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Induction and Isolation of a Minicell-Producing Strain of *Salmonella typhimurium* (37898)

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A mutant of *Escherichia coli* K12 which produces small, spherical, anucleate bodies (minicells) was isolated by Adler *et al.* (1) after treatment of a log-phase culture of the normal strain with triethylenemelamine (TEM). The mutant (P678-54) which differs from the parent strain only in its resistance to X-irradiation and in its ability to produce minicells has proved valuable in many bacterial genetics studies (2–4). Lacking DNA, minicells do not grow in size or undergo cell division, and they can be separated from parental cells by a combination of differential and density gradient centrifugation.

Our investigation was directed toward inducing, isolating, and characterizing a stable minicell-producing mutant in *Salmonella typhimurium* to be used in future serological and immunological studies.

Materials and Methods. Induction of mutant. The culture of *S. typhimurium* from which the minicell-producing mutant was induced was isolated from a clinical specimen at Fort Sanders Presbyterian Hospital, Knoxville, TN, and has been used in this laboratory since 1967. The mutant was induced after repeated exposure of the original culture to increasing concentrations of TEM in nutrient agar. Initially, cultures grown on TEM agar (0.1 mg/ml) were largely filamentous, but they also produced a very small number of small spherical cells, some of which were still attached to normal, rod-shaped parents. Isolates from these plates were then transferred serially on nutrient agar containing TEM in concentrations of 0.2 mg/ml and 0.4 mg/ml, respectively. All cultures were incubated at 37°C for 18 hr. Colonies isolated from the latter plates were restreaked on TEM agar medium (0.4 mg/ml) four additional times with incubation as before. A single minicell-producing colony was isolated from the final TEM agar plate and transferred to nutrient agar slants. This isolate was used throughout our studies as the stock culture for production of minicells. Filamentation disappeared after several transfers on nutrient agar, resulting in a stable culture morphologically similar to the parent strain of *S. typhimurium* except for the production of minicells.

Growth and isolation of minicells. Cultures of the minicell-producing mutant were grown on nutrient agar. After incubation at 37°C for 12–18 hr, the cultures were removed from the agar surface by washing with sterile physiological saline and centrifuged for 10 min at 2500*g*. The minicell-rich supernatants were carefully decanted and centrifuged for 15 min at 22,000*g*. The resulting pellets of minicells, which contained approximately 1% parental cells, were resuspended in 0.75 ml of physiological saline, carefully layered on 30 ml sucrose density gradients (10–30% w/v), and centrifuged for 35 min at 1025*g*. Density-gradient separations resulted in a large dense band of minicells and a more diffuse band of parental cells slightly below the former. The minicells were carefully pipetted from the sucrose gradients, and after diluting the sucrose with physiological saline to facilitate pelleting, they were recovered by centrifuging at 22,000*g* for 15 min. To

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obtain more highly purified preparations of minicells, a second sucrose density-gradient separation was performed for 30 min at 1600*g*. All centrifugation was conducted under refrigeration at 4°C.

Protein determination. Protein content of minicells or normal cells was determined by the Folin reagent method of Lowry (5).

DNA analysis. Samples of approximately 2×10^{10} normal cells or 2×10^{11} minicells were hydrolyzed in 5% trichloroacetic acid, the digests pelleted, and the DNA contents determined colorimetrically by the Burton modification of the diphenylamine reaction (6).

Respiration activity. Oxygen consumption of normal cells or minicells was determined in a Warburg respirometer at 37°C by the method of Umbreit, Burris, and Stauffer (7).

Production of specific antisera. Three injections of 2×10^8, 5×10^8, and 1×10^9 killed normal cells, or equivalent protein of purified minicells in Freund's Complete Adjuvant were administered by intraperitoneal injections to adult hybrid rabbits at 4-day intervals. Two weeks after the last injections the rabbits were bled by cardiac puncture for preparation of antisera.

Adsorption of antisera. Rabbit antisera prepared against minicells or normal cells were reciprocally adsorbed with homologous or heterologous antigens using a modification of the double-adsorption method of Edwards and Ewing (8).

Results. Microscopically, the minicell-producing strain of *S. typhimurium* appeared similar to normal cultures except for the presence of minicells. After the culture was grown 12–18 hr, most of the minicells observed were free of parent cells, but invariably a few were observed still attached on one or on both poles of the parent organism. Minicells of *S. typhimurium,* like those of *E. coli,* appeared to be approximately one-tenth the size of normal cells. After final purification, contamination of the minicell suspensions with parental cells was minimal and was estimated from a ratio of viable cell counts to total cell counts to be less than 1 in 10^5 organisms. Usually, the contaminating cells were much shorter rods (cocco-bacilli) than normal *S. typhimurium* and were probably immature cells. As shown in Table I, purified minicell preparations contained about 0.007 the amount of DNA per cell as normal *S. typhimurium* cells. The small amount of DNA detected in the minicell preparations is attributed to contaminating parental cells and to minicells containing portions of nuclear material (9).

TABLE I. Macromolecular Components of Normal Cells and Minicells of *Salmonella typhimurium.*

	Normal cells $\mu g/10^9$	Minicells[a] $\mu g/10^9$
Protein	285.0	21.8
DNA	10.4	0.074

[a] Contamination with parental cells approximately 1 in 10^5 minicells.

Oxygen uptake by minicells respiring glucose (Fig. 1) was linear with time rather than exponentially as observed in replicating normal cells, which is in agreement with results obtained in previous studies (1).

Serological tests to detect immunogenic differences in the two types of cells proved negative. When commercially prepared specific *O* antisera for *S. typhimurium* were titered against normal cells and minicells, both types of cells were agglutinated equally by each specific antiserum (Table II). In addition, both test antigens were able to adsorb all detectable antibodies from homologous and heterologous rabbit antisera, and no immunogenic differentiation between the minicells and normal cells was possible.

Discussion. Although the induction procedures differed slightly, both the minicell-

TABLE II. Agglutinin Titers for Normal Cells and Minicells in Commercially Prepared Specific O Antisera for *Salmonella* Group B.

	Test antigen	
Antiserum	Normal cells	Minicells
Anti-F_1	1:20	1:20
Anti-F_4	1:320	1:320
Anti-F_5	1:10	1:10
Anti-F_{12}	1:80	1:80

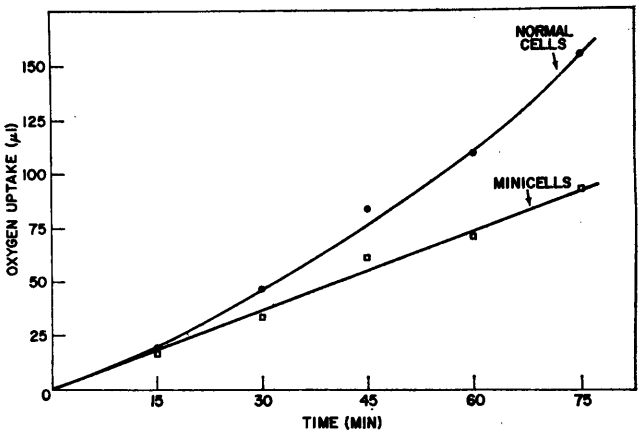

FIG. 1. Respiration of glucose at 37°C by normal cells and minicells of *S. typhimurium*.

producing strain of *E. coli,* and the minicell-producing strain of *S. typhimurium* described in this paper were induced from normal strains after treatment with TEM. This mutagen is used in the manufacture of resinous textile-finishing agents and has also been employed experimentally as an antineoplastic agent. We found it to be quite toxic for our organism which failed to grow from heavy inocula when exposed to 0.5 mg TEM/ml in nutrient agar. At present we can suggest no mechanism for the mode or site of action of TEM in producing the genetic lesion resulting in the ability to form minicells. It seems likely, however, that similar minicell-producing mutants might be induced in most species of gram-negative rod-shaped bacteria. Similarities observed between the *E. coli* minicells and those of *S. typhimurium* suggest that many of the findings established in these studies will be applicable to other types of minicells when, and if, they are induced.

All of our data suggest that the minicells are indeed units of parental bacilli resulting from asymmetrical binary fission with the consequential exclusion of DNA. Isolated minicells were shown to possess only a minute fraction of the amount of DNA in normal cells, and to respire for significant periods of time. No antigenic differences between the minicells and normal cells were detected by the methods we employed.

Other investigators (1–4) have used the P678-54 minicell strain in numerous studies on bacterial genetics, reproduction, and mutation. Our mutant of *S. typhimurium* has already been made available to several investigators for use in similar studies. We are investigating the use of minicells as a nonreplicative noninfectious bacterial vaccine in mice.

Summary. A mutant of *S. typhimurium* which produces small spherical anucleate bodies (minicells) was induced and isolated after treatment of a normal strain of *S. typhimurium* with increasing amounts of triethylenemelamine (TEM).

No differences were observed in growth characteristics or colonial morphology between the minicell-producing strain and the normal strain of *S. typhimurium.* The minicells were shown to be approximately one-tenth the size of normal cells of *S. typhimurium* and were easily isolated from the larger parent cells by a combination of differential and density-gradient centrifugations. Purified minicell preparations contained about 0.007 the amount of DNA per cell as normal cells. They utilized glucose linearly with time, and were serologically indistinguishable from normal cells.

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