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Identification of *Salmonella enterica* serovar Kentucky genes involved in attachment to chicken skin

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Abstract

Background: Regardless of sanitation practices implemented to reduce *Salmonella* prevalence in poultry processing plants, the problem continues to be an issue. To gain an understanding of the attachment mechanism of *Salmonella* to broiler skin, a bioluminescent-based mutant screening assay was used. A random mutant library of a field-isolated bioluminescent strain of *Salmonella enterica* serovar Kentucky was constructed. Mutants' attachment to chicken skin was assessed in 96-well plates containing uniform 6 mm diameter pieces of circular chicken skin. After washing steps, mutants with reduced attachment were selected based on reduced bioluminescence, and transposon insertion sites were identified.

Results: Attachment attenuation was detected in transposon mutants with insertion in genes encoding flagella biosynthesis, lipopolysaccharide core biosynthesis protein, tryptophan biosynthesis, amino acid catabolism pathway, shikimate pathway, tricarboxylic acid (TCA) cycle, conjugative transfer system, multidrug resistant protein, and ATP-binding cassette (ABC) transporter system. In particular, mutations in *S. Kentucky* flagellar biosynthesis genes (*flgA*, *flgC*, *flgK*, *flhB*, and *flgJ*) led to the poorest attachment of the bacterium to skin.

Conclusions: The current study indicates that attachment of *Salmonella* to broiler skin is a multifactorial process, in which flagella play an important role.

Keywords: *Salmonella*, Kentucky, Flagella, Poultry, Skin, Attachment

Background

Salmonella contamination is an important food safety concern in poultry processing plants. Recently, *Salmonella enterica* serovar Kentucky has been recognized as the most prominent *Salmonella* serovar in poultry processing [1]. According to the National Antimicrobial Resistance Monitoring System (NARMS), the prevalence of *S. Kentucky* isolates from broiler chicken has elevated from 25 % in 1997 to 50 % in 2007 [2]. Although this serovar is not considered a major source of human disease, high incidence of *Salmonella* Kentucky and the emergence of its recent multi-drug resistant strain outside US with high resistance level to ciprofloxacin,

indicates this serovar could be a potential threat to public health.

Salmonella contamination persists in all stages of chicken processing regardless of the hygienic steps taken. While poultry intestines are considered the most probable origin of contamination, abundant bacteria have been detected on the surface of the broilers. There have been numerous studies on *Salmonella* attachment to chicken skin, however, specific knowledge on the mechanism of attachment is lacking. Bacterial attachment, according to one study, was a result of bacterial retention in a network of fibers that forms when chicken muscle fascia is immersed in water [3]. In another study, *Salmonella* isolation from cervices and feather follicles suggested that the bacterium can be entrapped in water inside the follicles [4]. While cell charge was considered an important attachment factor [5], another study indicated that cell charge did not affect the attachment rate [6]. Bacterial concentration and inoculation time are

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other aspects that have been suggested as contributors to the attachment of bacterium to chicken skin [7]. There also has been conflicting information on the role of some surface structures (e.g., fimbriae, pili, and flagella) on the attachment of the bacterium to the broiler skin surface [8–10].

Several *Salmonella* surface proteins that appear to mediate adhesion are involved into chicken fascia, which is composed of collagen and elastin fibers interspread in the glycosaminoglycan (GAG) matrix by binding hyaluronan [11]. However, the exact characteristics and properties of these binding sites are not completely understood.

The recent emergence of a ciprofloxacin-resistant strain in a broiler prevalent serovar of *Salmonella* [12] highlights the need to expand our knowledge of the *S. Kentucky* attachment mechanisms broiler skin. The purpose of this current research was to identify *S. Kentucky* genes mediating the bacterial attachment to chicken skin. Identification of *Salmonella* attachment mechanisms to poultry skin could allow development of strategies to reduce carcass contamination during processing, which could assist the broiler processing industry in meeting regulatory concerns for pre- and post-harvest food safety.

Results

Identification of mutants with reduced skin attachment properties

In total 2,112 *S. Kentucky* mutants were screened for their ability to bind to poultry skin. In the first screening, 264 candidate mutants with decreased bioluminescence relative to wild type *S. Kentucky* strain SkTn7lux were identified, including candidate mutants with decreased bioluminescence on chicken skin before and after the 1 h washing step (Fig. 1). Of these candidates, 88 showed a reduction in bioluminescence even before the washing step. The remaining 176 mutants had similar bioluminescence to strain SkTn7lux before washing but they displayed reduced bioluminescence after washing with agitation. After the second screening, we identified 66 mutants with decreased bioluminescence on chicken skin compared to strain SkTn7lux. Of these, 44 had decreased binding after the final wash step and 22 had decreased binding prior to the final wash. Wild type *S. Kentucky* was not removed from chicken skin after an hour of washing with agitation, while *E. coli* DH5 α was completely removed (Fig. 1). A total of 66 mutants showing complete or reduced attachment were chosen for transposon end mapping.

Identification of transposon insertions in *S. Kentucky* genome

Transposon insertion sites of 66 mutants with attenuated attachment to chicken skin were identified (Tables 1

and 2). Mutants that demonstrated attachment attenuation were classified into two phenotypic groups. The first group showed reduced skin attachment compared to *S. Kentucky* SkTn7lux only after 1 h washing with agitation. This group had transposon insertions in various genes: lipopolysaccharide (LPS) biosynthesis, amino acid catabolism, shikimate pathway, TCA cycle, conjugative transfer system (*traD*), signaling and transportation system, phage tail fiber protein H, fimbrial export usher protein, membrane proteins, and several hypothetical proteins (Table 1). The second group had decreased skin attachment prior to 1 h washing. These mutants mostly had transposon insertions in different flagella structural genes (Table 2).

Discussion

In the current study, we used random transposon mutagenesis on bioluminescent *Salmonella Kentucky* strain SkTn7lux, to identify genes involved in attachment to chicken skin. Our results showed that *S. Kentucky* attachment to broiler skin is a multifactorial process requiring the expression of many genes. We identified two different phenotypic groups of mutants with decreased attachment on chicken skin. We expected to identify *S. Kentucky* mutants that were more susceptible to removal by washing, which mimics the washing process that occurs during the poultry processing. However, we also identified a group of mutants with more severe attachment defect, which had decreased skin binding after simple flushing with a pipette. Notably this second group included six mutants with transposon insertions in flagella genes highlighting the role of flagella in *S. Kentucky* attachment to poultry. These six mutants harbored two different transposon insertions in *flgA*, which encodes flagellar basal-body p-ring formation protein. Other mutated flagellar genes encode flagellar basal-body rod protein (*flgC*), flagellar hook-associated protein (*flgK*), a rod assembly protein (*flgJ*), and flagellar biosynthesis protein (*flhB*). Attachment defects in flagellar basal body protein mutants suggest that flagellar rotation contributes to skin attachment.

Previous studies have reported conflicting results on the role of flagella on attachment of *Salmonella* to broiler skin. In one study, attachment to broiler skin was dependent on the presence of flagella [10]. In later studies, it was concluded that under controlled conditions, non-flagellated bacteria attached as well as flagellated bacteria [9]. Similar to our results, attachment was found to be a complex reaction, and fimbria and flagella both contribute to the process [8].

Other attachment-defective mutations were in transporter and signaling systems, which have higher expression in attached bacteria, and may work as an efflux pump to help the bacterium resist environmental stress

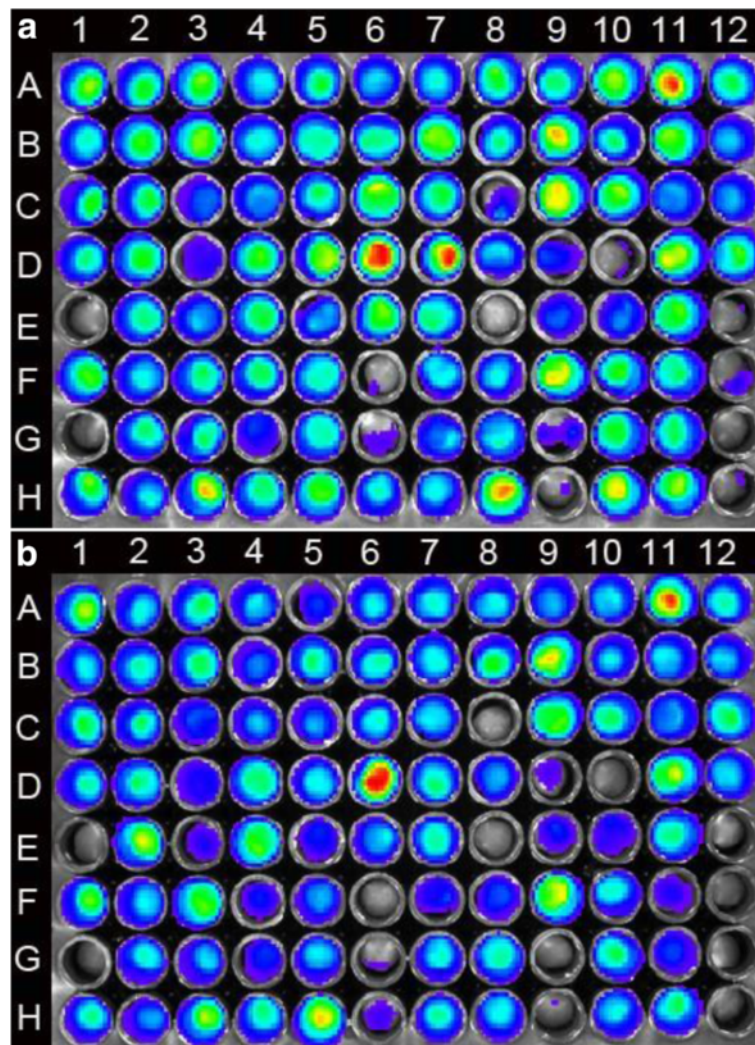


Fig. 1 Bioluminescence ($p/s/cm^2/sr$) of 96-well plate containing chicken skin cuts: **a** before washing and **b** after washing of chicken skin cuts. The first four wells in the last column (A12, B12, C12, D12) are wild type *S. Kentucky* strain SKTn7lux and the last four wells in the same column (E12, F12, G12, H12) are *E. coli* DH5a. The remaining wells are individual mutants that have been replicated in four plates. Examples of mutants with significantly decreased skin binding prior to the main wash are in wells E1, G1, F6, E8, D10 and H9

[13]. Thus mutation in these genes may make these mutants more susceptible to environmental conditions. In the current study, attachment-deficient mutant P07D05-P01C06 had an insertion in *lpp* which encodes the major outer membrane lipoprotein. Under specific environmental conditions, an *E. coli* outer membrane lipoprotein, NlpE, senses and generates an adhesion signal to the Cpx pathway that leads to stable adhesion [14]. Mutant P09H05-P01G06 had an insertion in the *waaG* gene, which encodes a LPS core biosynthesis protein. In *E. coli*, LPS is known to contribute to attachment. Some mutations in *E. coli* genes encoding lipopolysaccharide core biosynthesis enzymes showed decreased adhesion to solid surfaces [15]. In addition to *waaG*, two other mutants had insertions in LPS biosynthesis genes in the

current study: P09B04-P01D09 was mutated in *rffA* which encodes a LPS biosynthesis protein; and P09E05-P01F09 was mutated in *rfaF*, which encodes ADP-heptose: LPS heptosyltransferase II that contributes to synthesis of the inner core backbone of LPS.

P25D03-P01C11 had an insertion in *trpB*, which encodes tryptophan beta sub-unit synthase. Tryptophan is a major factor in forming *Salmonella enterica* serovar Typhimurium biofilms on food surfaces [16]. Amino acids metabolites, especially those involved in tryptophan biosynthesis are up-regulated at early stages of attachment. Although attachment of *Salmonella* to broiler skin is not considered a biofilm formation process, it is comparable with bacterial attachment to solid surfaces at an early stage of biofilm formation. In *E. coli*, over-

Table 1 Skin attachment attenuated mutants removed after washing step

| Mutant ^a | Protein ID | Location ^b |
|---------------------|---|--|
| P02F10-P01G01 | 3-dehydroquininate dehydratase | MAR2xT7 [^] TActgtccggtggttagcgcctgttcg |
| P04G08-P01E02 | Magnesium and cobalt transport protein CorA | MAR2xT7 [^] TAcgcgcaatcgctcgtcgtcccg |
| P07D05-P01C06 | Major outer membrane lipoprotein | MAR2xT7 [^] TAaataccggaagtaatagttactcg |
| P07G06-P01E06 | Dihydrolipoamide acetyltransferase | MAR2xT7 [^] TAgtccgtcaccagaacacgcaaca |
| P07G09-P01F06 | Dihydrolipoamide succinyl transferase | MAR2xT7 [^] TAgtcttcagtttccccgacgtatac |
| P08F01-P01A08 | Poly nucleotide phosphorylase/polyadenylase | MAR2xT7 [^] TAagcatggatgacaccgctgttcg |
| P08C05-P01B08 | Type IV conjugative transfer system coupling protein TraD | MAR2xT7 [^] TAaccaggaacgtccaaagtggcgccg |
| P09B04-P01D09 | Lipo polysaccharide biosynthesis protein RffA | MAR2xT7 [^] TAgtaacgtttaaagcgcggtgtt |
| P09E05-P01F09 | ADP-heptose:LPS heptosyl transferase II | MAR2xT7 [^] TAaaccgaattggcaacaccagcgcg |
| P10H10-P01D10 | Anti-terminator-like protein | MAR2xT7 [^] TAatattgataaacctcacgccgctta |
| P10D11-P01G11 | DNA helicase IV | MAR2xT7 [^] TAatgtcccgtatctcaaacggcg |
| P04H01-P01F02 | Phage tail fiber protein H | MAR2xT7 [^] TAactcagctggaaccaggttaccgg |
| P06F05-P01H04 | Precorin-4C11-methyl transferase | MAR2xT7 [^] TAgtccggttcgctgataatccgaa |
| P10F06-P01B11 | NADH pyro phosphatase | MAR2xT7 [^] TAgtgatcgtataattgaaaaattaga |
| P10D07-P01G10 | Conserved protein with nucleoside triphosphate hydrolase Domain | MAR2xT7 [^] TAgtgttcaagcagttgaccatcgcg |
| P08F09-P01H07 | Oligoribonuclease | MAR2xT7 [^] TActaaacgctttaccgatctgaaa |
| P12F08-P02D02 | Glutamyl-Q tRNA (Asp) synthetase | MAR2xT7 [^] TActccaccgcccgcgacggactgtt |
| P13H05-P02A03 | Chaperone protein HscA | MAR2xT7 [^] TAaccaactctggttgccgaggtt |
| P14B06-P02H03 | Chaperone protein HscA | MAR2xT7 [^] TAactgatcgtcggcgccggtggtt |
| P16D03-P02A05 | Shikimate 5-dehydrogenase AroDI gamma | MAR2xT7 [^] TAcgaagcgtggtatctcaattatctc |
| P16H02-P02C05 | Fatty acid oxidation complex sub-unit alpha | MAR2xT7 [^] TAcagcgggcccaggtgtgatactgc |
| P17C05-P02A07 | SppA | MAR2xT7 [^] TAatgcttatctccaaggtacaa |
| P18H08-P02C07 | NADH dehydrogenase sub-unit H | MAR2xT7 [^] TAattgggtggtggccgatttaacat |
| P23E10-P02E10 | Ribulose-phosphate 3-epimerase | MAR2xT7 [^] TAactttgacgtcattgataactct |
| P25D03-P02C11 | Tryptophan synthase beta sub-unit | MAR2xT7 [^] TAgtgccgagatcctgatgctg |
| P15C06-P02G04 | ATP-dependent RNA helicase DeaD | MAR2xT7 [^] TAaccgattgaagtgccgctgatgt |
| P11H11-P02B02 | Putative regulatory protein | MAR2xT7 [^] TAactgcagcaatgcccgaagaaagga |
| P15C03-P02H04 | Glutathione reductase | MAR2xT7 [^] TAactcaccgacaacgtgctgggcaa |
| P21E02-P02C09 | Aldolase | MAR2xT7 [^] TAgtggtatccagcaattcctggc |
| P12C04-P02F02 | Putative sodium/sulfate transporter, partial | MAR2xT7 [^] TAcagaatattggcgccgctttggct |
| P18C07-P02F07 | GTP-binding protein | MAR2xT7 [^] TAactatcctgctaaaacaccgctat |
| P23F01-P02D10 | Ornithine decarboxylase | MAR2xT7 [^] TAgtggcctctggtgattcactg |
| P16E01-P02B05 | Hypothetical protein STY0758 | MAR2xT7 [^] TAaccaggggactgacggcctgtgcag |
| P19F07-P02H08 | Oxidoreductase | MAR2xT7 [^] TAatattgagtcctctccggcgttcg |
| P25G02-P02F11 | Intramembrane serine protease GlpG | MAR2xT7 [^] TAatatactgtattttgatgga |
| P19A07-P02A08 | Fimbrial outer membrane usher protein | MAR2xT7 [^] TAcgttcggttcaatagcgtttcaat |
| P23C06-P02B10 | Pyruvate dehydrogenase sub-unit E1 | MAR2xT7 [^] TAcatcaacactattgcccgtgaagac |
| P20C11-P02B09 | Alpha ribazole-5'-P phosphatase | MAR2xT7 [^] TAcaataatcatacagtcggacgata |
| P18D02-P02G07 | 4-hydroxythreonine-4-phosphate dehydrogenase | MAR2xT7 [^] TAactctgtaggtgctcccagcccg |
| P22G01-P02A10 | Permease protein SitC | MAR2xT7 [^] TAagccatgcccagaaaactggtca |
| P13B03-P02E03 | Putative sensor kinase protein | MAR2xT7 [^] TAcaacaagaatcgccgagcggag |
| P10C09-P02C01 | Exoribonuclease II | MAR2xT7 [^] TAataaccagtcgcccagatcgcctcc |
| P22E10-P02G09 | Phosphorpyruvate hydratase | MAR2xT7 [^] TAcacaccagggcagccgaccggag |
| P19H03-P02B08 | High-affinity zinc transporter periplasmic protein | MAR2xT7 [^] TAaaaccacgctacaagcgttgact |

^aMutants are listed according to the degree of attachment attenuation

^bMAR2xT7, mariner transposon; ^, insertion point; TA, two-base TA duplication; lowercase letters, 25-bp flanking unique gene sequences of *S. enterica*

Table 2 Attachment attenuated mutants removed before the washing step

| Mutant ^a | Protein ID | Location ^b |
|---------------------|---|---|
| P09G05-P03F06 | Flagellar basal-body P-ring formation protein FlgA | MAR2xT7 [^] TAAttcatcgctgaccttccgcattga |
| P03G04-P03G01 | Flagellar basal-body rod protein FlgC | MAR2xT7 [^] TAgctgcgcaggctgacatcggttg |
| P24B04-P03D10 | Unnamed protein product | MAR2xT7 [^] TAttccctggatgattttttacgcag |
| P21C09-P03G09 | Flagellar biosynthesis protein FlhB | MAR2xT7 [^] TAttccgtggcgtcagatgacgaa |
| P05D08-P03D03 | Multidrug resistance protein, SMR family | MAR2xT7 [^] TAcgcggcttaaaggccaattcccg |
| P05H05-P03C03 | Cysteine/glutathione ABC transporter membrane/ATP-binding comp. | MAR2xT7 [^] TAgttaaaactgtaattcccgcaag |
| P09H05-P03G06 | Lipopolysaccharide core biosynthesis protein | MAR2xT7 [^] TAgcctgttctggcgctgacagaaga |
| P15B11-P03C08 | Flagellar hook-associated protein FlgK | MAR2xT7 [^] TAgaacagtaataatgccgataaac |
| P22D04-P03H09 | tRNAuridine5-carboxymethylaminomethyl modification enzyme GidA | MAR2xT7 [^] TAacgaatcacgctatgggtttctca |
| P25E10-P03E10 | Flagellar rod assembly protein/muramidase FlgJ | MAR2xT7 [^] TAcgttatagctgggttcgccattctc |
| P16F02-P03F8 | DamX protein | MAR2xT7 [^] TAtttccgcacatgctgcgagataaa |
| P13F05-P03G07 | NitrogenregulationproteinNR2, partial | MAR2xT7 [^] TAcgtggcgcggcgagctgcagagca |
| P05E06-P03F02 | Dimethyl adenosine transferase | MAR2xT7 [^] TAAttatcagcaggacgcatgacat |
| P17E05-P03D09 | 1-acyl-glycerol-3-phosphateacyltransferase | MAR2xT7 [^] TAgaatgccgggctttaggccttcag |
| P12H05-P03F07 | Chain A, DNA-binding transcriptional repressor AcrR | MAR2xT7 [^] TAagcaacgcgatggcgcgtaaaacca |
| P25F11-P03C11 | Cystathionine beta-lyase | MAR2xT7 [^] TAatagaccagccggctgctgcgaca |
| P08C02-P03E05 | Flagellar basal body P-ring biosynthesis protein FlgA | MAR2xT7 [^] TAAttcatcgctgaccttccgcattga |
| P13C07-P03H07 | ParB gene product | MAR2xT7 [^] TAcgaactaaactcataagtaaacgtac |
| P02E02-P03B01 | Two-component sensor kinase SsrA | MAR2xT7 [^] TAActtcgagatggctggataaaacaa |
| P16F04-P03G08 | Hemelyase sub-unit NrfE | MAR2xT7 [^] TAtagcccgccagtaccacctgctgac |
| P06D02-P03F04 | Hypothetical proteinSel_A3977 | MAR2xT7 [^] TAaacactcaaaactcttggtattcg |
| P05F11-P03H02 | Membrane protein suppressor for copper sensitivity ScsD | MAR2xT7 [^] TAatccgtgtcggcgccggacattct |

^aMutants are listed according to the degree of attachment attenuation

^bMAR2xT7, mariner transposon; ^, insertion point; TA, two-base TA duplication; lowercase letters, 25-bp flanking unique gene sequences of *S. enterica*

expression of tryptophan biosynthesis and increased production of tryptophan and its precursor, indole, prepares the bacteria for nutrient-poor environments and increases catabolism of amino acids. Indole also up-regulates detoxifying genes (e.g., drug exporters) to make the bacterium more resistant to toxic compounds and increases bacterial adherence to surfaces [16]. These characteristics can be vital in *Salmonella* adherence to chicken skin. Also, P16D03-P01A05 and P02F10-P01G01 had insertions in *aroDI* and *aroD* which encode 5-dehydrogenase gamma and 3-dehydroquinase dehydratase, respectively. These compounds are both enzymes in the Shikimate pathway and are involved in the biosynthesis of aromatic amino acids. These mutations also emphasize the importance of tryptophan in the attachment process.

Conclusions

Bioluminescence mutant screening of *S. Kentucky* was applied to identify mutants that are defective in attachment to chicken skin. Results indicate that flagella have an important role in attachment of *S. Kentucky* to broiler skin. Some other pathways that are important for skin

adherence include LPS biosynthesis, aromatic amino acid biosynthesis, outer membrane lipoprotein, and transport/secretion systems. Further investigations, especially in flagella structure and basal body genes, could lead to a better understanding of the exact molecular mechanism of *Salmonella* attachment to poultry skin.

Methods

Bacterial strains, plasmids and growth conditions

Escherichia coli SM10 λ pir [17] was used as the donor strain in conjugations for transfer of pMAR2xT7 [18] into bioluminescent *S. Kentucky* strain SkTn7lux [19] originally isolated from a broiler processing plant [20]. Bioluminescent *E. coli* DH5 α (Thermo Fisher Scientific, Waltham, MA) and bioluminescent wild type *S. Kentucky* strain SkTn7lux [19] were used as controls. *E. coli* and *Salmonella* strains were grown on Luria-Bertani (LB) broth and agar plates at 37 °C. Gentamicin and streptomycin added to LB agar plates at 50 μ g/ml⁻¹ as appropriate.

Construction of transposon insertion library

pMAR2xT7 was transferred from *E. coli* SM10 λ pir into bioluminescent *S. Kentucky* strain SkTn7lux by conjugal

mating [21]. Briefly, a colony of *E. coli* SM10 λ pir carrying pMAR2xT7 and bioluminescent *S. Kentucky* SkTn7lux were inoculated in 5 ml of LB broth at 37 °C overnight at 200 rpm. Equal amounts of overnight culture of donor and recipient (1.5 ml) were pelleted separately by centrifugation, washed three times with LB broth, and then re-suspended in 1 ml of LB broth. Donor and recipient strains were mixed in a 1:3 ratio based on their volume. The mixture was centrifuged at 12,100 x g for 2 min. The harvested cells were diluted in 10 μ l of LB broth and transferred to a 0.45 μ m sterile filter paper, which was placed on LB agar and incubated at 37 °C for 18 h. The filter was washed with 5 ml LB broth, and 50 μ l of the washed bacteria was spread on the LB agar containing gentamicin and streptomycin. Bioluminescence of colonies on agar plates were confirmed using an IVIS 100 Imaging System. A batch of gentamicin-resistant colonies was tested for random transposon insertion using single-primer PCR [22] and sequencing. More than 2,000 colonies of mutant *S. Kentucky* were picked using a pipette tip and inoculated in 150 μ l of LB plus gentamicin broth in 96-well plates and incubated in an incubator shaker overnight. Plates were sealed, and the mutant library was stored in 20 % glycerol at -80 °C [21].

Chicken skin attachment assay

In our previous studies, a chicken skin attachment assay was established, which indicated that the number of attached bacteria to chicken skin can be measured by the bioluminescence intensity of the correlated bacteria [23]. In this work, the chicken skin attachment assay was performed twice. Primary screening was accomplished with one replicate for a total of 2,112 mutants. Those mutants showing reduced attachment went through the final skin attachment assay with four replicates. Each 96-well plate contained four replicates of bioluminescent wild type *S. Kentucky* strain SkTn7lux and *E. coli* DH5 α . Plates were covered with Breath-Easy film (Diversified Biotech, Boston, MA) and grown at 37 °C overnight at 250 rpm on a shaker incubator. The OD and bioluminescence of each well were measured to ensure the growth and bioluminescence of each mutant. Five microliters from overnight cultures were used to inoculate fresh 96-well plate containing 100 μ l LB broth, which were incubated at 37 °C for 2 h to reach the log phase.

The chicken skin was obtained from a commercial broiler processing plant inspected by USDA Food Safety Inspection Service. Chicken skins were cut into uniform, circular sections by 6 mm skin biopsy punch and placed into clear-bottomed 96-well black cell culture plates. 100 μ l of log phase mutant culture with known OD and bioluminescence were added to each well and after a brief spin, plates were incubated at 25 °C for 1 h to allow

bacterial attachment to the broiler skin. Following incubation, bacterial suspensions were removed by vacuum suction, and the wells were washed with 200 μ l of distilled water by pipetting twice to remove unattached bacteria. Plates were incubated at 37 °C for 10 min. and bioluminescent imaging was recorded for 15 s of exposure at 37 °C with an IVIS 100 Imaging System. Bioluminescence was quantified using Living Images software as described [19].

To determine the effect of washing on bacterial attachment properties, plates were filled with 200 μ l of water and placed in a rotating platform incubator at 700 rpm for 1 h. After the removal of excess solution, bioluminescence on skin sections was measured and recorded for 15 s of exposure. This stage was considered as the main washing step.

Determination of mutants with attenuated attachment

Bioluminescence (p/s/cm²/sr) was measured twice in each assay: prior to and after the final 1 h washing step. In the primary screening, percent bioluminescence reduction was calculated from each mutant, which were then ranked from highest to lowest reduction. In the secondary screening, mutants with highest bioluminescence reduction went through another skin attachment assay with four replicates. Mutants were considered deficient in attachment if their attachment percentage fell out of the lower 95 % confidence limit calculated from attachment rate of wild type strain SkTn7lux replicates. Mutants with decreased attachment either before or after the final wash were chosen for transposon end mapping.

Identification of transposon insertion site

Transposon insertion sites for the 66 mutants with reduced attachment to chicken skin were identified by overlap extension PCR [24]. Briefly, genomic DNA was prepared from overnight cultures using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Each 25 μ l of PCR contained 0.2 μ M forward or reverse transposon specific primer (MAR2xT7F: TACAGTTTACGAACCGAACAGGC or MAR2xT7R TCTATACAAAGTTGGGCATACGG) 0.2 mM dNTPs, 1.5 mM MgCl₂ and 1.25 U of *Taq* polymerase (Promega, Madison, WI). The PCR was performed using a PTC-100 thermocycler (MJ Research, Water town, MA) with the following cycling steps: initial denaturation (2 min at 94 °C) followed by 25 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), and elongation (3 min at 72 °C) followed by 30 cycles of denaturation (30 s at 94 °C), annealing (30 s at 30 °C), and elongation (2 min at 72 °C) followed by 30 cycle of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), and elongation (2 min at 72 °C). A final extension of 10 min at 72 °C was also applied. PCR

products were cleaned with ExoSAP-IT enzyme mix (USB Corp. Cleveland, Ohio) and used as template in sequencing reactions using BigDye Terminator v1.1 and 0.5 μ M of a nested transposon specific primer (MAR2xT7FSeq: GGACCGAGATAGGGTTGAGTG or MAR2xT7R3Seq: AACAAATTCGTTCAAGCCGAGA). Transposon specific sequences were trimmed and the remaining sequences were checked against the protein database of the National Center for Biotechnology Information (NCBI) using BLAST+, which revealed the location of transposon insertion [21].

Abbreviations

ABC, ATP-binding cassette; *flg*, flagellar; GAG, glycosaminoglycan; LPS, lipopolysaccharide; NARMS, National Antimicrobial Resistance Monitoring System; TCA, tricarboxylic acid.

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Availability of data and material

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Conceived and designed the experiments: SS, KH, RHB, JB, MLL, AK. Performed the experiments: SS. Analyzed the data: SS. Contributed reagents/materials/analysis tools: RHB, JB, MLL, AK. Wrote the paper: SS, KH, RHB, JB, MLL, AK. All authors have read and approved of the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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