Effect of Iron Deprivation on the Production of Siderophores and Outer Membrane Proteins in *Klebsiella aerogenes*

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The outer membrane (OM) protein profile of *Klebsiella aerogenes* grown in an iron rich chemically defined medium (Fe+CDM) showed three major proteins of 32.5, 35.5 and 39.0 kDal. The 35.5 and 39.0 kDal proteins were non-covalently associated with peptidoglycan. At least six new iron regulated outer membrane proteins (IRMP) of 69, 70, 73, 75, 78 and 83 kDal, which were not peptidoglycan associated, were apparent in the OM of *K. aerogenes* grown in iron restricted (serum) or iron deficient (Fe-CDM) media. An 18.5 kDal protein was also present in the OM of stationary phase *K. aerogenes* following growth in Fe+CDM, in iron saturated serum and in citrate supplemented CDM but was repressed in Fe-CDM or in serum. Enterochelin but not aerobactin was detected in the spent supernates of iron deficient *K. aerogenes*. Inoculation of iron replete *K. aerogenes* into low iron CDM (<17 × 10⁻⁷ M-Fe³⁺) produced IRMP and enterochelin within two generations, and several generations before the end of exponential phase. Inoculation of iron depleted cells into Fe+CDM resulted in dilution rather than active excretion from the OM of the IRMP, 1-5 generations being required for the initial level to decrease by one-half and 4 generations for it to return to that observed after growth to stationary phase in Fe+CDM. The appearance of the IRMP of *K. aerogenes* grown under iron depletion was unaffected by prior growth of the inoculum in a gross excess of iron which suggested that whether or not *K. aerogenes* was capable of storing iron, it rapidly responded to the extracellular iron concentration.

INTRODUCTION

Specific nutrient depletion not only reduces microbial growth but also influences major surface characteristics (Brown, 1977; Gilbert & Brown, 1978; Ombaka et al., 1983). Iron availability is of major importance in bacterial pathogenesis (Schade & Caroline, 1944; for a recent review see Griffiths, 1983). In body fluids such as serum, the high affinity iron binding compounds transferrin and lactoferrin ensure that little free ionic iron is available to the invading micro-organism (Bullen, 1981). As bacterial pathogens need to multiply to establish an infection they must possess mechanisms for acquiring this protein bound iron. Gram-negative bacteria growing *in vitro* under iron depleted conditions have been shown to express a number of iron sequestering mechanisms (Bullen, 1981; Griffiths, 1983; Weinberg, 1984). *Escherichia coli* both *in vivo* and *in vitro* under iron restricted conditions synthesized the phenolic iron chelator enterochelin together with several new outer membrane (OM) proteins in the 74-0-83-0 kDal range (Klebba et al., 1982; Griffiths, 1983). A second high affinity iron uptake system is often expressed by invasive *E. coli* harbouring the CoV plasmid (Stuart et al., 1982). This system

*Abbreviations*: Fe+CDM, iron rich chemically defined medium; Fe-CDM, iron deficient chemically defined medium; IRMP, iron regulated outer membrane proteins; OM, outer membrane.
employs the citrate hydroxamate derivative aerobactin. *Klebsiella* species are capable of producing both enterochelin and aerobactin (Neilands, 1981) in response to iron stress. They also appear to be able to utilize exogenously supplied iron chelators such as desferrioxamine B (Khimji & Miles, 1978). Sciortino & Finkelstein (1983) found that *Vibrio cholerae* grown in the intestines of rabbits expressed iron regulated OM proteins. The first report to show similar results in humans was for *Pseudomonas aeruginosa* isolated from the lungs of cystic fibrosis patients (Brown *et al.*, 1984).

Nutrient depletion alters the cell envelope in ways important in bacterial pathogenesis and in resistance to antimicrobial drugs (Brown, 1977; Anwar *et al.*, 1983; Costerton *et al.*, 1981; Ombaka *et al.*, 1983; Turnowsky *et al.*, 1983). The understanding of the effect of iron starvation on these surface structures in modifying the response of the organism to host defences and antibiotics is of vital importance. A study of the effect of iron deprivation on *Klebsiella aerogenes* was made particularly with respect to the relationship between growth medium iron content and the production of new outer membrane proteins and siderophores.

**METHODS**

*Bacteria.* *Klebsiella aerogenes* DL1 was a clinical isolate of capsular type 1 (Williams *et al.*, 1983). It was maintained by monthly subculture on nutrient agar and stored at 4°C.

**Chemicals.** All chemicals and reagents used were of analytical grade. Glassware was immersed overnight in 5% (w/v) Extran 300 (BDH), rinsed once in distilled water, once in 1% (v/v) HCl and finally rinsed thoroughly in double distilled water. Glassware for the collection of samples for atomic absorption analysis was subjected to an additional step of soaking overnight in 0-01% (w/v) EDTA before the final rinsing.

**Growth media.** A chemically defined medium (CDM) was designed by the method of Klemperer *et al.* (1979) such that sufficient of each essential nutrient was present to enable exponential growth of the organism to an OD470 of 10 before the onset of stationary phase. This medium enabled the production of cultures depleted of any specific essential nutrient, all other nutrients being in controlled excess. The iron deficient CDM (Fe-CDM) consisted of glucose, 35 mm; NH₄Cl, 25 mm; KCl, 1-5 mm; NaCl, 45-2 mm; MgSO₄·7H₂O, 0-4 mm; sodium phosphate buffer pH 7-4, 66-67 mm and was supplemented with CaCl₂·2H₂O, 0-5 μM; H₃BO₃, 0-5 μM; CoCl₂·6H₂O, 0-05 μM; CuSO₄·7H₂O, 0-05 μM; ZnSO₄·7H₂O, 0-05 μM; MnSO₄·0-1 μM; (NH₄)₆M₀₇O₂₄·4H₂O, 0-005 μM. FeSO₄·7H₂O (20 μM) was added to obtain an iron rich medium (Fe + CDM). NaCl was included in both Fe + CDM and Fe-CDM to render them isotonic with serum. To avoid precipitation the basal medium was sterilized by autoclaving and phosphates and FeSO₄·7H₂O were added aseptically immediately prior to inoculation. Sterile heat inactivated horse serum (Gibco, Paisley, Strathclyde, UK) was diluted to 50% (v/v) with 0-9% (w/v) NaCl before use. In one experiment 80 μM-FeSO₄ was added to alleviate the iron restriction imposed by serum transferrin.

**Iron deprivation and growth phase experiments.** Cultures of *K. aerogenes* in CDM (250 ml) were grown for 16 h at 37°C in 1 litre Erlenmeyer flasks in an orbital incubator at a shaking rate of 200 r.p.m. Optical density measurements were made at 470 nm and the cultures harvested by centrifugation at 30000 g for 15 min; the pellet was washed twice in 0-9% (w/v) NaCl unless otherwise stated and inoculated into fresh prewarmed Fe + CDM or Fe-CDM to an OD470 of 0-02 or 0-2. Cells were harvested at intervals along the growth curve by a replicate flask method in order to obtain sufficient cell mass for outer membrane (OM) preparation. At each time point, flasks were removed from the incubator, cooled rapidly in ice, the cells harvested and supernates retained.

**Outer membrane preparations.** Harvested bacteria were resuspended in 10 ml distilled water, broken by sonication (3 × 60 s pulses from a Dawe Soniprobe) at 4°C followed by the addition of 1 ml 20% (v/v) sodium N-lauryl sarcosinate (Sarkosyl, Sigma) which solubilizes the cytoplasmic membrane but leaves the outer membrane essentially intact (Filip *et al.*, 1973). After 30 min at room temperature, OMs were pelleted by centrifugation at 50000 g for 60 min at 4°C, washed once in distilled water, resuspended in 0-5 ml distilled water and stored at −20°C.

**Non-covalent peptidoglycan associated proteins.** Cells of *K. aerogenes* grown in Fe-CDM and in Fe + CDM + 1 mm-citrate were resuspended in 10 mM-sodium phosphate buffer, sonicated as above, and the proteins non-covalently bound to peptidoglycan isolated by the method of Mizuno & Kageyama (1979).

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).** OM proteins were separated on 10% gels according to the procedure described by Lugtenberg *et al.* (1975) using purified SDS (specially pure, BDH). Comparable OM protein concentrations were achieved by resuspending cells obtained at different stages along the growth curve to the same OD470 before sonication, treatment with Sarkosyl and resuspension of the isolated OM in the same volume of water. Gels were fixed and stained for protein with Coomassie Brilliant Blue R250 (Sigma) in 50% (v/v) methanol, 10% (v/v) glacial acetic acid in water, and scanned with a Bio-Rad Scanning Densitometer (model 1650).
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**Siderophore detection.** Enterochelin and related phenolates were assayed by the method of Arnow (1937). *K. aerogenes* was tested for its ability to produce hydroxamates by the method described by Csaky (1948) using the modified hydrolysis conditions of Gibson & Magrath (1969).

**Estimation of iron.** The iron concentration of Fe–CDM was measured by flameless atomic absorption spectrophotometry using a Perkin-Elmer model 360 fitted with a deuterium background corrector.

**RESULTS**

**Iron requirement of *K. aerogenes* in batch culture**

The addition of increasing concentrations of iron to a CDM in which all other ingredients were present in excess resulted in an extended exponential phase and a greater growth yield as determined by OD₄₇₀ measurements. A linear relationship was found between the concentration of added iron and the OD₄₇₀ at the end of the exponential phase, up to OD₄₇₀ 6.2. If no iron was added to the medium, the organism grew exponentially to an OD₄₇₀ of 2.1 (Fig. 1). The mean doubling time for *K. aerogenes* in both Fe⁺CDM and Fe⁻CDM during the exponential phase was 33 min (0.55 h) and therefore the growth rate was 1.26 h⁻¹. In 50% (v/v) heat inactivated horse serum, the organism doubled at the same rate as that in CDM up to OD₄₇₀ 0.5 (Fig. 1). A reduction in the contaminating concentration of iron in Fe⁻CDM could be achieved by passage of the sodium phosphate buffer (the major iron contributor) through the cationic exchange resin Chelex 100 (Bio-Rad). The resulting CDM supported exponential growth to OD₄₇₀ 0.7 (Fig. 1) and atomic absorption analysis showed that this medium contained 8 × 10⁻⁷ M-Fe³⁺.

**Effect of iron deprivation on the outer membrane protein profile**

The pattern of the major OM proteins obtained from Sarkosyl insoluble membranes of *K. aerogenes* grown to stationary phase in Fe⁺CDM, Fe⁻CDM, and Fe⁺CDM + 1 mM-citrate is shown in Fig. 2(a–c). Profiles similar to Figs 2(b) and 2(a), respectively, were obtained following growth in serum and in serum + iron (data not shown). The OM profile of *K. aerogenes* is closely related to that of *E. coli* in that three major bands of 32.5, 35.5 and 39.0 kDal are observed (Lugtenberg & Van Alphen, 1983). They were always present and appeared to be independent of the growth conditions employed. The 35.5 and 39.0 kDal proteins remained

![Fig. 1. Growth of *K. aerogenes* in Fe⁻CDM before (■) and after (▲) treatment with Chelex 100, and in serum (●). The curves are superimposable; they have been offset for clarity and do not imply the occurrence of different lag phases.](image-url)
Fig. 2. SDS-PAGE of the OM proteins of *K. aerogenes* after growth in (a) Fe + CDM, (b) Fe-CDM and (c) Fe+CDM + 1 mM-citrate; (d) peptidoglycan associated proteins of *K. aerogenes*. The pointers indicate molecular sizes in kDal.

bound to the peptidoglycan after incubation at 60 °C in 2% (w/v) SDS but were released after heating in denaturing buffer (containing 2% w/v, SDS) for 10 min at 100 °C prior to electrophoresis (Fig. 2d). These proteins are therefore bound tightly although not covalently to peptidoglycan (Mizuno & Kageyama, 1979) and are probably related to the OmpF and OmpC porin proteins of *E. coli*. Other workers have reported the presence of two major OM proteins in *Klebsiella* of 35.0 and 38.0 kDal, the 38.0 kDal protein being peptidoglycan associated (Lugtenberg et al., 1977; Hofstra & Dankert, 1979). The 32.5 kDal protein was not peptidoglycan associated and is therefore akin to the *E. coli* OmpA protein which plays a role in F pilus mediated conjugation (Van Alphen et al., 1977).

In iron restricted (serum) or iron deficient (Fe-CDM) conditions, at least six new OM proteins, which were not bound to peptidoglycan and were of 69, 70, 73, 75, 78 and 83 kDal were apparent. These proteins are only just detectable in the OM of *K. aerogenes* grown in Fe + CDM or in iron saturated serum. They will therefore be referred to as the iron regulated membrane proteins (IRMP). Klibba et al. (1982) reported *E. coli* to produce four such proteins, of 83, 81, 78 and 74 kDal. The 81 kDal protein functions as the ferric enterochelin receptor and the 78 kDal protein is involved in the uptake of iron from the fungal siderophore, ferriochrome. The role of the 74 and 83 kDal proteins in iron transport is as yet undefined. As *K. aerogenes* and *E. coli* are closely related members of the *Enterobacteriaceae* it seems reasonable that their IRMP may be related.

Hancock et al. (1976) have shown that the OM of *E. coli* strains grown in the presence of 1 mM-citrate contain a polypeptide of 81 kDal that appears to be involved in citrate mediated iron uptake. However, *K. aerogenes* did not induce the formation of a similar protein in CDM supplemented with 1 mM-citrate.

The OM of stationary phase *K. aerogenes* contained a protein of 18.5 kDal after growth in Fe + CDM, in iron saturated serum and in citrate supplemented CDM. This protein was not peptidoglycan associated and was repressed after growth in Fe–CDM or in serum. Its function is unknown, although the involvement of low molecular weight OM proteins in iron metabolism in
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**Siderophore production by *K. aerogenes***

Under conditions of iron stress *K. aerogenes* secreted enterochelin but not aerobactin. To follow the appearance of enterochelin during the growth cycle, cells grown overnight in Fe+CDM were washed and resuspended to OD$_{470}$ 0·02 in Fe–CDM. The growth at 37°C was followed by the change in OD$_{470}$ at fixed time intervals. Bacteria were harvested after cooling to 4°C, OM prepared and supernates assayed for enterochelin. Enterochelin biosynthesis paralleled the growth of the organism (Fig. 3), and a maximum concentration of 27 µg ml$^{-1}$ was detected in late stationary phase. Thus the synthesis of enterochelin takes place several generations before onset of stationary phase. The supernates obtained from iron deficient cultures of *K. aerogenes* were yellow due to a dialysable pigment, not extractable by ethyl acetate, with fluorescence maxima at 444 and 375 nm, which correspond to those of riboflavin. Cultures of *K. aerogenes* grown in Fe–CDM made approximately three times more riboflavin than those grown in Fe+CDM, indicating that iron is involved in the control of riboflavin synthesis (Demain, 1972).

**Effect of growth phase on the appearance of the IRMP**

To follow the rate at which the IRMP accumulated in the OM, *K. aerogenes* grown in Fe+CDM was resuspended to OD$_{470}$ 0·2 in Fe–CDM. Outer membranes could then be prepared from cells harvested at approximately half-generation intervals. The IRMP were clearly visible 64 min (1·95 generations) after inoculation into Fe–CDM (Fig. 4). A sharp increase in the ratio occurred some 53 min (1·8 generations) after inoculation, corresponding to...
the accumulation of the IRMP in the OM. Thus in the presence of a low extracellular iron concentration, the IRMP were rapidly de-repressed and appeared in the OM within two generations. Therefore both the siderophore enterochelin and the IRMP, which presumably include the enterochelin receptor, are produced several generations before the end of the exponential phase.

**Growth of iron deprived K. aerogenes in Fe+CDM**

The effect on the OM protein profile of inoculating late exponential phase Fe−CDM grown K. aerogenes into Fe+CDM is shown in Fig. 4. The time required for the relative level of the IRMP to decrease by one half was estimated to be 49.5 min, which is close to 1.5 cell divisions. After four generations (approximately 132 min) the ratio was reduced to that observed after growth to stationary phase in Fe+CDM. Therefore, the IRMP appeared to be diluted exponentially rather than actively excreted from the OM as the iron depleted organism multiplied in an iron replete environment. The reduction in the level of IRMP following restoration of iron to the medium is analogous to the loss of protein H1 from magnesium depleted Pseudomonas aeruginosa upon the addition of magnesium to the growth medium (Nicas & Hancock, 1983). However, since it took one-and-a-half rather than one cell division, after inoculating late exponential phase iron depleted K. aerogenes into Fe+CDM, to reduce the relative level of IRMP by half, it appears that K. aerogenes continues to manufacture IRMP for half a generation after its subculture into an iron replete environment. This may reflect the relatively long half lives of the mRNAs for outer membrane proteins (Halegoua & Inouye, 1979).

**Growth of iron replete K. aerogenes in Fe+CDM**

The changes occurring in the OM protein composition of K. aerogenes throughout the growth cycle of iron replete cells in Fe+CDM are shown in Fig. 4, which reveals that the IRMP never completely disappear but remain in the OM in amounts (relative to the 39 kDal protein) just detectable by Coomassie Blue staining of the SDS polyacrylamide gels. The 18.5 kDal protein which was virtually absent from Fe−CDM and serum grown K. aerogenes was induced in the exponential phase in Fe+CDM and was retained throughout the remainder of the growth cycle.

**Relationship between the extracellular iron concentration and de-repression of the IRMP**

Klebsiella aerogenes manufactured an OM complete with its high affinity iron transport systems within two generations of subculture into Fe−CDM even after growth in a gross excess of iron (20 μM). The subculture of K. aerogenes grown in a range of iron concentrations between 20 and 100 μM into Fe−CDM and its subsequent effect on the appearance of the IRMP was investigated. The de-repression of the IRMP and their incorporation into the OM still occurred within two generations in Fe−CDM despite prior growth in up to 100 μM-iron (data not shown).

The relationship between the initial extracellular iron concentration and the rate at which the IRMP appeared in the OM was investigated as follows: after growth in Fe+CDM, K. aerogenes was subcultured into fresh CDM to which a range of iron concentrations between 0 and 200 × 10⁻⁷ m-FeSO₄·7H₂O had been added. After two generations, only the OM from cells grown in Fe−CDM (no added iron) expressed the IRMP. The iron content of this Fe−CDM was calculated to be 12 × 10⁻⁷ m-Fe³⁺. The experiment was repeated with a narrower range of added iron concentrations (0 to 5 × 10⁻⁷ m). An initial total iron content of <17 × 10⁻⁷ m-Fe³⁺ enabled K. aerogenes, after growth to stationary phase in Fe+CDM, to induce the formation of its high affinity iron transport system so that the IRMP were clearly visible in stained gels within two generations.

**DISCUSSION**

In an iron restricted environment, e.g. in the presence of iron binding proteins such as transferrin, the doubling times of Gram-negative bacteria may be increased (Griffiths & Humphreys, 1978; Bullen et al., 1974). In contrast, K. aerogenes doubled in serum at the same
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rate as in Fe+CDM. As differences in doubling times will affect the attainment of critical bacterial populations, they may be crucial in determining the outcome of an infection. The ability of K. aerogenes to multiply rapidly in serum without a long lag phase or an increased doubling time may contribute to the virulence of the organism in vivo during an infection.

When subjected to conditions of iron stress K. aerogenes synthesized at least six new OM proteins together with the iron chelator, enterochelin. At least one of the new OM proteins of iron depleted K. aerogenes is presumably involved in ferric enterochelin uptake (Ichihara & Mizushima, 1978). The function of the others requires further investigation. As E. coli can utilize exogenous chelators such as ferrichrome (Wayne & Neilands, 1975), it is conceivable that the K. aerogenes IRMP may also act as receptors for exogenous siderophores. Some evidence in support of this was obtained by Khimji & Miles (1978) who reported that Klebsiella infections could be enhanced by supplying the Streptomyces iron chelator desferroxamine B.

Much is known about the enterobacterial iron chelators and IRMP although little is known of the mechanisms by which these high affinity transport systems are regulated. In E. coli the biosynthesis of the 83, 81 and 74 kDal IRMP and enterochelin are co-ordinately regulated by the intracellular iron concentration (McIntosh & Earhart, 1977). There also appears to be a regulatory mechanism which covers all iron uptake systems and is superimposed on the regulation of individual systems (Hantke, 1981). The synthesis of enterochelin and the IRMP of K. aerogenes is repressed in the presence of an excess of free iron in both Fe+CDM and in serum. The effect of iron deprivation on the regulation of these iron uptake systems was followed throughout the growth cycle. The high affinity iron uptake system was expressed at least two generations before onset of stationary phase. Enterochelin synthesis paralleled growth and the IRMP were present in the OM some two generations (66 min) following the inoculation of iron replete cells into a CDM containing <17 x 10^{-7} M-Fe^{3+}. Klebsiella is therefore capable of rapidly responding to low iron levels in the extracellular environment by the co-ordinate synthesis of enterochelin and the IRMP, an ability which may contribute to the virulence of the organism. Inoculation of iron depleted K. aerogenes into fresh Fe+CDM resulted in the exponential dilution of the IRMP from the OM as the cells divided. However, a few copies of these proteins appeared to be present throughout the growth cycle in Fe+CDM, a phenomenon which may arise as a result of the methods employed or which may reflect the biochemical basis of regulation of the IRMP. Changes in envelope properties several generations before onset of stationary phase due to magnesium depletion have been reported for P. aeruginosa (Brown & Melling, 1969).

According to the conditions employed, E. coli can store iron intracellularly during periods of iron repletion and use it during periods of iron stress (Klebba et al., 1982). The appearance of the IRMP of K. aerogenes was unaffected by the prior growth of the organism in high concentrations of iron. A number of possible explanations can be made: K. aerogenes may be unable to store iron; de-repression may depend on the organism's ability to sense the extracellular iron concentration independently of the stored or free intracellular concentration; de-repression may be dependent on the free as opposed to the stored intracellular iron concentration. For E. coli it has been suggested that once the intracellular iron falls below a specific level, synthesis of iron transport components becomes de-repressed, and in iron replete cells the stored iron becomes accessible during the induction sequence, increasing the concentration of free intracellular iron and therefore retarding induction kinetics (Klebba et al., 1982; McIntosh & Earhart, 1977).

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REFERENCES


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