Isopycnic Separation of *Escherichia coli* Cultures Possessing Colonization Factor Antigen I

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A culture of *Escherichia coli* possessing colonization factor antigen I was subjected to isopycnic separation on Percoll gradients. The results demonstrated successful division of the culture into two populations: (i) bacteria which cause mannose-resistant hemagglutination and (ii) bacteria which lack the ability to hemagglutinate in the presence of mannose.

*Escherichia coli* possessing colonization factor antigen I (CFA/I), which has been correlated to virulence, causes mannose-resistant hemagglutination (MRHA) with human type A erythrocytes (1, 3, 5, 7). Passage of these bacteria on laboratory media results in a culture containing a mixed population of *E. coli*: (i) bacteria which are capable of MRHA and (ii) bacteria which have lost this property (3, 7). We investigated the use of Percoll in density gradient centrifugation to separate these two populations of bacteria. Percoll is a colloidal suspension of polyvinylpyrrolidone-coated silica particles and has been used successfully in the separation of mammalian and bacterial cells (2, 8, 9, 12). By using Percoll gradients, separation of the two *E. coli* populations present in culture was achieved.


**MATERIALS AND METHODS**

*Escherichia coli* strain H10407 (O78:H11) was used in all experiments. This strain, originally isolated by Evans and Evans (6), possesses CFA/I and was generously provided by Stanley Falkow. Bacteria were stored in a 1% Casamino Acids medium supplemented with 15% (vol/vol) glycerol at −70°C. Before an experiment, 0.1 ml of the thawed bacterial suspension was inoculated into 1.0 ml of Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) at pH 7.2. Bacteria were statically incubated for 75 min at 37°C before being layered on the Percoll gradient. Incubation for this period of time provided cultures that were in the middle of the exponential growth phase and had attained a viable cell density of 1.7 × 10^8 colony-forming units per ml as determined by standard plate counts.

An isososmotic stock solution of Percoll (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was prepared by combining 90 ml of undiluted Percoll with 10 ml of 10×-concentrated balanced salt solution. A final Percoll concentration (50%, vol/vol) was prepared from the stock solution, using 1× balanced salt solution (pH 7.2). A preformed continuous gradient was generated by centrifugation of 9.0 ml of the 50% (vol/vol) Percoll solution for 10 min at 20,000 × g and 4°C, using a Sorvall RC-B2 centrifuge with an SS-34 rotor. A preformed gradient was used since many identical gradients can be formed simultaneously and stored at 4°C for weeks without change in gradient shape (Separation News, 1980, Pharmacia Fine Chemicals). Density separation was achieved by layering 1.0 ml of the bacterial suspension on the gradient and centrifuging at 3,000 × g and 4°C for 30 min, using the same rotor. Concurrently, a tube containing a simultaneously prepared gradient was layered with a 1.0-ml suspension of density marker beads (Pharmacia Fine Chemicals). This was included as an external standard to ascertain the density levels of the gradient.

In some experiments, 0.1 ml of 2(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) solution (0.15% [wt/vol] INT, 0.9% sodium chloride, 2% glucose, 1% methyl alcohol) was added to the bacterial suspension. This vital dye stained bacteria red without detectable loss of viability (unpublished data) and enhanced the visibility of the bands in the gradient.

By utilizing the Beckman fraction recovery system, eight 1.25-ml fractions were collected from the top with a hollow cone cap and a 60% (wt/vol) sucrose solution as the displacement fluid. Bacteria from each fraction were immediately assayed for MRHA and hemadsorption (HAD) properties.

MRHA activity was determined as previously described (10). Briefly, a sample from each fraction was obtained and the total bacterial count was determined by using counting chamber. Subsequently, each fraction was diluted to a bacterial concentration of 10^8 cells per ml, and 50 μl of the bacterial suspension was serially diluted in microtiter plates, using low-ionic-strength medium [200 mM d-sorbitol, 50 mM d-mannose, 25 mM 2-(N-morpholino)ethanesulfonic acid]. An equal volume of 3% (vol/vol) erythrocytes in low-ionic-strength medium was added to each well, and they were incubated for 1 h at 37°C. The titers, defined
as the reciprocal of the highest dilution of bacterial suspension which produced distinct macroscopic MRHA, were determined after incubation.

The number of MRHA bacteria in each fraction was determined by HAd assay (11). Basically, a sample from each fraction was serially diluted, plated on Casamino Acids agar, and incubated for 48 h at 37°C. After incubation, plates containing well-isolated colonies were chilled on ice for 5 min and gently flooded for 2 min with 10 ml of 10% (vol/vol) erythrocytes in low-ionic-strength medium. This suspension was aspirated, and 10 ml of a 1.0% (wt/vol) tannic acid solution was added and immediately removed. Colonies registering red to pink were considered HAd⁺. Colonies which remained white were regarded as HAd⁻. For confirmation of the HAd reaction, individual colonies were picked, and slide MRHA was performed (3, 10). There was a 99% correlation between HAd and slide MRHA reactions (unpublished data).

FIG. 2. Diagrammatic presentation of the banding pattern of E. coli H10407 in the Percoll gradient.

RESULTS AND DISCUSSION

Calibration of the gradient after each separation was achieved by measuring the distance from the meniscus to each band of marker beads. This distance was plotted against the respective densities of the beads. The plot was then superimposed on the graph of recovered bacteria from each fraction (Fig. 1). The results showed that viable bacteria banded at two densities. The first occurred at 1.040 to 1.050 g/ml, and the second occurred at 1.085 to 1.140 g/ml. Enhanced detection of these bands was attained by adding INT solution to the bacterial suspension before centrifugation (Fig. 2). Based on five experiments, the average total recovery of viable bacteria from the gradient was 90 ± 4%.

Testing each fraction for MRHA showed an eightfold increase in activity in fraction 2 and no detectable titers in fractions 6 through 8 (Fig. 3). Analysis of bacterial distribution within the gradient by means of the HAd assay (Fig. 4) showed that 73% of the viable bacteria which banded in fraction 2 were HAd⁺. In contrast, bacteria banding in fractions 7 and 8 were >99% HAd⁻.

The results supported previous observations (3) that E. coli H10407, when grown in suspension, grows as a mixture of bacteria with regard to the expression of CFA/I. This is due to the spontaneous loss of the plasmid mediating the expression of CFA/I, which is accompanied by the loss of MRHA activity and CFA/I fimbriae and the retention of type 1 pili (3-5, 7). We considered the issue of multiple expression of pili and looked for type 1 fimbriae by checking CFA/I⁺ colonies for agglutination of guinea pig erythrocytes. No agglutination was detected (unpublished data), suggesting no expression of type 1 pili on CFA/I⁺ colonies. Based on these observations, we assumed that the culture grew as a mixture of CFA/I⁺ and CFA/I⁻ bacteria. Although Percoll separation of CFA/I⁺ bacteria was possible, it remains to be determined wheth-

FIG. 3. Demonstration of MRHA activity within each fraction of the gradient after isopycnic separation. For comparative purposes, all samples were standardized to a total bacterial concentration of 10⁸ cells per ml before being tested.
er this technique is capable of separating a mixture of *E. coli* expressing two or more antigenically distinct fimbriae. Our results imply that the expression of CFA/I pili conferred sufficiently different properties to permit physical separation from bacteria which did not possess CFA/I fimbriae. The use of Percoll gradients provided a simple and rapid means for separation of bacteria possessing CFA/I present in a mixed culture of *E. coli*.

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LITERATURE CITED

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- Fig. 4. Distribution of *E. coli* H10407 after fractionation of $1.7 \times 10^8$ colony-forming units per ml on the Percoll gradient.

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