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Leakage of Periplasmic Enzymes by Mutants of *Escherichia coli* and *Salmonella typhimurium*: Isolation of "Periplasmic Leaky" Mutants

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Mutants of *Escherichia coli* and *Salmonella typhimurium* were selected on the basis of their spontaneous leakage of ribonuclease I. The mutants also leaked several other periplasmic enzymes into the medium during active growth but did not leak the intracellular enzymes glucose-6-phosphate dehydrogenase or phosphoglucose isomerase.

The studies of Mitchell and of Heppel and his co-workers (reviewed in reference 6) have led to the concept that a cellular domain, the periplasmic space, exists external to the cytoplasmic membrane in *Escherichia coli* and other gram-negative bacteria. This is based in large part on the observation that a number of proteins are released when intact bacteria are treated in various ways which do not liberate most of the soluble proteins of the cell, suggesting a localization within the cell envelope, and on histochemical studies indicating that alkaline phosphatase and several other enzymes of the easily released group are localized in the cell envelope region (18).

The cell envelopes of gram-negative bacteria contain two membranous structures, an inner membrane (the cytoplasmic membrane) and an outer membrane. The region between the two membranes contains the rigid peptidoglycan component of the cell, and it is likely that the periplasmic proteins also reside somewhere in this region. Since different periplasmic enzymes are released to different extents when the conditions of osmotic shock are varied (12), the proteins may be bound to different components within the periplasmic region.

Several observations suggest that the outer membrane may play a role in preventing escape of the periplasmic proteins. These include the observation that many enzymes that are periplasmic in gram-negative bacteria are excreted directly into the medium by gram-positive bacteria, whose cell envelopes lack an outer membrane. In addition, the osmotic shock procedure used to release periplasmic proteins is known to result in loss of significant amounts of lipopolysaccharide, a major structural component of the outer membrane (2). It therefore seemed reasonable to predict that abnormalities in the structure or assembly of the outer membrane or of other cell envelope components that serve as binding sites in the periplasmic region would result in spontaneous leakage of periplasmic proteins and that mutants with outer membrane defects could be selected on this basis.

Based on these assumptions, we have isolated mutants of *E. coli* and *Salmonella typhimurium* that spontaneously leak ribonuclease I and other periplasmic enzymes in the absence of leakage of intracellular enzymes. The isolation and some of the general characteristics of these "periplasmic leaky" mutants are described in this report.

MATERIALS AND METHODS

Organisms and media. *E. coli* C90 (alkaline phosphatase constitutive) was kindly provided by A. Garen. *S. typhimurium* G30 is a uridine diphosphate (UDP)-galactose-4-epimerase-negative derivative of strain LT2 (13). *E. coli* R1 and R5 and *S. typhimurium* R18 and R19 are ribonuclease-excreting mutants of strains C90 and G30, respectively, isolated as described below. The following culture media were used: L broth (medium 1; reference 8) and low phosphate containing 0.2% glucose (medium 2; reference 10). Yeast ribonucleic acid (RNA) and ^{14}C-polyriboadenylic acid (poly-A) were purchased from Schwarz/Mann and Miles Laboratories, respectively. Tritiated *E. coli* deoxyribonucleic acid (DNA) was obtained from M. Deutscher and was prepared by growth of a thymine auxotroph in the presence of ^{3}H-thymidine.

Procedure. Protein synthesis was estimated by adding a mixture of ^{14}C-labeled arginine, lysine, leucine, and valine to exponentially growing cells and measuring incorporation of radioactivity into the hot trichloroacetic acid-insoluble fraction (14). Assays for histidine-binding proteins were kindly performed by Giovanna Ames (1).

Ribonuclease I was assayed in a reaction mixture (0.3 ml) containing 30 mM potassium-phosphate buffer, *p*H 7.0; 2 mM ethylenediaminetetraacetic acid (EDTA); and ^{14}C-poly-A (20 nmoles of adenine, 500 counts per min per nmole of adenine). After incubation for 30 min at 37 C, the mixture was chilled, and 0.05 ml of 0.1% yeast RNA and 1 ml of cold 3% perchloric acid were added. After 15 min at 4 C, the mixture was centrifuged at 10,000 $\times$ *g* for 10 min, and 0.5 ml of the supernatant fraction was used for determination of radioactivity (1 unit = 1 nmole of ^{14}C-adenine released into the acid-soluble fraction in 30 min). The assay was linear for 60 min for 0 to 85 units of enzyme activity. Endonuclease I (1 unit = 1,000 counts/min released in 20 min) was assayed as described by Nossal and Heppel (12), except that tritiated *E. coli* DNA (1,600 counts per min per nmole of thymine) was used. Alkaline phosphatase (1 unit = 1 nmole of *p*-nitrophenyl phosphate hydrolyzed per min), phosphoglucose isomerase [1 unit = 1 nmole of reduced nicotinamide adenine dinucleotide phosphate (NADPH) formed per min], and glucose-6-phosphate dehydrogenase (1 unit = 1 nmole of NADPH formed per min) were assayed as described by Garen and Levinthal (4), Noltmann (11), and Langdon (7), respectively. The enzyme activities were shown to be stable for at least 2 hr when cell extracts were incubated at 30 or 42 C on a rotary shaker in medium 1 or (for alkaline phosphatase) in medium 2.

Isolation of mutants. Ribonuclease-excreting mutants were isolated by a modification of the method used by Gesteland (5) to isolate ribonuclease-negative mutants. Cells were collected after overnight growth in medium 1 or 2, washed with 0.9% sodium chloride, and suspended to the original volume in 50 mM tris(hydroxymethyl)aminomethane (Tris)-maleate, *p*H 6.0, containing 1 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml. After shaking at 30 C for 30 min, the cells were collected by centrifugation, washed three times with the Tris-maleate buffer, and suspended in 1 ml of nutrient broth. After standing at 4 C overnight, the suspension was incubated for 3 hr at 30 C and diluted 20-fold with nutrient broth. The preparation was incubated at 42 C for 30 min, penicillin G (to 10,000 units per ml) was added, and incubation was continued until visible lysis of cells occurred (approximately 1 hr). The cells were washed five times with cold 0.9% sodium chloride, resuspended in nutrient broth, and plated directly on nutrient agar. (The penicillin selection at 42 C was included in an effort to enrich for temperature-sensitive mutants on the assumption that some defects leading to leakage of periplasmic enzymes might also result in inhibition of growth.)

Colonies that grew at 30 C were replica-plated onto nutrient agar, and those colonies excreting ribonuclease were identified by their ability to digest RNA on the plates. The plates were overlaid with 2.5 ml of soft agar containing 1.5% RNA (*p*H adjusted to 7.0). After incubation at 42 C for 2 hr to permit digestion, the plates were flooded with 0.1 N hydrochloric acid. The surface became opaque as a result of precipitation of the RNA, except in the vicinity of the mutant clones, which were easily recognized by the clear halo that surrounded each colony (Fig. 1).

RESULTS

Selection of "periplasmic leaky" mutants. After mutagenesis with nitrosoguanidine, a large number of "leaky" mutants of *E. coli* and *S. typhimurium* was isolated, with an incidence of approximately 5×10^{-5} (1% of the survivors of the nitrosoguanidine treatment). Two *E. coli* and two *S. typhimurium* mutants were selected for further study because they differed in growth characteristics. As described below, excretion of ribonuclease I by all four strains was confirmed by quantitative assays in liquid media.

Excretion patterns. When grown at 30 C all of the mutants excreted ribonuclease I into the culture fluid when compared with the parental strains (Table 1). In two of the mutants (R5 and R18), the excretion was significantly increased when the cells were grown at 42 C (Fig. 2). Strain R19 was temperature-sensitive in growth but failed to excrete detectible amounts of ribonuclease at the elevated temperature. In the "leaky" mutants, leakage of ribonuclease was linear over several hours of observation (Fig. 2), but synthesis of new enzyme appeared to keep pace with the leakage, since total cellular ribonuclease I was approximately the same in the leaky mutants and in the parental strains.

Two other periplasmic enzymes were also assayed in these initial studies, and these were excreted to various degrees in the different mutants. Thus, strain R5 leaked significant amounts of ribonuclease I and alkaline phosphatase but failed to excrete endonuclease I at 30 C, whereas all three enzymes were leaked when R5 was grown at 42 C. In contrast, strain R18 excreted large amounts of ribonuclease but excreted only small amounts of endonuclease I. In strain R19, the leakage was limited to ribonuclease.

The class of periplasmic proteins also includes several binding proteins which have been implicated in entry of amino acids and sugars into the cell. Studies of the histidine-binding protein showed significant leakage in strains R18 and R19 (Table 2).

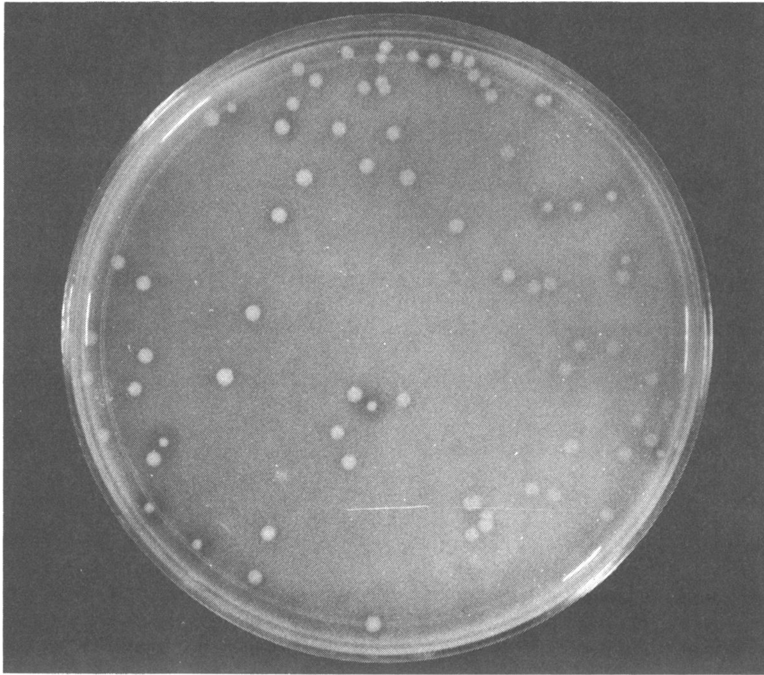

FIG. 1. *Plate assay for excretion of ribonuclease. A mixture of Salmonella typhimurium leaky strain R18 and nonleaky strain G30 cells were tested for ribonuclease excretion by the RNA-overlay technique described in the text. Clear haloes are visible around the leaky colonies from strain R18.*

None of the strains excreted the intracellular enzymes phosphohexose isomerase or glucose-6-phosphate dehydrogenase.

In addition to the leakage of periplasmic enzymes, a variety of other physiological defects resulted from the nitrosoguanidine treatment, none of which seemed responsible for the "leaky" phenotype. These included temperature sensitivity of growth in strain R19. This was associated with a temperature-sensitive defect in protein synthesis, as evidenced by the prompt cessation of incorporation of ^{14}C-labeled amino acids into hot trichloroacetic acid-insoluble material when the cells were switched to 42 C without any defect in uptake of the labeled amino acids. Spontaneous revertants of strain R19 that grew normally at 42 C continued to leak ribonuclease I.

Electron micrographs of mutant and parental strains showed no visible abnormalities of the cell envelope region.

DISCUSSION

The plate assay technique provides a convenient method of identifying mutant clones which leak ribonuclease during growth. The studies in liquid media confirmed the validity of the plate assay technique and also demonstrated that several other periplasmic enzymes are excreted by the "leaky" mutants. It is of interest that the pattern of enzyme excretion differed among the mutants selected for these initial studies. This is consistent with the findings of Nossal and Heppel (12), who found that changes in conditions of the osmotic shock procedure resulted in different rates of release of the different periplasmic enzymes. Thus, two factors may be involved in maintaining these proteins in their periplasmic location: first, a permeability barrier in the cell envelope, presumably the outer membrane, and second, nonequivalent binding of the various enzymes to different structural components of the cell envelope.

It is likely that a variety of abnormalities of cell envelope structure can result in leakage of periplasmic proteins. One can predict that these will involve components of the outer membrane such as lipopolysaccharide, proteins, and phospholipids, as well as other cell envelope components involved in binding the proteins within the cell envelope structure. Abnormalities in several membrane and cell envelope components have been identified in a number of the "periplasmic leaky" mutants.

TABLE 1. *Release of enzymes in culture fluid*[a]

Organism	Ribonuclease I	Endonuclease I	Alkaline phosphatase	Phospho-glucose isomerase	Glucose-6-phosphate dehydro-genase
Incubated at 30 C					
Escherichia coli C90	<0.01	<0.01	<0.4	<0.4	<0.3
	(35)	(2.6)	(16)	(161)	(20)
E. coli R1	1.5	<0.01	<0.4	1.8	<0.3
	(44)	(1.7)	(<0.4)	(332)	(31)
E. coli R5	1.1	<0.01	6.1	<0.4	<0.3
	(50)	(3.1)	(26)	(213)	(35)
Salmonella typhimurium G30	0.1	<0.01	ND[b]	<0.4	<0.3
	(433)	(9.6)		(177)	(38)
S. typhimurium R18	19.4	0.33	ND	<0.4	<0.3
	(312)	(11.4)		(212)	(39)
S. typhimurium R19	2.3	<0.01	ND	1.3	<0.3
	(335)	(14.3)		(186)	(46)
Incubated at 40 C					
E. coli C90	0.4	<0.01	<0.4	<0.4	<0.3
	(47)	(7.5)	(19)	(150)	(15)
E. coli R1	5.7	0.15	<0.4	2.9	<0.3
	(45)	(4.4)	(<0.4)	(412)	(17)
E. coli R5	19.7	1.3	9.3	<0.4	<0.3
	(50)	(10.1)	(26)	(166)	(11)
S. typhimurium G30	1.8	0.2	ND	<0.4	<0.3
	(301)	(16.6)		(134)	(11)
S. typhimurium R18	45.2	0.44	ND	<0.4	<0.3
	(283)	(16.8)		(145)	(11)
S. typhimurium R19	26.0	ND	ND	ND	ND
	(85)				

[a] Cells in mid-exponential growth phase were collected by centrifugation and suspended in fresh medium. Incubation was resumed on a rotary shaker at the indicated temperatures. After 1 hr, the cultures were chilled and centrifuged at 10,000 $\times$ *g* for 10 min at 4 C, and supernatant fluids were removed to be assayed for the enzymes shown in the table. The cells were suspended in the same volume of fresh medium, disrupted by sonic treatment in a Branson sonifier for a total of 5 min at a temperature below 10 C, and centrifuged at 10,000 $\times$ *g* for 20 min at 4 C. The supernatant fraction of this centrifugation was used to determine total cellular enzyme activities, which are indicated in parentheses. L broth was used for all experiments except those involving assays for alkaline phosphatase, in which low phosphate containing 0.2% glucose was used. Total cellular enzyme is expressed as units per 10^9 cells. Enzyme in the culture fluid is expressed as units appearing in the culture fluid in 1 hr per 10^9 cells.

[b] Not done.

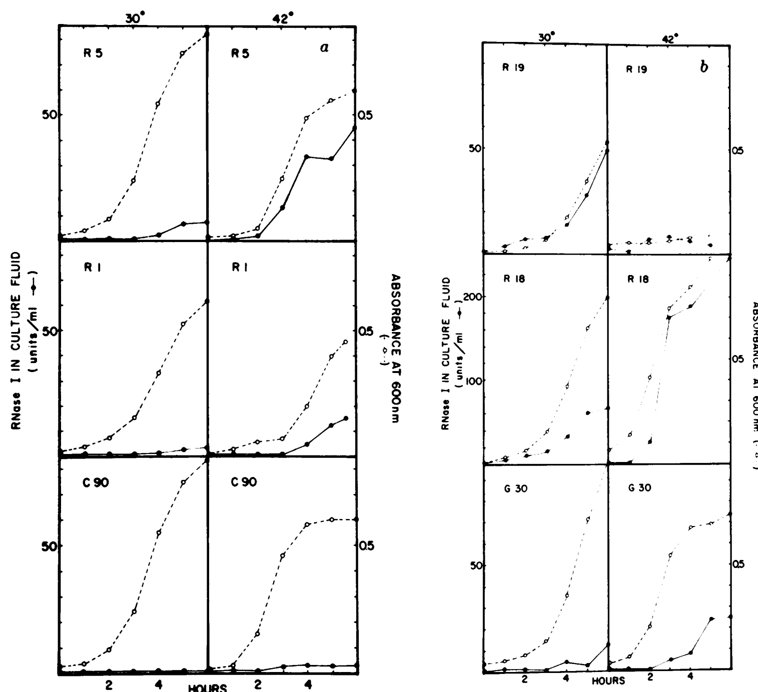

FIG. 2. *Time course of excretion of ribonuclease I. Cells were grown at 30 or 42 C on a rotary shaker by inoculating 0.5 ml of an overnight culture into 25 ml of medium 1. Samples were removed at intervals, and cells were immediately removed by centrifugation at 10,000 × g for 10 min at 4 C. Samples of the supernatant fractions were assayed for ribonuclease I as described in the text. Absorbance at 600 nm was measured as an estimate of cell growth. a, E. coli strains; b, S. typhimurium strains.*

TABLE 2. *Release of histidine-binding protein*[a]

Temp (C)	Organism	Histidine-binding protein
37	*Salmonella typhimurium* G30	12(388)
	S. typhimurium R18	231(548)
30	*S. typhimurium* G30	0(351)
	S. typhimurium R19	35(488)

[a] Cells were grown overnight at the indicated temperatures in minimal medium (17). After centrifugation at 4 C, the supernatant fluid was concentrated by ultrafiltration, sterilized by filtration through a membrane filter (Millipore Corp., HA 0.45), and assayed for histidine-binding activity as described by Ames and Lever (1). Results are expressed as units per gram of dry weight of cells. The total histidine-binding protein released from the cells by the osmotic shock procedure of Neu and Heppel (10) is indicated in parentheses.

These will be described in subsequent reports.

Several examples are known of genetic abnormalities that affect the barrier function of the outer membrane. In these cases, the defective barrier function has been reflected by increased permeability to external compounds. These examples include the increased sensitivity to antibiotics, deoxycholate, and dyes of mutants defective in lipopolysaccharide biosynthesis (3, 15; R. J. Roantree et al., Bacteriol. Proc., p. 79, 1969); the increased sensitivity to antibiotics of certain *E. coli* mutants (16); and the increased sensitivity to dyes and detergents of some colicin-tolerant mutants (9). Studies of the leakage of periplasmic proteins were not described in these reports. Recently, however, Sanderson and his colleagues (K. E. Sanderson, T. MacAlister, and M. Fenn, *personal communication*) observed that certain mutants of *S. typhimurium* with defects in synthesis of the heptose-containing backbone of their lipopolysaccharide showed increased sensitivity to deoxycholate and to various antibiotics and also excreted several periplasmic enzymes into the culture fluid. Since lipopolysaccharide is located primarily in the outer membrane, this is consistent with the idea that an outer membrane barrier is important in preventing escape of components of the periplasmic region.

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