Effect of Osmotic Shock and Shearing Forces on Ferric Enterochelin Transport in *Escherichia coli* K12

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The *fes* mutation in *Escherichia coli* K12, which inactivates enterochelin esterase, allows the cell to accumulate ferric enterochelin. The ferric complex of enterochelin was released in significant quantities from a *fes* mutant after osmotic shock. Analysis of the effects of the individual stages of the shock procedure in wild-type cells showed that prior exposure of cells to sucrose and EDTA was not required, careful dilution of cells into a hypo-osmolar medium being sufficient to induce efflux of Fe^{3+} . Prior treatment with EDTA or exposure to shearing forces served either to enhance efflux or to induce efflux in isotonic media. Neither vitamin B_{12} nor 5'-nucleotidase was released from the periplasm by these procedures. The release observed under mild conditions was stimulated specifically by Co^{2+} , did not occur at 0 °C, and was inhibited by 2,4-dinitrophenol at 37 °C. From these observations, it was concluded that the efflux of Fe^{3+} represents a physiological response of the cell to exposure to a hypo-osmolar medium. Such changes may enhance survival following physicochemical stressing of the bacterial outer membrane.

INTRODUCTION

Iron deficiency in *Escherichia coli* arises after growth either in media containing submicromolar concentrations of iron or in body fluids such as serum or milk. In the latter, iron is present as stable complexes with the iron-binding proteins transferrin and lactoferrin. In each case, the ferric enterochelin transport system is induced in the bacterium (Braun, 1985; Rogers, 1983). Enterochelin, the cyclic trilactone of 2,3-dihydroxy-*N*-benzoyl-L-serine, is secreted into the medium (O'Brien & Gibson, 1979; Rogers, 1973) where, by virtue of its extremely high affinity for Fe^{3+} , the Fe^{3+} complex is formed by exchange reactions with Fe^{3+} complexes present in the medium (Carrano & Raymond, 1979). The complex so formed is first adsorbed by a specific receptor (Fiss *et al.*, 1982), the *fepA* gene product (Pierce *et al.*, 1983), located in the bacterial outer membrane. The complex is then internalized by a process involving the *tonB* function (Kadner & McElhaney, 1978). The next stage of the process is not clearly understood but the *fepB* gene product may serve as a receptor for the complex on the cytoplasmic membrane (Pierce *et al.*, 1983). Wild-type strains of *E. coli* possess an enterochelin esterase which degrades the lactone ring of the complex (Greenwood & Luke, 1978) and may be involved in making iron available for incorporation into, for example, haem (Porra *et al.*, 1972).

For some time we have been interested in the antibacterial effects of the indium and scandium complexes of enterochelin (Rogers *et al.*, 1980, 1982). During the course of a detailed study of the mechanism of action of these compounds (Plaha & Rogers, 1983; Plaha *et al.*, 1984) it was found that a *fes* mutant, which lacks enterochelin esterase, tended to lose label spontaneously after incubation for 30 min or longer with either $^{59}\text{Fe}^{3+}$ -enterochelin or $^{46}\text{Sc}^{3+}$ -enterochelin. No such loss was observed from wild-type cells. The *fes* mutant accumulates considerable quantities of intact Fe^{3+} complex, as shown by the pink colour of cells exposed to Fe^{3+} -enterochelin. The

Abbreviation: DNP, 2,4-dinitrophenol.

transport system for vitamin B₁₂ bears a number of similarities to that of ferric enterochelin, namely the presence of specific outer- and cytoplasmic-membrane receptors and the involvement of the *tonB* function. On subjecting *E. coli* to osmotic shock (suspension in sucrose/EDTA followed by dilution), both vitamin B₁₂ (Reynolds *et al.*, 1980) and a number of enzymes (Nossal & Heppel, 1966) are rapidly released from the periplasmic space. In view of these findings it was decided to employ the osmotic shock technique in an attempt to determine the site of accumulation of ferric enterochelin.

METHODS

Chemicals. All chemicals used were of analytical grade. ⁵⁹FeCl₃, [⁵⁷Co]cyanocobalamin (vitamin B₁₂) and [U-¹⁴C]protein hydrolysate were obtained from Amersham. The preparation of [¹⁴C]enterochelin was described earlier (Plaha & Rogers, 1983).

Bacteria. The two *E. coli* K12 strains used were the wild-type HfrH *thi* and AN273 *fes* (Porra *et al.*, 1972). Iron-deficient bacteria were used throughout. They were obtained by growing the cells in the presence of conalbumin (Sigma), 0.5 mg ml⁻¹, in either trypticase soy broth or medium 56 (Monod *et al.*, 1951) containing appropriate supplements (Porra *et al.*, 1972) (medium A).

Preparation and measurement of uptake of ⁵⁹Fe³⁺-enterochelin and Fe³⁺-[¹⁴C]enterochelin. These procedures have been described previously (Plaha & Rogers, 1983). All radioactive samples were counted in duplicate. The experimental results have been checked for reproducibility; the use of early exponential phase cultures appeared to be particularly important in this respect.

Uptake of [⁵⁷Co]vitamin B₁₂. After addition of [⁵⁷Co]vitamin B₁₂ at a concentration of 10 nM to cells at 37 °C, uptake of the label was determined as for the uptake of enterochelin complexes. Membrane filters were soaked before use for 30 min in 10 µM-vitamin B₁₂ solution and finally washed three times in distilled water.

The counting efficiency of ⁵⁷Co was 90% and the specific activity was 7.14 µCi µg⁻¹ (264.2 kBq µg⁻¹).

Osmotic shock treatment. After uptake of the labelled complexes of enterochelin had proceeded to 20–60 min, the cells were subjected to osmotic shock (Reynolds *et al.*, 1980). Basically, this involved addition of solid sucrose to the culture to give a concentration of 10% (w/v), followed, after 5 min, by 2 mM-EDTA and then immediate dilution of 0.1 ml (obtained with a Gilson P200 micropipette fitted with a C20 tip) of the culture into 10 ml distilled water at 37 °C. In some experiments, bacteria were collected by centrifugation and then subjected to osmotic shock at 4 °C (Nossal & Heppel, 1966). Duplicate samples (0.5 ml) were removed during the uptake phase and immediately before the application of osmotic shock. Samples (1 ml) of the shocked cell suspension were also removed at intervals over a 30 min incubation period at 37 °C. The cells were collected on membrane filters (0.45 µm) and washed with 1 ml saline (0.85% NaCl) or 10% (v/v) TSB in 0.85% NaCl (broth saline) (depending on whether the uptake was in medium A or TSB). Radioactive counting of dried membranes in toluene scintillation fluid was used to measure the amount of label associated with the cells (Plaha & Rogers, 1983).

Effect of EDTA and dilution. After a 30 min period of uptake of the complex, 2 mM-EDTA was added to the culture and samples were diluted 100-fold at 37 °C. Loss of label from the cells was determined as described above.

5'-Nucleotidase assay. After growing bacteria in the appropriate medium, 5 × 10⁸ cells suspended in 5.0 ml 0.85% NaCl were subjected to either osmotic shock, sonication (12 × 10 s; MSE 100 W ultrasonic disintegrator), treatment with EDTA followed by dilution, or dilution alone. Each treatment was followed immediately by membrane filtration (0.45 µm). The filtrates were assayed according to Sigma Technical Bulletin no. 675 except that 0.01 M-CaCl₂ and CoCl₂ were added for optimum activity (Neu & Heppel, 1965).

Gel electrophoresis of membrane proteins after osmotic shock. The *fes* mutant was grown to the exponential phase in medium A (Plaha & Rogers, 1983) supplemented with 0.25 µCi ml⁻¹ ¹⁴C-labelled amino acids [57 mCi (2.1 MBq) per milliatom carbon]. To a 10 ml cell suspension (2 × 10⁸ cells ml⁻¹) was added Fe³⁺-enterochelin (final concn 1 µM), and the culture was stirred at 37 °C for 20 min. EDTA (2 mM) was then added and the cells were immediately harvested by centrifugation in the cold (12000 g for 2–3 min). A control sample containing no EDTA was also prepared. The bacterial pellets were resuspended in distilled water at 37 °C and incubation was continued with shaking for 1 h. The cells were then harvested as above and finally resuspended in 2 ml 0.01 M-Tris/HCl, pH 8.5, containing 2 mM-EDTA and to this was added 3 ml of unlabelled carrier cells (2 × 10⁹ ml⁻¹) in the same buffer. Bacterial outer membranes were obtained as the insoluble fraction after treatment of the cells with 0.5% Sarkosyl NL97 (Lugtenberg *et al.*, 1975). The supernatants obtained after centrifugation of cells incubated in distilled water were membrane filtered (0.45 µm) and freeze-dried in 2 ml volumes. These were reconstituted in 0.2 ml of non-radioactive outer-membrane protein preparations in 0.0625 M-Tris/HCl, pH 6.8, containing appropriate supplements for application to gels (Lugtenberg *et al.*, 1975). After SDS polyacrylamide gel electrophoresis, staining was done with 0.25% Coomassie blue in methanol. Gels were dried on Whatman 3 MM paper prior to autoradiography. The autoradiographs were developed after one week.

RESULTS

Release of label after osmotic shock

After uptake of $^{59}\text{Fe}^{3+}$ -enterochelin by wild-type *E. coli* K12 from a $1\text{ }\mu\text{M}$ solution in TSB for 60 min, cells were subjected to sucrose/EDTA treatment and the suspension was diluted 100-fold into water by micropipette. This osmotic shock resulted in 25% loss of label from the cells over a period of 30 min. When the salts medium A (Plaha & Rogers 1983) was used rather than TSB as suspending medium, 17% of the label was lost from the cells. Corresponding representative figures for the *fes* mutant were 11% and 59% respectively. The results obtained using medium A are in accord with the idea that in this mutant, the intact complex accumulates within the bacterial cell (Greenwood & Luke 1978) whilst the relatively low figure obtained with TSB is in accord with previous observations that Fe^{3+} -enterochelin is degraded, to some extent, by this mutant suspended in a rich medium (Plaha & Rogers 1983). It should be noted that with the *fes* mutant in medium A, a significant proportion (17%) of the label was lost spontaneously after 30 min of uptake and well before the application of osmotic shock at 60 min. When the cells were shocked after a 20 min uptake period, a larger amount (38%) of the label was lost.

It was observed in each of three experiments that when cells were treated with sucrose/EDTA, collected by centrifugation and resuspended in diluting fluid at 4°C as described by Nossal & Heppel (1966), little or no loss of label occurred. On raising the temperature to 37°C , however, label was lost from the cells, although the loss was only half that detected following micropipetting and dilution of the culture. The rate of release of Fe^{3+} -enterochelin observed in these experiments is slow compared to that of either vitamin B_{12} (Reynolds *et al.*, 1980) or certain periplasmic enzymes (Nossal & Heppel, 1966), where equilibration with the medium is almost complete within 1–2 min.

As a control for the osmotic shock technique, the supernatant was assayed for the presence of 5'-nucleotidase, one of several enzymes released by subjecting the cells to osmotic shock (Nossal & Heppel, 1966). When represented as a percentage of the maximum amount of enzyme released after sonication, the release of 5'-nucleotidase following osmotic shock of *E. coli* K12 wild-type and *fes* cells was 36% and 32% respectively (data not shown). These values agree fairly well with the figure of 46% given by Nossal & Heppel (1966).

Release of label after addition of EDTA followed by dilution

The unexpectedly slow release of label after osmotic shock prompted an examination of the effect of each stage of the procedure. Addition of 2 mM-EDTA followed by a 100-fold dilution by micropipetting into distilled water at 37°C after uptake of iron from $1\text{ }\mu\text{M}$ - $^{59}\text{Fe}^{3+}$ -enterochelin by wild-type *E. coli* K12 in TSB or *E. coli fes* in medium A resulted in 18% and 50% release of the label respectively. These values are similar to those obtained when the bacteria were subjected to the complete osmotic shock procedure which requires the initial addition of 10% sucrose. Changing the diluting medium from distilled water to 0.85% NaCl or TSB (the uptake medium) made little difference to the release of label from wild-type *E. coli* K12 (14% and 12% respectively). However, dilution into TSB containing 2 mM-EDTA resulted in no release, although dilution into either aqueous EDTA alone or 4 mM-2,4-dinitrophenol (DNP) did not significantly inhibit the release of radioactivity from cells (21% and 11% respectively). The 35% release of label from *E. coli fes* after dilution into the same medium as that used for the uptake (medium A) was slightly less than that after dilution into distilled water (50%).

In order to differentiate between the release of iron and that of the Fe^{3+} -enterochelin complex, *E. coli fes* was allowed to accumulate label from $1\text{ }\mu\text{M}$ - Fe^{3+} - $[^{14}\text{C}]$ enterochelin in medium A, after which 2 mM-EDTA was added and the cells were harvested by centrifugation and resuspended in distilled water at 37°C . There was 21% release of label after incubation for 30 min. The same treatment during uptake of label from $1\text{ }\mu\text{M}$ - $^{59}\text{Fe}^{3+}$ -enterochelin released 24% of the accumulated label, suggesting that the intact ferric enterochelin complex is released from the mutant. Since the specific activity of the $[^{14}\text{C}]$ enterochelin was low, the technique of Nossal & Heppel (1966), which involves centrifugation followed by resuspension of the pellet, was used in this case.

Table 1. *Effect of cations on the release of $^{59}\text{Fe}^{3+}$ -enterochelin from E. coli K12*

Cation	Radioactivity released (%) [*]	
	Wild-type [†]	<i>fes</i> mutant [‡]
None (control)	18	50
Fe^{3+}	15	50
Zn^{2+}	13	51
Mn^{2+}	21	57
Mg^{2+}	22	—
In^{3+}	15	61
Cd^{2+}	17	—
Co^{2+}	45	60

^{*} Percentage radioactivity released at 37 °C 30 min after the addition of 2 mM-EDTA and immediate dilution of 0.1 ml into 10 ml 40 μM -cation solution in water (100% represents not less than 2×10^3 c.p.m. per 10^8 cells).

[†] After 60 min uptake in TSB.

[‡] After 20 min uptake in medium A.

Control experiments showed that addition of 2 mM-EDTA to *E. coli fes* followed by 100-fold dilution by micropipetting into distilled water at 37 °C did not cause any release of periplasmic 5'-nucleotidase. Likewise, this treatment caused no release of label from *E. coli fes* during uptake of 10 nM-[^{57}Co]vitamin B₁₂ in medium A (data not shown). It seemed possible, therefore, that either a significant proportion of the Fe^{3+} -enterochelin may have been released from the outer-membrane receptor or that the receptor itself was lost from the cells (Pugsley & Reeves, 1977). However, treatment of *E. coli fes* labelled with ^{14}C -labelled amino acids with 2 mM-EDTA followed by resuspension in distilled water at 37 °C did not appear to release either the outer-membrane receptor for ferric enterochelin or any other proteins of $M_r > 32000$, as determined by SDS polyacrylamide gel electrophoresis of the outer-membrane proteins and the supernatant (Lugtenberg *et al.*, 1975) (data not shown).

Effect of cations on the release of label

The results presented above suggested that the release of label from cells after addition of EDTA followed by dilution was not solely a consequence of osmotic difference between the uptake and the diluting medium. In particular, the prevention of release into TSB by the presence of EDTA suggested that some cation present in the broth may have been aiding the release of label. This possibility was examined by supplementing the diluting medium with various cations. Table 1 illustrates their effect on the release of label after addition of EDTA and dilution by micropipetting during uptake of iron from 1 μM - $^{59}\text{Fe}^{3+}$ -enterochelin by *E. coli* K12 in TSB and *E. coli fes* in medium A. The cations were present at twice the final concentrations of EDTA. Of all the ions tested, only Co^{2+} (40 μM) had a marked stimulatory effect on the release of label from wild-type cells; this effect was observed consistently in five experiments. None of the ions tested had a significant effect on the release of label from the *fes* mutant. The loss of label from wild-type cells after dilution into the Co^{2+} solution was reduced by the presence in the solution of either 2 mM-EDTA or 4 mM-DNP (Fig. 1), suggesting that the release is specifically related to the Co^{2+} ion and requires an energized cytoplasmic membrane.

Effect of shearing forces on the release of label

Comparison of the results obtained by dilution using a micropipette with those obtained by centrifugation and resuspension (Nossal & Heppel, 1966) showed consistently that more label was released by the first of these techniques. It was considered that shearing forces may have been responsible for this difference. Further, since EDTA actually inhibited the subsequent loss of label from cells after dilution into TSB, it was decided to omit this reagent. The release of radioactivity following a 100-fold dilution into various solutions at 37 °C during uptake of iron from 1 μM - Fe^{3+} -enterochelin by wild-type *E. coli* K12 in TSB is illustrated in Fig. 2. The dilutions were made either by adding a 0.1 ml sample by micropipette to 10 ml medium (method

Fig. 1

Fig. 2

Fig. 1. Effect of dilution on Fe^{3+} -enterochelin transport in wild-type *E. coli* K12 suspended in $1 \mu\text{M}$ - $^{59}\text{Fe}^{3+}$ -enterochelin in TSB (specific activity of $^{59}\text{Fe}^{3+}$ $13.3 \mu\text{Ci} \mu\text{g}^{-1}$). ●, Uptake phase; at 60 min (arrow) 2 mM -EDTA was added and 0.1 ml samples were diluted into 10 ml of: water (○), $40 \mu\text{M}$ - CoCl_2 (▲), $40 \mu\text{M}$ - CoCl_2 + 4 mM -DNP (△), or $40 \mu\text{M}$ - CoCl_2 + 2 mM -EDTA (■) (all maintained at 37°C).

Fig. 2. Effect of dilution technique on Fe^{3+} -enterochelin transport in wild-type *E. coli* K12 suspended in $1 \mu\text{M}$ - $^{59}\text{Fe}^{3+}$ -enterochelin in TSB (specific activity of $^{59}\text{Fe}^{3+}$ $13.3 \mu\text{Ci} \mu\text{g}^{-1}$). ●, Uptake phase; at 60 min (arrow) samples were diluted 100-fold into TSB (○), water (△), 0.85% NaCl (□, ■), or $20 \mu\text{M}$ - CoCl_2 (◇, ◆). Open symbols refer to 0.1 ml samples diluted by micropipette into 10 ml diluent (method A); filled symbols refer to 100 ml diluent added carefully to 1.0 ml culture (method B).

A) or by a gentle addition of 100 ml medium to a 1 ml sample (method B). When TSB and saline were used as diluting media, there was no release of label by method A, although further uptake of ferric enterochelin was inhibited. However, dilution into saline by method B caused neither release of label nor inhibition of its uptake. By comparison with the results described above, it appears that release into saline or TSB requires prior treatment of cells with EDTA. Loss of radioactivity from cells after dilution into distilled water was greater using method A than by method B, the release by method A being similar to that after osmotic shock or EDTA-dilution (Fig. 1). The results suggest that the release of Fe^{3+} is partly due to the osmotic difference between the uptake and the diluting medium and partly a result of shearing during pipetting. However, the effect of pipetting is not important in the release of label when the diluting medium is supplemented with $20 \mu\text{M}$ - Co^{2+} , since in this case the loss of radioactivity from the cells was the same whether the dilutions were made according to method A or B (Fig. 2). The release of label was large and similar to that resulting from addition of EDTA followed by dilution into Co^{2+} solution. Dilution by method A into TSB supplemented with $20 \mu\text{M}$ - CoCl_2 resulted in no release of label (data not shown), suggesting that the enhancement of release by Co^{2+} occurs only in a hypo-osmotic medium.

Control experiments showed that dilution by method A into $20 \mu\text{M}$ - Co^{2+} solution caused no significant release of periplasmic $5'$ -nucleotidase from wild-type *E. coli* K12. Similarly, dilution into distilled water, 0.85% saline or $20 \mu\text{M}$ - Co^{2+} solution during uptake of 10 nM - $[^{57}\text{Co}]\text{vitamin B}_{12}$ by wild-type cells in TSB did not result in any significant loss of radioactivity from the cells (data not shown). It was concluded that the release of Fe^{3+} following dilution into Co^{2+} solution appears to be a specific effect.

DISCUSSION

Recently published work has shown that *E. coli* can accumulate a pool of Fe^{3+} -enterochelin. This appears to be present in the periplasmic space since it undergoes exchange with extracellular complex and is released from the cell following osmotic shock (Ecker *et al.*, 1986; Matzanke *et al.*, 1986). We began our work using a *fes* mutant of *E. coli* K12 in which the lack of enterochelin esterase allows the cell to accumulate Fe^{3+} -enterochelin. The site of this accumulation was examined by the application of osmotic shock. The ferric complex of enterochelin was released in significant quantities from the mutant. Analysis of the effects of the individual stages of the shock procedure on wild-type cells showed that prior exposure of cells to sucrose and EDTA was not required, careful dilution of cells into a hypo-osmolar medium being sufficient to induce efflux of Fe^{3+} . Prior treatment with EDTA or exposure to shearing forces served either to enhance efflux or to induce efflux in isotonic media. Efflux was temperature dependent. Control experiments showed that neither vitamin B_{12} nor 5'-nucleotidase was released from the periplasm by these procedures. The release of Fe^{3+} from wild-type cells under mild conditions was stimulated specifically by Co^{2+} , but Co^{2+} did not stimulate the release of Fe^{3+} -enterochelin from the mutant. The observation (Fig. 1) that efflux in the presence of Co^{2+} was abolished by DNP suggested that in this case the release is an active process, dependent upon the proton-motive force, rather than passive diffusion. Shearing forces encountered during micropipetting enhanced release of Fe^{3+} from the cells but failed to release either [^{57}Co]vitamin B_{12} (Reynolds *et al.*, 1980) or 5'-nucleotidase (Nossal & Heppel, 1966), both well-characterized markers of the periplasm. Thus, the release of $^{59}\text{Fe}^{3+}$ could have arisen through the combined action of shearing forces and a physiological response to a hypo-osmolar medium. The mildest conditions giving maximal release consisted of careful dilution into water containing $20\text{ }\mu\text{M}$ - Co^{2+} .

It is necessary to form a working hypothesis based on these observations. Clearly, osmotic shock in the accepted sense (Neu & Heppel, 1965; Nossal & Heppel, 1966) is not involved. Further, the effect of shearing forces introduces a complication but even so it is interesting that these forces immediately inhibit uptake of Fe^{3+} -enterochelin (Fig. 1), presumably as a result of the loss or disorganization of some outer-membrane component. However, these shearing forces only induce an actual loss of label from cells previously exposed to EDTA. It seems possible therefore that the efflux which occurs on careful dilution into a hypo-osmolar medium could be a normal physiological response to a shift down to a dilute medium. The effects of exposing *E. coli* to hyperosmotic solutions have been studied quite thoroughly (LeRudulier *et al.*, 1984). From the present work, the response to hypo-osmolarity appears to be quite rapid (Figs 1 and 2), and thus may be under the control of an allosteric effector which responds to the osmolarity of the medium. The fact that the efflux is driven by an energized membrane also suggests that it is a physiological response. Co^{2+} appears to be an essential cofactor for the release of Fe^{3+} from the cell.

The consequences of the loss of Fe^{3+} -enterochelin on the physiology of the cell must be considered. As iron-deficient cells were used throughout this work, the efflux of either Fe^{3+} -enterochelin or Fe^{3+} seems to represent the loss of a short-term storage form of iron. This loss implies an enhancement of iron deficiency on exposure to a hypo-osmolar, and in the present context, nutritionally poor medium. Although iron-deficient bacteria are known to lack a number of enzymes for which iron is an essential cofactor (Coughlan, 1971), the rapid response encountered here implies the presence of an active control system rather than passive dilution of iron during repeated cell division cycles. Such systems have been described by Buck & Griffiths (1982), who found both increased expression of the *trp* operon and enhanced transport of aromatic amino acids due to the presence of undermodified tRNAs in iron-deficient *E. coli* (Buck & Griffiths, 1981). The phenotypic changes in iron metabolism which occur on exposure of enterobacterial pathogens to body fluids (Griffiths *et al.*, 1983; Rogers, 1973) point to a central role for iron metabolism in bacterial survival under adverse conditions. The present results also suggest an adjustment in iron metabolism which may enable the organism to survive under hypo-osmolar conditions. It is known, for example, that *Salmonella typhi* can survive in small numbers for at least two weeks at ambient temperature in river water (Wilson, 1983).

Boyd & Holland (1977) showed that simple filtration and resuspension of *E. coli* K12 can induce the synthesis of the outer-membrane receptor for Fe^{3+} -enterochelin. This observation, together with those presented above on the effect of shearing, suggest that the physical stressing of the outer membrane of *E. coli* leads to rapid and marked changes in the Fe^{3+} -enterochelin transport system.

Bacterial iron metabolism is known to be an important determinant of virulence. It may be that exposure of cells to shearing forces during the process of injection into experimental animals may result in a temporary change in virulence. Such an effect would represent yet another complication when comparing *in vitro*- and *in vivo*-grown bacteria (Penn *et al.*, 1976).

REFERENCES

- BOYD, A. & HOLLAND, I. B. (1977). Protein D, an iron-transport protein induced by filtration of cultures of *Escherichia coli*. *FEBS Letters* **76**, 20–24.
- BRAUN, V. (1985). The unusual features of the iron transport systems of *Escherichia coli*. *Trends in Biochemical Sciences* **10**, 75–78.
- BUCK, M. & GRIFFITHS, E. (1981). Regulation of aromatic amino acid transport by tRNA; role of 2-methylthio- N^6 -(Δ^2 -isopentenyl)-adenosine. *Nucleic Acids Research* **9**, 401–414.
- BUCK, M. & GRIFFITHS, E. (1982). Iron mediated methylthiolation of tRNA as a regulator of operon expression in *Escherichia coli*. *Nucleic Acids Research* **10**, 2609–2624.
- CARRANO, C. J. & RAYMOND, K. N. (1979). Ferric iron sequestering agents. 2. Kinetics and mechanism of iron removal from transferrin by enterochelin and synthetic tricatechols. *Journal of the American Chemical Society* **101**, 5401–5404.
- COUGHLAN, M. P. (1971). The role of iron in microbial metabolism. *Science Progress* **59**, 1–23.
- ECKER, D. J., MATZANKE, B. F. & RAYMOND, K. N. (1986). Recognition and transport of ferric enterobactin in *Escherichia coli*. *Journal of Bacteriology* **167**, 666–673.
- FISS, E. H., STANLEY-SAMUELSON, P. & NEILANDS, J. B. (1982). Properties and proteolysis of ferric enterobactin outer membrane receptor in *Escherichia coli* K12. *Biochemistry* **21**, 4517–4522.
- GREENWOOD, K. T. & LUKE, R. K. J. (1978). Enzymatic hydrolysis of enterochelin and its iron complex in *Escherichia coli* K12. *Biochimica et biophysica acta* **528**, 209–218.
- GRIFFITHS, E., STEVENSON, P. & JOYCE, P. (1983). Pathogenic *Escherichia coli* express new outer membrane proteins when growing *in vivo*. *FEMS Microbiology Letters* **16**, 95–99.
- KADNER, R. J. & MCELHANEY, G. (1978). Outer membrane dependent transport systems in *Escherichia coli*: turnover of *tonB* function. *Journal of Bacteriology* **134**, 1020–1029.
- LERUDULIER, D., STROM, A. R., DANDELIAR, A. M., SMITH, L. T. & VALENTINE, R. C. (1984). Molecular biology of osmoregulation. *Science* **224**, 1064–1068.
- LUGTENBERG, B., MEIJERS, J., PETERS, R., VAN DER HOEL, P. & VAN ALPHEN, L. (1975). Electrophoretic resolution of the major outer membrane proteins of *Escherichia coli* K12 into four bands. *FEBS Letters* **58**, 254–258.
- MATZANKE, B. F., ECKER, D. J., YANG, T.-S., HUYNH, B. H., MULLER, G. & RAYMOND, K. N. (1986). *Escherichia coli* iron enterobactin uptake monitored by Mossbauer spectroscopy. *Journal of Bacteriology* **167**, 674–680.
- MONOD, J., COHEN-BAZIRE, G. & COHEN, M. (1951). Sur la biosynthese de la β -galactosidase (lactase) chez *Escherichia coli*. La spécificité de l'induction. *Biochimica et biophysica acta* **7**, 585–599.
- NEU, H. C. & HEPPEL, L. A. (1965). The release of enzymes from *Escherichia coli* by osmotic shock during the formation of spheroblasts. *Journal of Biological Chemistry* **240**, 3685–3692.
- NOSSAL, N. G. & HEPPEL, L. A. (1966). The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. *Journal of Biological Chemistry* **241**, 3055–3062.
- O'BRIEN, I. G. & GIBSON, F. (1979). The structure of enterochelin and related 2,3-dihydroxy-*N*-benzoylserine conjugates from *Escherichia coli*. *Biochimica et biophysica acta* **215**, 393–402.
- PENN, C. W., SEN, D., VEALE, D. R., PARSONS, N. J. & SMITH, H. (1976). Morphological, biological and antigenic properties of *Neisseria gonorrhoeae* adapted for growth in guinea pig subcutaneous chambers. *Journal of Bacteriology* **97**, 35–43.
- PIERCE, J. R., PICKETT, C. L. & EARHART, C. F. (1983). Two *fes* genes are required for ferrienterochelin uptake in *Escherichia coli* K12. *Journal of Bacteriology* **155**, 330–336.
- PLAHA, D. S. & ROGERS, H. J. (1983). Antibacterial effect of the scandium complex of enterochelin. Studies of the mechanism of action. *Biochimica et biophysica acta* **760**, 246–255.
- PLAHA, D. S., ROGERS, H. J. & WILLIAMS, G. W. (1984). Studies of the antibacterial effect of the scandium complex of enterochelin. *Journal of Antibiotics* **37**, 588–595.
- PORRA, R. J., LANGMAN, L., YOUNG, I. G. & GIBSON, F. (1972). The role of ferric enterochelin esterase in enterochelin mediated iron transport and ferrochelatase activity in *Escherichia coli*. *Archives of Biochemistry and Biophysics* **153**, 74–78.
- PUGSLEY, A. P. & REEVES, P. (1977). Uptake of ferrienterochelin by *Escherichia coli*: energy dependent stage of uptake. *Journal of Bacteriology* **130**, 26–36.
- REYNOLDS, P. R., MOTTUR, G. P. & BRADBEER, C. (1980). Transport of vitamin B_{12} in *Escherichia coli*. *Journal of Biological Chemistry* **255**, 4313–4319.
- ROGERS, H. J. (1973). Iron binding catechols and virulence in *Escherichia coli*. *Infection and Immunity* **7**, 445–456.

- ROGERS, H. J. (1983). Role of iron chelators, antibodies and iron binding proteins in infection. In *Microbiology—1983*, pp. 334–337. Edited by D. Schlesinger. Washington, DC: American Society for Microbiology.
- ROGERS, H. J., SYNGE, C. & WOODS, V. E. (1980). Antibacterial effect of scandium and indium complexes of enterochelin on *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* **18**, 63–68.
- ROGERS, H. J., WOODS, V. E. & SYNGE, C. (1982). Antibacterial effect of the scandium and indium complexes of enterochelin on *Escherichia coli*. *Journal of General Microbiology* **128**, 2389–2394.
- WILSON, G. (1983). The bacteriology of air, water and milk. In *Topley and Wilson's Principles of Bacteriology, Virology and Immunity*, 7th edn, vol. 1, p. 262. Edited by G. Wilson & H. M. Dick. London: Edward Arnold.