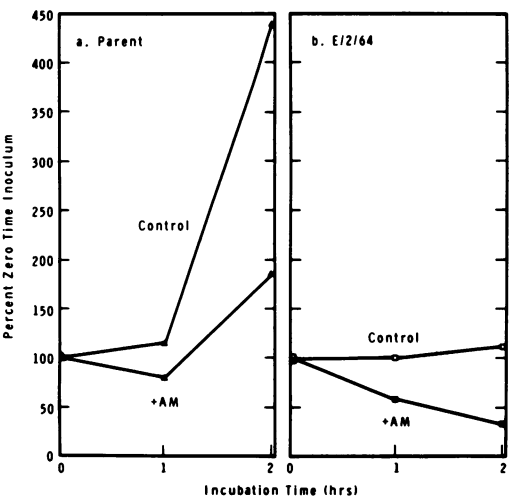

FIG. 4. *Bactericidal assays with rabbit alveolar macrophages (AM) as effector cells. (a) Strain Easter as the target organism; (b) strain E/2/64 as the target organism. Details of the assay are given in the text.*

TABLE 2. *Effect of temperature on the incorporation of radiolabeled precursors of ribonucleic acid and protein in E/2/64 and its parent strain*

Expt no.	Temp (°C)	Time after temp shift (min)	Incorporation[a]					
			Uridine			Tyrosine		
			cpm[b]		% Inhibition[c]	cpm[b]		% Inhibition[c]
			Parent	Mutant		Parent	Mutant	
1	25		595,700	628,420		50,360	64,160	
	37	3	499,600	160,130	70	43,810	6,155	89
	37	20	578,500	88,600	85	59,780	7,768	90
2	25			1,577,516			70,228	
	37	3		285,342	78		961	98
	37	20		156,674	90		476	99

[a] Cells were grown in TDC-Y medium (Oeschger, J. Bacteriol., in press) at 25°C, and 0.4-ml portions were transferred to 37°C. At 3 and 20 min after transfer, 20 μl of a 5:1 mixture of [^{3}H]uridine and [^{14}C]tyrosine was added to the cultures. After 1 min, 1 ml of 10% trichloroacetic acid was added to the cultures, the samples were chilled on ice, and the precipitates were collected, washed, and counted as previously described (Oeschger, J. Bacteriol., in press). The 25°C cultures were labeled 20 min after the transfer with a 2-min pulse and prepared for counting in a similar way.

[b] Counts per minute are corrected for ^{14}C contribution in the ^{3}H channel. The counting efficiencies were approximately 10% for ^{3}H and 50% for ^{14}C.

[c] Percent inhibition was determined by designating incorporation by the mutant for 2 min at 25°C as 100%.

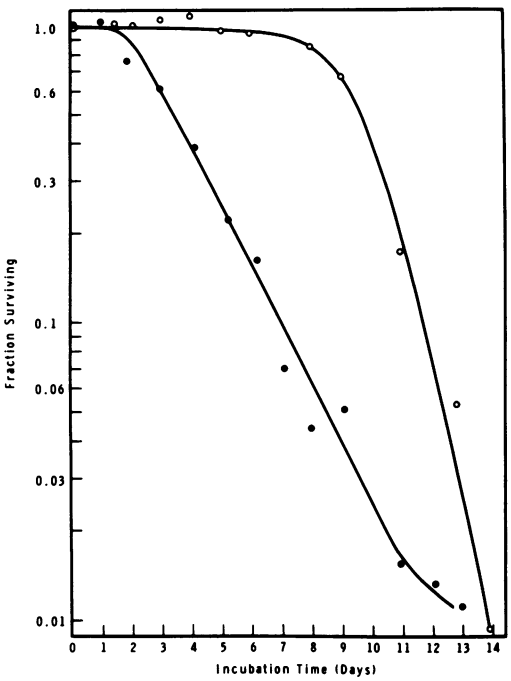

FIG. 5. *Viability of E/2/64 incubated at 37°C. (●) Log-phase cultures of E/2/64 that were transferred to and maintained at 37°C in Krebs-Ringers phosphate buffer containing 5% heat-inactivated fetal calf serum; samples were taken at the times indicated and plated on G agar plates at 25°C for quantitation. (○) E/2/64 spread on G agar plates and incubated at 37°C; at the times indicated, plates were returned to 25°C for quantitation.*

Isolation of the mutant strain. To isolate those mutants that ceased to grow at 37°C but recovered at 25°C, penicillin and D-cycloserine were used as enriching agents. Because these antibiotics only kill growing cells, those mutants whose growth is inhibited at 37°C are spared. The relative number of desired mutants is thus increased by several logs. Restrictive temperatures of 34 to 35°C were used in the enrichment steps to provide a wider range of working temperatures with the final isolate. Bactericidal assays are usually performed in the presence of serum; it is therefore important that the temperature-sensitive lesion generated should not be susceptible to nutritional correction. To prevent this from happening, a rich medium was used during mutagenesis, enrichment, and selection. Evidence that the lesion cannot be corrected by nutritional factors is provided by the inability of the mutant to grow at the restrictive temperature on blood or chocolate agar or in the presence of serum.

Properties of the mutant. The mutant E/2/64 rapidly ceases growth when the temperature is raised to 34°C and resumes growth when the temperature is returned to 25°C. The strain retains full colony-forming potential for long periods of incubation at the restrictive temperature. Reversion analysis suggests that a single mutation is responsible for the temperature sensitivity. The lesion apparently affects an essential function, for growth cannot be restored at the nonpermissive temperature by the addition of nutritional factors. Other significant mutations resulting from treatment with nitrosoguanidine have not been detected. More importantly, the surface antigenicity and susceptibility to the bactericidal activity of phagocytic cells remain unchanged.

Applications. The temperature-sensitive mutant E/2/64 is an ideal target organism for bactericidal assays. Complications introduced by adding antibiotics to arrest the growth of bacteria during the assay are avoided. When necessary, assays can be carried out for prolonged periods without the background overwhelming the experimental results. This mutant has allowed the study of the appearance of bactericidal activity in rabbit alveolar macrophages during postnatal development (13).

E/2/64 can be exploited for other in vitro and in vivo experiments. The replication of the bacterium is blocked at the body temperature of most experimental animals, and E/2/64 can therefore be used for investigations with whole animals. The role of bacterial replication in the induction of host responses to microbial invasion can be evaluated by comparative studies with E/2/64 and its parent strain. The properties of E/2/64 make it eminently suitable for use in the further dissection of these responses at both the in vivo and in vitro levels.

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