Microbial Pathogenesis 104 (2017) 146–150

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Identification by PCR signature-tagged mutagenesis of attenuated *Salmonella* Pullorum mutants and corresponding genes in a chicken embryo model

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ARTICLE INFO

Article history:
Received 19 May 2016
Received in revised form
30 June 2016
Accepted 5 January 2017
Available online 12 January 2017

Keywords:
Salmonella Pullorum
Signature-tagged mutagenesis
Chicken embryo

ABSTRACT

A key feature of the fowl-specific pathogen *Salmonella* Pullorum is its vertical transmission to progeny via the egg. In this study, PCR signature-tagged mutagenesis identified nine genes of a strain of *S.* Pullorum that contributed to survival in the chicken embryo during incubation. The genes were involved in invasion, cell division, metabolism and bacterial defence. The competition index *in vivo* and in vitro together with a virulence evaluation for chicken embryos of all nine mutant strains confirmed their attenuation.

1. Introduction

The *Salmonella* genus contains two species, *Salmonella enterica* and Salmonella bongori, and comprises 2610 serovars that are common vertebrate pathogens causing disease ranging from enterocolitis to systemic infections [1]. Although most serovars have a wide host range, a small number are adapted to specific hosts, such as *Salmonella* enterica serovar Typhi in humans, *S.* Dublin in cattle and *S.* Gallinarum and *S.* Pullorum in poultry.

As an avian pathogen, *Salmonella* Pullorum continues to produce severe systemic diseases of domestic poultry with economic losses worldwide arising from high morbidity and mortality and reduction in egg production [2–4]. S. Pulloram is thus a major factor restricting the growth of the poultry industry, especially in developing countries [5]. In addition to horizontal transmission in very young birds, *S.* Pullorum in infected hens may be transmitted vertically via the ovary to the egg, with subsequent extensive horizontal transmission in the hatchery. Little is known about bacterial factors that allow *Salmonella* Pullorum to survive in the chicken embryo during incubation without causing death of the embryo.

We employed PCR signature-tagged mutagenesis in use in our laboratory [6] to identify the genes associated with the survival of *S.* Pullorum in the chicken embryo.

2. Materials and methods

2.1. Bacterial strains and chicken embryo

S. Pullorum SP S06004 is virulent for newly hatched chickens, and both this and a set of 12 donor *E. coli* χ7213 strains containing the tagged-plasmid pUTminiTn5 were grown in Luria-Bertani (LB) broth (Difco,USA) [6]. When required, this medium was supplemented with 1.5% (w/v) agarose, ampicillin (Ap, 100 mg/ml), kanamycin (Km, 50 mg/ml), and chloramphenicol (Cm, 34 μg/ml).

2.2. Construction of the transposon mutant library

Following a previous protocol [6], each tagged-transposon with ampicillin, kanamycin and chloramphenicol resistant genes in the

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http://dx.doi.org/10.1016/j.micpath.2017.01.014
0882-4010/

E. coli χ7213-pir strain, which requires 2,6-diaminopimelic acid (DAP) for growth, was transferred into the recipient S06004 by conjugation, with each donor containing the specific tag. The transformants of 12 mutant libraries were generated on selective LB agar plates containing 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 34 μg/ml chloramphenicol without DAP.

2.3. Identification of attenuated S. Pullorum mutants and corresponding genes

One mutant strain was selected randomly from each mutant library, and 12 mutant strains were pooled together to make the input pool. From the input pool, 100 CFU/100 μL and 200 CFU/100 μL were injected separately into the allantoic cavity in 5 16-day-old embryos. Five days later the chickens were hatched, and all were killed humanely. The spleens and livers were removed aseptically and homogenized in deionized water. The homogenates were plated on selective media to form the output pool.

Chromosomal DNA was extracted from the input and output pools. PCR identification of the mutants was carried out, and PCR amplicons from the output pools were compared with those from the input pools. Any mutant not represented in the output pool was identified tentatively as an attenuated strain.

2.4. Virulence analysis of attenuated mutants

2.4.1. Growth phenotypes

Newly cultured salmonella mutants (1×10^6 CFU/ml) and the parent strain S06004 were cultured again in new LB media. Every 2 h, the bacterial concentration according to the OD600 was detected, and growth curves of the 9 mutants together with the parent strain were made and compared to each other.

2.4.2. In vivo competitive virulence assay

All of the attenuated mutants from the primary screen were confirmed by repeating the screening procedure using the individual strains. In addition, each mutant and parent strain were mixed together in equal numbers (100 CFU/100 μL each) for an *in vivo* competition assay, in which the mixtures were administered to 5 16-day-old embryos via the allantoic cavity following a previously published protocol [6].

2.4.3. Virulence for 16-day-old embryos

Each attenuated mutant (100 CFU/100 μL) was inoculated separately into the allantoic cavity of 16-day-old embryos (n = 10). After 5 days, the degree of attenuation was measured by the hatch rate and absence of isolation of the mutant in the liver and spleen of the newly hatched chicken.

2.5. In vitro competitive index

For the in vitro assay, 5×10^3 CFU of mutant and parent were co-inoculated in 5 ml LB. Cultures were grown at 37 °C for 12 h with shaking (180 rpm), and the input and output ratios of the mutant and parent strains were determined by selective plating as described above. The competitive index (CI) was calculated as the ratio of the mutant (CFU at hour 0/CFU at hour 12) divided by the ratio of the parent (CFU at hour 0/CFU hour 12). For each mutant strain, the mean in vitro CI from two such experiments was recorded.

3. Results

3.1. Establishment of the chicken embryo infection model of S. Pullorum

Sixteen-day-old chicken HY-line embryos were inoculated using an 18-gauge needle with 200 CFU/μL of input pools via the allantoic cavity, which was found to be optimal, resulting in 50% hatch rates.

3.2. Identification by PCR signature-tagged mutagenesis of attenuated S. Pullorum mutants and genes in the chicken embryo infection model

Twelve mini-Tn5 transposon mutant libraries were constructed; each library contained specific signal tag-labelled sequences encoding Km^r and Cm^r in the *S.* Pullorum genome as biomarkers. One tag-labelled mutant strain from each library was included in each input pool.

To model the effect of the mutants on embryos, 2 groups of 5 16-day-old chicken embryos were inoculated via the allantoic cavity with 100 CFU and 200 CFU of input pools, respectively. After the chickens were hatched, bacteria isolated from their livers and spleens were taken as output pools in which mutants were screened by PCR. A total of 100 input pools including 1200 mutants were screened. Nine mutants were identified as attenuated. From sequences flanking the transposon, transposon-disrupted genes were identified by a BLAST comparison with the bacterial genome in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The genes identified in the 9 mutants shown in Table 1 are involved in invasion (*uvrY*::Tn5), cell division (*folA*::Tn5, *miaA*::Tn5, *ftsN*::Tn5), metabolism (*citC*::Tn5, *yabF*::Tn5, *rfbG*::Tn5, *unknown*::Tn5), and bacterial defence (*wza*::Tn5).

3.3. Growth phenotypes

Fig. 1a and b shows the growth of mutants in comparison with the parent strain in two experiments. The growth rates of all mutants were slower than that of S06004, further confirming the deleterious effects of the mutants on the in vitro metabolic characteristics.

3.4. Analysis of the virulence of individual mutants

The competitive index values obtained from the simultaneous inoculation of 16-day-old chicken embryos with each of the 9 mutants together with the parent strain are shown in Table 1. The virulence of these mutants for 16-day-old embryos is also shown.

The *in vivo* competitive index data, calculated as the change in CFUs of the mutant 5 days after inoculation divided by the same change in the parent strain, showed that none of the mutants performed as well as the parent. Mutants yabF, citC, folA and the unknown::Tn5 all have CI values greater than 0.1 and in some cases much higher than that.

The virulence of Salmonella Pullorum mutants was assessed for 16-day-old chicken embryos (n = 10) on the hatch rates and positive live chickens (Fig. 2). All 9 mutants showed different degrees of attenuation. The hatch rates for *rfbG*::Tn5, *yabF*::Tn5, *citC*::Tn5, *ftsN*::Tn5 and *folA*::Tn5 were all greater than 50%. Mutant *rfbG*::Tn5 was not isolated from any of the hatched chickens, whereas the isolation rates for mutants *ftsN*::Tn5 and *folA*::Tn5 were 87.5% (7/8) and 55.6% (5/9), respectively.

4. Discussion

Among poultry-related *Salmonella* serovars, *S.* Enteritidis, *S.*