Epidemiol. Infect. (1993), **110**, 41–47

Iron restriction and the growth of *Salmonella enteritidis*

H. CHART AND B. ROWE

Division of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Avenue, London, UK

(*Accepted 23 August 1992*)

SUMMARY

Strains of *Salmonella enteritidis* were examined for their ability to remove ferric-ions from the iron chelating agents ovotransferrin, Desferal and EDDA. Growth of *S. enteritidis* phage type (PT) 4 (SE4) in trypticase soy broth containing ovotransferrin resulted in the expression of iron regulated outer membrane proteins (OMPs) of 74, 78 and 81 kDa, and unexpectedly the repression of expression of OMP C. The 38 MDa 'mouse virulence' plasmid was not required for the expression of the iron-regulated OMPs (IROMPs). SE4 was able to obtain iron bound to the iron chelator Desferal and EDDA without expressing a high-affinity iron uptake system. Strains of *S. enteritidis* belonging to PTs 7, 8, 13a, 23, 24 and 30 were also able to remove ferric ions from Desferal and EDDA without expressing a high-affinity iron uptake system. We conclude that strains of SE4 possess a high-affinity iron sequestering mechanism that can readily remove iron from ovotransferrin. It is likely that iron limitation, and not iron restriction, is responsible for the bacteriostatic properties of fresh egg whites.

INTRODUCTION

Salmonella enteritidis is the most frequently isolated serotype from cases of human food poisoning in England and Wales, and strains belonging to phage type (PT) 4 predominate [1]. Chicken is a major reservoir of *S. enteritidis* PT4, and shell eggs and poultry meat represent important vehicles of infection [2, 3]. Eggs from naturally infected flocks may contain large numbers of SE4 [4] and these can multiply in both the egg yolk [5] and egg white [6].

The early studies of Schade and Caroline [7] demonstrated the bacteriostatic properties of egg white, subsequently identified as the iron chelating compound ovotransferrin [8]. Egg white contains high levels of ovotransferrin, as compared with the levels of, for example, transferrin in human serum or lactoferrin in human milk [8].

The levels of 'free' iron in egg white are considerably lower than those necessary for bacterial growth [9]. However, certain species of bacteria express high-affinity iron sequestering systems which can remove iron from these transferrins. These mechanisms generally involve the synthesis of high-affinity iron-carrying compounds called siderophores, and outer membrane proteins which act as receptors for ferric-siderophore complexes [10]. Previous studies [11] have shown that strains of SE4 can produce the siderophore enterobactin, but not aerobactin.

In the present study we examined the ability of strains of SE4 to utilize iron bound to ovotransferrin, and also consider the role of iron in the multiplication of SE4 within eggs.

MATERIALS AND METHODS

Bacteria

Strains of *S. enteritidis* used in the present study (Table 1), were stored on Dorset's egg slopes in the culture collection held by the Division of Enteric Pathogens.

Bacterial culture

Bacteria were grown in 10 ml of Trypticase Soy Broth (TSB, BBL Microbiology Systems, Cockeysville, MD) (37 °C, 16 h), and this entire starter culture was used to inoculate 150 ml TSB prior to incubation (37 °C, 3 h). The bacterial density of this second broth culture was determined by measuring the culture absorbance at 621 nm (A_{621}) and estimating the viable count using a graph plotting culture absorbance (A_{621}) against viable counts.

Bacteria were grown in 500 ml of TSB containing 250 mg apo-ovotransferrin (Sigma Chemical Co. Ltd) as described by Griffiths and Humphrey [12], in TSB containing desferrioxamine (Desferal, CIBA-Geigy; at a concentration of 1 and 5 mg/ml) and in TSB containing ethylenediamine dihydroxyphenylacetic acid (EDDA, Sigma Chemical Co. Ltd; at a concentration of 100 μg/ml). Bacteria growing in TSB–ovotransferrin were cultured in an atmosphere comprising 5% CO_2 and 95% air, and EDDA was made free from contaminating iron using the method of Rogers [13].

Preparation of outer membranes and SDS–PAGE

Outer membranes (OMs) were prepared from SE4 strains P132344 and P132344/1 as described previously [14]. OMs were also prepared using solutions containing the protease inhibitors phenylmethylsulphonylfluoride (10 mM PMSF, Sigma Chemical Co. Ltd) and ethylenediaminetetraacetic acid (1 mM $EDTANa_2$, Merck Ltd).

Outer membrane proteins (OMPs) were separated by SDS–PAGE as described previously [14]. 30 μg of OM preparations were applied to SDS–PAGE gels with a 12·5% separation gel and electrophoresed using constant current (50 mA) for 3·25 h. Profiles were stained with Coomassie blue [14].

RESULTS

Characteristics of growth under iron restriction

S. enteritidis PT4 strain P132344 was grown in TSB alone, and in TSB containing ovotransferrin, Desferal or EDDA. In TSB, strain 132344 grew rapidly over a 12 h incubation period with exponential growth commencing approximately 6 h post-inoculation (Fig. 1). Outer membranes prepared from these bacteria contained predominantly three major outer membrane proteins (MOMPs) of 33, 35 and 36 kDa (Fig. 2, lane 1) which appeared to be expressed in similar quantities.

Strain 132344 was consistently found to grow noticeably slower in TSB

Table 1. *Strains of* Salmonella enteritidis *used*

Strain no.	Phage type	Source	Ref
P132344	4	Chicken pericardium	[11]
P132344/1	4	Isogenic variant of P132344	[11]
P1492	7	Type strain	[18]
P2468	8	Type strain	[18]
P180216	13a	Avian	[18]
P186565	23	Chicken	[18]
P99768	24	Type strain	[18]
P159851	30	Chicken	[18]

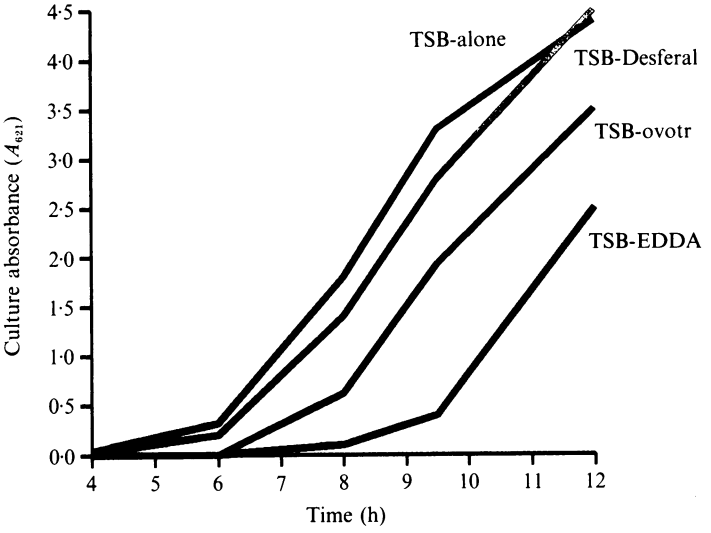

Fig. 1. *Salmonella enteritidis* strain P132344 grew rapidly in TSB alone or in TSB–Desferal. A 2 h lag phase was observed for bacteria growing in TSB–ovotransferrin, whilst bacteria in TSB–EDDA exhibited a 3·5 h lag in growth rate.

containing ovotransferrin with exponential growth commencing approximately 2 h later than bacteria growing in TSB alone (Fig. 1). This experiment was repeated several times and iron-restricted bacteria were consistently found to enter exponential growth phase approximately 2 h later than iron-replete organisms. Growth in TSB containing ovotransferrin resulted in the reduced expression of the 36 kDa MOMP (Fig. 2, lane 2), and the expression of iron-regulated outer membrane proteins (IROMPs) of 74, 78 and 81 kDa. The IROMPs of strain P132344 were found to migrate as fuzzy bands following very limited freezing and thawing of outer membrane preparations. To investigate the possible involvement of bacterial enzymes in the degradation of these IROMPs, OMs were prepared from strain P132344, grown in TSB-ovotransferrin, using solutions containing PMSF and $EDTANa_2$. Following this procedure IROMPs were found to migrate in SDS–PAGE gels as sharp bands, regardless of repeated freezing and thawing. SE4 'virulence' plasmid-free strain P132344/1 was also found to express iron-regulated outer membrane proteins of 74, 78 and 81 kDa showing that the

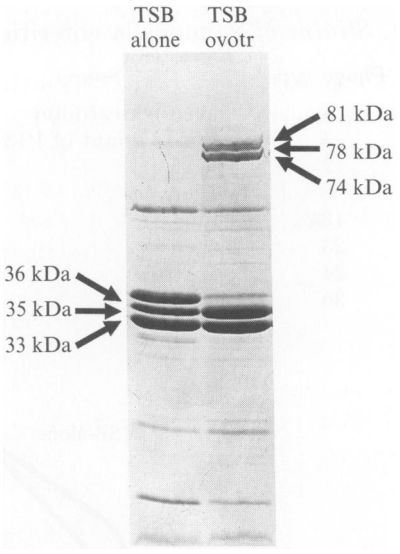

Fig. 2. *Salmonella enteritidis* strain P132344 grown in TSB alone expressed OMPs of 33, 35 and 36 kDa; in contast, growth of this strain in TSB–Desferal resulted in the expression of OMPs of 74, 78 and 81 kDa and the reduced expression of the 36 kDa OMP. 30 μg of protein was loaded per lane.

mechanisms involved in the acquisition of iron from ovotransferrin were not encoded on the 38 MDa 'virulence' plasmid.

Strains of *S. enteritidis* were also grown in TSB containing the iron chelating agent Desferal and EDDA. In the presence of 5 mg/ml Desferal, a concentration known to cause iron restriction for strains of *Escherichia coli* [15], SE4 strain P132344 grew very rapidly and repeatedly IROMPs were found not to be expressed. When the Desferal concentration was increased to 10 mg/ml (Fig. 1), SE4 strain P132344 exhibited kinetics of growth similar to those shown by this organism growing in TSB alone (Fig. 1). IROMPs were not expressed and the three MOMPs were expressed in apparently similar amounts. When SE4 strain P132344 was grown in TSB containing EDDA, the growth rate was retarded (Fig. 1) with exponential growth commencing almost 3·5 h later than bacteria growing in TSB alone. However, growth in TSB–EDDA did not result in the expression of IROMPs and the three MOMPs were expressed in similar amounts.

Poultry associated strains of *S. enteritidis* belonging to phage types 7, 8, 13a, 23, 24 and 30 were also found to grow in TSB containing Desferal (10 mg/ml) without showing a reduction in growth rate.

DISCUSSION

S. enteritidis PT4 strain P132344 showed a typical delay in growth rate when cultured in TSB containing ovotransferrin as compared to TSB alone. This lag in growth rate was thought to be caused by SE4 adapting to an iron restricted environment, by expressing iron-regulated outer membrane proteins and the siderophore enterobactin, since similar phenomena have been demonstrated for bacterial species such as *E. coli* [15]. In the presence of ovotransferrin, strain P132344 expressed IROMPs of 74, 78 and 81 kDa, which correspond to the 74 kDa

CIR protein, the 78 kDa ferrichrome receptor protein and the 81 kDa ferric-enterobactin receptor protein described for *E. coli* [16]. The protease involved in the degradation of the IROMPs remains unknown; although, since the protease inhibitor PMSF inhibited the enzyme, this protease was thought to belong to the family of 'serine' proteases. Studies concerning the IROMPs of *E. coli* [15], *Vibrio vulnificus* [19] and *Aeromonas salmonicida* [20] have shown that these proteins are generally not proteolytically degraded suggesting that SE4 might express cell-associated proteases which are released following cell disruption. Nevertheless, workers involved with the examination of IROMPs expressed by strains of salmonella should perhaps consider the use of protease inhibitors when performing studies of this nature.

Growth of SE4 in TSB containing ovotransferrin also resulted in the repression of the 36 kDa porin protein, OMP C. Although growth under conditions of iron restriction can result in the repression of certain outer membrane proteins, the effect on OMP C we describe here appears to be unusual and has not been encountered previously for other organisms. All strains of *S. enteritidis* tested were able to remove ferric iron from Desferal without expressing a high-affinity iron uptake mechanism; the processes involved are unknown and may involve the physical breaking or distortion of the Desferal molecule. The ability of bacteria to remove iron from desferrioxamine has been reported for strains of *V. vulnificus* [17], where this organism has been shown to use Desferrioxamine as a siderophore; whether SE4 can use this molecule as a siderophore remains to be established.

Growth of strain P132344 in TSB–EDDA resulted in a considerable lag in the growth rate. The growth rate of strains of *Escherichia coli* has been shown to be retarded by EDDA [15]; however, in contrast to *S. enteritidis*, strains of *E. coli* were found to express IROMPs under these growth conditions. The strains of SE4 examined were unusual in having the ability to remove iron from Desferrioxamine and EDDA, although the mechanisms involved in these processes are unknown and require further investigation.

Strains of SE4, like strains of *S. typhi* [18] and other members of the genus *Salmonella*, have been shown not to express an aerobactin mediated iron uptake mechanism [11]; consequently, we concluded that an enterobactin mediated iron uptake system was used. Enterobactin has an extremely high binding affinity for iron (formation constant = 10^{52}) as compared to that of ovotransferrin (formation constant = 10^{32}) and strains of SE4 should readily be able to remove ferric ions from egg ovotransferrin. Hen egg contents contain considerable amounts of iron, most of which is present in the egg yolk, whilst the levels of 'free' iron in the egg white are very low because of the presence of ovotransferrin.

Strains of SE4 appear to grow more rapidly in eggs that have been stored as opposed to freshly laid eggs [5]. Since the contents of eggs change during storage [6] and SE4 can migrate within eggs [6], the multiplication of SE4 within stored eggs may relate to chemical changes in egg contents which might also include the movement of iron into the egg white. As shown here strains of SE4 possess a high-affinity iron-sequestering mechanism that can readily remove iron from ovotransferrin, and it is therefore likely that iron restriction is not responsible for the bacteriostatic properties of fresh egg whites. However, bacteria are unable to multiply in an iron-limited environment and the inability of strains of SE4 to grow

in fresh egg whites may result from an absence of iron in the egg white. The work of Lock and Board [21] showed that SE4 grows very rapidly in egg whites following the injection of iron into infected eggs; however, rapid growth of bacteria in preparations containing iron-saturated transferrins has been reported for many bacterial species [22–25] and this phenomenon cannot be considered unique to SE4. We conclude that the growth of SE4 in stored eggs may result from iron migrating from egg yolk to egg white, and that the high-affinity binding of iron by ovotransferrin would not prevent SE4 from acquiring iron diffusing from the egg yolk to the egg white.

REFERENCES

1. Threlfall EJ, Rowe B, Ward LR. Subdivision of *Salmonella enteritidis* phage types by plasmid typing. Epidemiol Infect 1989; **102**: 459–65.
2. Coyle EF, Palmer SR, Riberio CD, et al. *Salmonella enteritidis* phage type 4 infection: association with hens' eggs. Lancet 1988; **ii**: 1295–7.
3. Smith JGW. Memorandum of evidence to the agricultural committee enquiry on salmonella in eggs. Public Health Service Microbiology Digest 1989; **6**: 1–9.
4. Humphrey TJ, Whitehead A, Gawler AHL, Henley A, Rowe B. Numbers of *Salmonella enteritidis* in the contents of naturally contaminated hens' eggs. Epidemiol Infect 1991; **106**: 489–96.
5. Humphrey TJ, Baskerville A, Mawer S, Rowe B, Hopper S. *Salmonella enteritidis* phage type 4 from the contents of intact eggs: a study involving naturally infected hens. Epidemiol Infect 1989; **103**: 415–23.
6. Clay C, Board R. Growth of *Salmonella enteritidis* in artificially contaminated hens' shell eggs. Epidemiol Infect 1991; **106**: 271–81.
7. Schade A, Caroline L. Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae*. Science 1944; **100**: 14–15.
8. Bezkorovainy A. Iron proteins. In: Bullen JJ, Griffiths E, eds. Iron and infection: molecular, physiological and clinical aspects. John Wiley 1987: 27–68.
9. Griffiths E. The iron-uptake systems of pathogenic bacteria. In: Bullen JJ, Griffiths E, eds. Iron and infection: molecular, physiological and clinical aspects. John Wiley 1987: 69–137.
10. Griffiths E, Chart H, Stevenson P. High-affinity iron uptake systems and bacterial virulence. In: Roth, ed. Virulence mechanisms of pathogenic bacteria. ASM Publications 1988: 121–37.
11. Chart H, Threlfall EJ, Rowe B. Virulence studies of *Salmonella enteritidis* phage types. Letts Appl Microbiol 1991; **12**: 188–91.
12. Griffiths E, Humphrey J. Alterations in tRNAs containing 2-Methylthio-N^6-(Δ^2-isopentenyl)-adenosine during growth of enteropathogenic *Escherichia coli* in the presence of iron-binding proteins. Eur J Biochem 1978; **82**: 503–13.
13. Rogers H. Iron-binding catechols and virulence in *Escherichia coli*. Infect Immun 1973; **7**: 445–56.
14. Chart H, Griffiths E. Antigenic and molecular homology of the ferric enterobactin receptor protein of *Escherichia coli*. J Gen Microbiol 1985; **131**: 1503–9.
15. Chart H, Buck M, Stevenson P, Griffiths E. Iron regulated outer membrane proteins of *Escherichia coli*: Variations in expression due to the chelator used to restrict the availability of iron. J Gen Microbiol 1986; **132**: 1373–8.
16. Chart H, Stevenson P, Griffiths E. Iron-regulated outer membrane proteins of *Escherichia coli* strains associated with enteric or extraintestinal diseases of man and animals. J Gen Microbiol 1988; **134**: 1549–59.
17. Wright A, Simpson L, Oliver J. Role of iron in the pathogenesis of *Vibrio vulnificus* infections. Infect Immun 1981; **34**: 503–7.
18. Faundez G, Aron L, Cabello FC. Chromosomal DNA, iron-transport systems, outer membrane proteins, and enterotoxin (heat labile) production in *Salmonella typhi* strains. J Clin Microbiol 1990; **28**: 894–7.

19. Chart H, Griffiths E. The availability of iron and the growth of *Vibrio vulnificus* in sera from patients with haemochromatosis. FEMS Microbiol Letts 1985; **26**: 227–31.
20. Chart H, Trust TJ. Acquisition of iron by *Aeromonas salmonicida*. J Bact 1983; **156**: 758–64.
21. Lock JL, Board RG. Persistence of contamination of hens' egg albumen *in vitro* with salmonella serotypes. Epidemiol Infect 1992; **108**: 389–96.
22. Tucker RG. Iron and infection. Aust Microbiologist 1986; **7**: 380–3.
23. Payne SM. Iron and virulence in the family Enterobacteriaceae. CRC Crit Revs Microbiol 1988; **16**: 81–111.
24. Payne SM. Iron and virulence in *Shigella*. Mol Microbiol 1989; **3**: 1301–6.
25. Hershko C, Peto TEA, Weatherall DJ. Iron and infection. B M J 1988; **296**: 660–4.