Epidemiol. Infect. (1993), **111**, 449–454

Outer membrane characteristics of *Salmonella enteritidis* phage type 4 growing in chickens

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(*Accepted 16 June 1993*)

SUMMARY

Strains of *Salmonella enteritidis* belonging to phage type 4 (SE4) were grown in the peritoneal cavities of chickens, and without subculture on laboratory media examined for inducible *in vivo* phenotypic characteristics. These bacteria expressed three major outer membrane proteins (OMPs) of 33, 35 and 36 kilodaltons (kDa), and iron regulated OMPs of 74, 78 and 81 kDa. Bacteria growing *in vivo* did not express flagella, or fimbriae with a subunit molecular mass of 14 kDa (14 kDa fimbriae). Two OMPs of 55 and 23 kDa, expressed during culture in nutrient broth, were repressed during growth in chickens. Possession of a 38 MDa 'mouse virulence' plasmid did not influence the expression of OMPs, flagella or fimbriae. It was concluded that strains of SE4 growing in chicken tissues, use an enterobactin mediated iron uptake system to obtain ferric ions, do not express flagella or 14 kDa fimbriae and appear not to express novel OMPs involved in survival *in vivo*.

INTRODUCTION

Salmonella enteritidis remains the most frequently isolated serotype from cases of human food poisoning in England and Wales, and strains belonging to phage type 4 (SE4) continue to predominate [1]. Chickens are a major reservoir of SE4, and shell eggs and poultry meat represent important vehicles of infection [2, 3]. Chickens have been shown to harbour SE4 extra-intestinally [4, 5], and the presence of SE4 in hens' reproductive tissue has been suggested as the probable cause of infected eggs [6, 7]. Chickens appear to tolerate the presence of large numbers of SE4 in body tissues. This may occur due to an inadequate chicken immune system or it may result from strains of SE4 having specialized mechanisms for avoiding the chicken immune system.

Virulence studies involving SE4 and BALB/c mice have shown the possession of a 38 MDa 'mouse-virulence' plasmid and expression of long-chain lipopolysaccharide is essential for virulence. Strains of SE4 have been shown to express fimbriae with subunit molecular masses of 14, 17 and 21 kDa [8–10] which may be involved in attachment to eukaryotic cells, although a role for these putative virulence mechanisms remain to be established.

Certain bacterial virulence mechanisms are known to be inducible and the possibility arises that SE4 may possess virulence mechanisms which are only expressed during growth in chicken tissues. For example, siderophore mediated high affinity iron sequestering systems are expressed by certain bacteria during growth *in vivo* [11, 12], and can only be demonstrated *in vitro* using specialized culture media. Previous studies have shown that strains of SE4, growing in media containing ovotransferrin, express a high affinity iron sequestering system involving the siderophore enterobactin [13]; however, mechanisms enabling SE4 to survive within chicken tissues have not been investigated. In the present study we grew SE4 in the peritoneal cavities of chickens and examined these bacteria for inducible outer membrane proteins which might be involved in the extra-intestinal survival of SE4 in poultry.

MATERIALS AND METHODS

Bacteria

Strains of SE4 used in the present study were P132344, a strain carrying a 'mouse virulence' plasmid and expressing long-chain lipopolysaccharide (LPS) [14] and P132344/1, an isogenic variant of P132344 which also expresses long-chain LPS but had been cured of the 'mouse virulence' plasmid [14]. Bacteria were stored on Dorset's egg agar slopes at room temperature in the culture collection held by the Laboratory 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 used to inoculate 150 ml TSB prior to incubation (37 °C, 3 h). The bacterial density of the 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 sedimented (5000 ***g***, 10 min, 15 °C), washed twice in saline and suspended in 1 ml of saline prior to use for implantation.

Bacteria were also grown on Sensitest Agar (Oxoid Ltd; 37 °C, 16 h). Each strain was grown on one 15 cm diameter agar plate.

Bacterial implants

Bacterial suspensions, containing approximately 10^{10} bacteria, were placed into sterile 1·5 ml screw-cap Eppendorf tubes, sealed and immersed in 10 ml of 70% aqueous methanol for 10 min. Eppendorf tubes were washed in sterile saline and placed into sterile dialysis tubing (20 cm long × 1·5 cm diameter), when both ends of the tube were sealed with a double knot, Eppendorf tubes were uncapped and the bacteria released into the dialysis tube.

Insertion of implants

White leghorn hens were used for the present study. A 1 ml preoperative blood sample was obtained by venepuncture of the alar vein, and used to screen birds for serum antibodies to SE4. Each bird received buprenorphine (Temgesic. Reckitt

and Colman Ltd) 4 h prior to anaesthesia using halothane and nitrous oxide. A 5 cm incision was made on the medial aspect of the abdomen just below the rib cage to allow insertion of the implant into the abdominal cavity and the incision was sutured. Buprenorphine was administered as a post-operative analgesic every 12 h for 36 h. The birds were individually housed and received a commercial pelleted diet (HPG, Special Diet Services) and water *ad libitum*. After 2 days, birds were sacrificed by cervical dislocation and the implants retrieved.

ELISA and bacterial agglutination test

Enzyme-linked immunosorbent assays (ELISA) were performed as described previously [15]. ELISA plates were coated with LPS prepared from SE4 and serum antibodies of the IgG class assayed for using an alkaline phosphatase-conjugated anti-chicken IgG immunoglobulin. Bacterial agglutination tests were performed as described elsewhere [15], using a *S. pullorum* strained antigen (Central Veterinary Laboratories, Weybridge). Sera from experimental birds were examined alongside known anti-LPS antibody-positive and antibody-negative sera obtained in the course of previous studies [15].

Preparation of outer membranes and SDS–PAGE

Outer membrane (OMs) were prepared from SE4 strains P132344 and P132344/1 as described previously [16], using the protease inhibitors phenylmethylsulphonylfluoride (10 mM PMSF, Sigma Chemical Co. Ltd) and ethylenediaminetetraacetic acid (1 mM $EDTANa_2$, Merck Ltd) [13].

Outer membrane proteins (OMPs) were separated by SDS–PAGE as described previously [16]. 30 μg of OM preparations were applied to SDS–PAGE gels with a 15% separation gel and electrophoresed using a constant current (50 mA) for 3·25 h. Profiles were stained with Coomassie blue [16].

Extraction of flagella and fimbriae

Bacteria were scraped from Sensitest Agar, transferred to a 1·5 ml Eppendorf tube and suspended in saline. Following incubation at 60 °C (30 min), preparations were centrifuged (12000 ***g***, 10 min) and the supernatant containing fimbriae and flagella were removed for analysis.

RESULTS

ELISA

Sera prepared from chickens prior to experimentation were examined for serum antibodies to the LPS of SE4. Antibodies to SE4 LPS were not detected by an LPS ELISA, and agglutinating antibodies were not found using the '*S. pullorum*' agglutination test.

Characteristics of growth in vitro

During growth in nutrient broth, strain P132344 and P132344/1 expressed predominantly three major outer membrane proteins (MOMPs) of 33, 35 and 36 kDa (for SE4 strain P132344 see Fig. 1, lane 1). Extracts of bacteria grown on

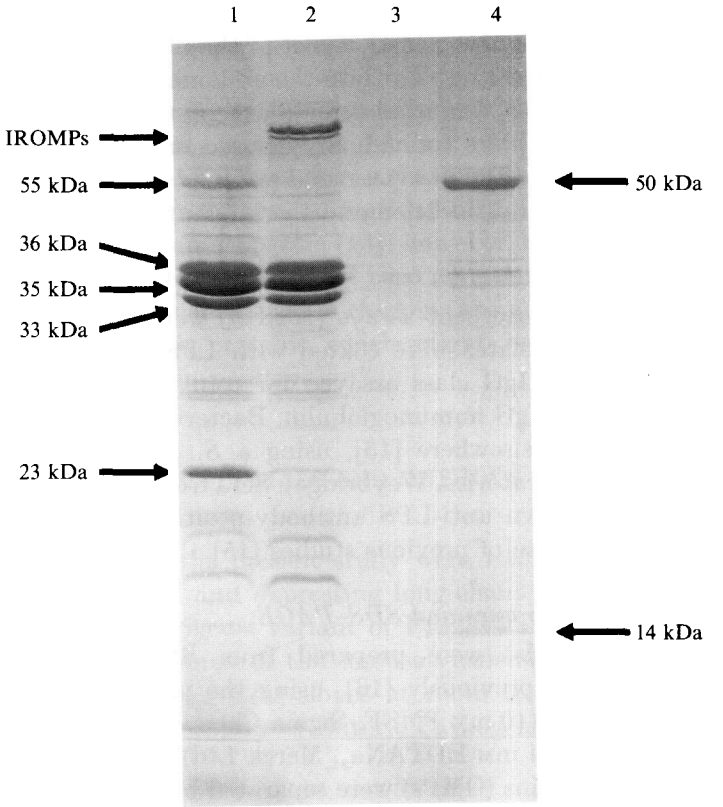

Fig. 1. During growth *in vitro*, SE4 strain P132344 expressed OMPs of 33, 35 and 36 kDa (lane 1). In addition to these OMPs, bacteria obtained from chicken peritoneal cavities expressed OMPs of 74, 78 and 81 kDa (IROMPs), but did not express an OMP of 55 kDa and expressed reduced amounts of an OMP of 23 kDa (lane 2). SE4 strain P132344 growing *in vivo* did not express flagella or '14 kDa' fimbriae (lane 3) as detected in bacteria growing on sensitest agar (lane 4). 30 μg of protein was loaded per lane.

sensitest agar contained flagella with subunits of 50 kDa (for SE4 strain P132344 see Fig. 1, lane 4).

Characteristics of growth in vivo

SE4 strains P132344 and P132344/1 were also grown in the peritoneal cavity of chickens. At the termination of experimentation, chickens were killed and the implants retrieved. Following brief washing of intact implants with sterile saline. dialysis tubes were opened and bacteria harvested prior to preparation of outer membranes and flagella/fimbrial extraction. Bacteria obtained from implants were devoid of any pigment and appeared bleached. From optical density measurements obtained with bacteria retrieved from implants, the bacterial density of both strains had increased by factor of approximately tenfold. Growth in chickens resulted in the expression of three MOMPs of 33, 35 and 36 kDa. and iron regulated OMPs (IROMPs) (for SE4 strain P132344 see Fig. 1, lane 2). Loading 15 μg protein per lane showed that the IROMPS comprised proteins of 74, 78 and 81 kDa (not shown). Extracting *in vivo* grown bacteria for flagella or

https://doi.org/10.1017/S0950268800057174 Published online by Cambridge University Press

fimbriae failed to detect these structures (for SE4 strain P132344 see Fig. 1, lane 3), although flagella with a subunit molecular mass of 50 kDa and fimbriae with a subunit molecular mass of 14 kDa were detected on sensitest agar grown bacteria (Fig. 1, lane 4).

DISCUSSION

In the present study we examined strains of SE4 for inducible outer membrane proteins which might facilitate the survival of this organism when growing in chicken tissues. The increase in bacterial density of inocula used for implantation, showed that bacteria were able to survive and multiply within the peritoneal cavities of chickens. The expression of iron regulated outer membrane proteins, *in vivo*, suggested that an enterobactin mediated iron sequestering system was used by strains of SE4 to obtain ferric ions. The ability of bacteria to obtain iron from host iron transport proteins has been considered as a major virulence factor [11], and the results of our study suggests that the iron uptake system expressed by strains of SE4 constitutes a major virulence mechanism.

Strains of SE4 grown in chickens expressed three major OMPs including one of 36 kDa, previous studies [13] have shown that growth of SE4 in TSB containing ovotransferrin resulted in the repression of this 36 kDa OMP. From these earlier studies it was thought that the 36 kDa OMP might be negatively iron regulated; however, the expression of a 36 kDa OMP by SE4 growing in chickens suggests that iron may not play a role in the expression of this protein. Outer membrane proteins of 23 and 55 kDa, observed in bacteria grown in nutrient broth, were poorly expressed during growth *in vivo*. The function of these proteins is unknown and the regulation of their expression has still to be determined.

Strains of *S. enteritidis* have been reported to express three different types of fimbriae, with subunit molecular weights of 14, 17 and 21 kDa [8–10], although their role in adhesion has not been demonstrated. Fimbriae with a subunit molecular mass of 14 kDa were expressed by bacteria growing on sensitest agar, but this structure was not produced by SE4 during multiplication within chickens. Fimbriae are primarily structures associated with attachment to mucosal membranes and may only be produced when growing on solid substrates; nevertheless, SE4 seems to repress the expression of these fimbrial structures *in vivo*.

The observation that SE4 growing in chickens did not express flagella may explain the absence of a serological response to this structure by birds infected with SE4 [15], where sera from infected birds were reacted with purified flagellar protein, but serum antibodies to SE4 flagella were not detected. Flagella are the main structures involved in bacterial motility, and the observation that SE4 do not assemble flagella *in vivo* suggest that motility was not essential for existence in chicken tissues.

From this study it was concluded that for strains of SE4, growing in chickens, flagella and 14 kDa fimbriae are not required for multiplication *in vivo*. Furthermore, SE4 appears not to produce novel membrane associated proteins involved with survival in chicken tissues. However, strains of SE4 use an enterobactin-mediated high affinity iron sequestering mechanism for obtaining ferric ions.

ACKNOWLEDGEMENTS

The experiments carried out in this study were performed under Home Office project licence number 70/01210.

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