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Identification of *Salmonella pullorum* Genomic Sequences Using Suppression Subtractive Hybridization

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Pullorum disease affecting poultry is caused by *Salmonella enterica* serovar Pullorum and results in severe economic loss every year, especially in countries with a developing poultry industry. The pathogenesis of *S.* Pullorum is not yet well defined, as the specific virulence factors still need to be identified. Thus, to isolate specific DNA fragments belonging to *S.* Pullorum, this study used suppression subtractive hybridization. As such, the genome of the *S.* Pullorum C79-13 strain was subtracted from the genome of *Salmonella enterica* serovar Gallinarum 9 and *Salmonella enterica* serovar Enteritidis CMCC(B) 50041, respectively, resulting in the identification of 20 subtracted fragments. A sequence homology analysis then revealed three types of fragment: phage sequences, plasmid sequences, and sequences with an unknown function. As a result, several important virulence-related genes encoding the IpaJ protein, colicinY, tailspike protein, excisionase, and Rhs protein were identified that may play a role in the pathogenesis of *S.* Pullorum.

Keywords: *Salmonella enterica* serovar Pullorum, suppression subtractive hybridization (SSH), poultry

Salmonella enterica can essentially be divided into two groups on the basis of pathogenesis and infection biology [32]. One group, including *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonela enterica* serovar Enteritidis (*S.* Enteritidis), causes gastrointestinal diseases in a range of hosts, including humans, whereas the other group causes systemic typhoid-like diseases in a restricted range of host species, such as *Salmonella enterica* serovar Typhi (*S.* Typhi) in humans, *Salmonella enterica* serovar Dublin (*S.* Dublin) in cattle, and *Salmonella enterica* serovars Pullorum (*S.* Pullorum) and Gallinarum (*S.* Gallinarum) in poultry. *S.* Pullorum is the causative agent of pullorum disease in poultry, an acute systemic disease that results in a high mortality rate in young chicks, yet rarely causes significant clinical disease in adult birds [26]. The infected adult birds show a loss of weight, decreased laying, diarrhea, lesions, and abnormalities of the reproductive tract [26]. *S.* Pullorum localizes in the reproductive tract, and can be transmitted vertically to chicks through eggs [26]. Although *S.* Pullorum has been effectively eradicated from intensively reared commercial flocks in Europe and North America using diagnostic tests and control measures, it remains a big threat in developing countries.

S. Gallinarum causes fowl typhoid, an acute septicemic disease, in both young and adult chickens [4], and multilocus enzyme electrophoresis and comparative sequence analyses have revealed that *S.* Gallinarum and *S.* Pullorum are closely related [5, 21]. *S.* Enteritidis produces systemic disease in young chickens and can be transmitted vertically to progeny through eggs, plus gene contents analyses have shown that *S.* Enteritidis is closely related to *S.* Gallinarum and *S.* Pullorum [5, 23]. Nonetheless, despite the close relationship of *S.* Pullorum, *S.* Gallinarum, and *S.* Enteritidis, they cause different diseases in chickens, and since the pathogenesis of *S.* Pullorum is not well defined, it is very important to find new virulence genes in this pathogen.

Suppression subtractive hybridization (SSH) is a simple and efficient method for isolating differentially expressed genes based on a suppression PCR and subtractive hybridization [1, 13]. This method has already been used to identify previously unknown virulence genes in pathogens and analyze microbial genetic diversity and evolution [1]. Accordingly, the purpose of this study was to find *S.* Pullorum-specific sequences and identify the sequences that may be involved in virulence. Thus, two subtractive libraries were constructed, one between the *S.* Pullorum C79-13 strain and *S.* Gallinarum 9 (SG9) strain, and another

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between the *S.* Pullorum C79-13 strain and *S.* Enteritidis CMCC(B) 50041 strain.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The *S.* Pullorum C79-13 was provided by the China Institute of Veterinary Drug Control, and the *S.* Gallinarum 9 (SG9) was supplied by Dr. Barrow [20]. The *S.* Enteritidis CMCC(B) 50041 and *Escherichia coli* DH5α were from the Jiangsu Key Laboratory of Zoonosis at Yangzhou University. The bacterial cells were grown in a Lennox broth (LB). Agar was added to a final concentration of 1.5% for all the solid media. Ampicillin was used to maintain the recombinant plasmids in *E. coli* DH5α, as indicated in the next section.

Suppression Subtractive Hybridization

The genomes of the three *Salmonella* strains were extracted according to the instructions for a Bacterial DNA Mini kit (Waston, China). The genomic subtraction between strain C79-13 and strain SG9 or *S.* Enteritidis CMCC(B) 50041 was performed using a Clontech PCR-Select Bacterial Genome Subtraction Kit (Clontech, Takara, Japan) with some modifications. Two rounds of PCR were conducted and the secondary PCR products then cloned into a PCR 2.1 cloning vector (Invitrogen) and transformed into *E. coli* DH5α competent cells, followed by plating onto agar plates containing ampicillin, X-gal, and IPTG. White clones were selected randomly and grown overnight in an LB medium containing ampicillin (100 ng/ml) at 37°C, and the plasmids were extracted using the alkaline lysis method. The length of the inserted fragment was confirmed by a PCR.

Dot-Blot Screening of Subtracted Fragments

A dot-blot was used to screen the specific fragments of strain C79-13 in the subtracted library according to the protocol of a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany).

Clone Sequencing and Analysis

The DNA sequencing was performed by the Shanghai Bioasia Company (http://bioasia.cn.busytrade.com/) and Shanghai Sangon Biological Engineering Technology&Services Co., Ltd. (http://www.sangon.com/). The companies used an ABI 3730 DNA sequencer and ABI 377 DNA sequencer, and M13 forward or M13 reverse primers were used in all the sequencing reactions. The DNA sequences were then edited manually to remove the vector sequences and analyzed in GenBank using BLASTN and BLASTX [2].

Identification of Positive Clones by PCR

Primer pairs specific to the sequenced fragments were synthesized for their identification in *S.* Pullorum. The genomic DNA of strain C79-13 and SG9 or *S.* Enteritidis CMCC(B) 50041 was used as the template, respectively. The PCR products were subjected to electrophoresis on a 1.5% agarose gel in a TAE buffer (0.04 mol/l Tris–acetic acid and 0.001 mol/l EDTA) and then visualized using ethidium bromide staining.

GenBank

The nucleotide sequences of the 20 subtractive hybridization fragments reported in this paper have been deposited in the GenBank database under accession numbers FI495644 to FI495663.

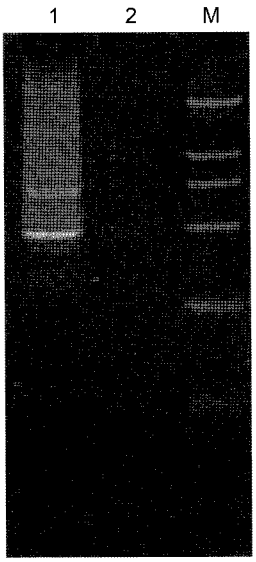

Fig. 1. Isolation of *S.* Pullorum C79-13 DNA fragments not present in SG9 using subtractive hybridization.
Lane 1: Final PCR product of specific *S.* Pullorum C79-13 DNA sequences. Lane 2: Unsubtracted C79-13 DNA for comparison. Lane M: DL 2000 marker (Takara).

RESULTS

The genomic subtraction resulted in the enrichment of the tester-specific sequences, as shown in Fig. 1, and gel electrophoresis of the amplified products showed fragments ranging from 200 to 2,000 bp. The subtraction efficiency analysis of the SSH was evaluated by a PCR of the *fliC* gene, which exists in *S.* Pullorum, *S.* Gallinarum, and *S.* Enteritidis. The *fliC* PCR product was detected in the unsubtracted DNA after 18 cycles. However, no *fliC* PCR

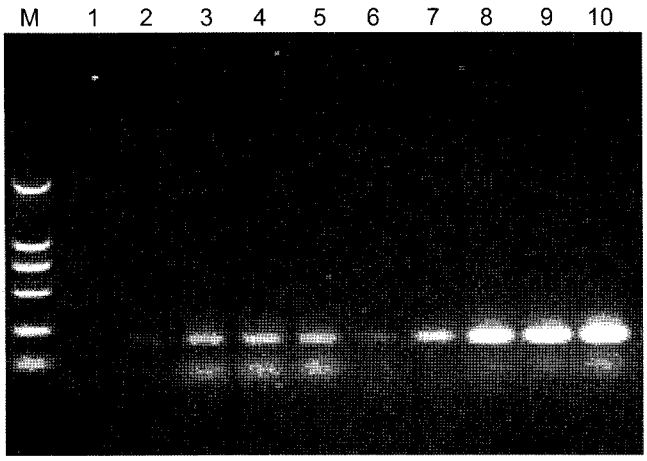

Fig. 2. Evaluation of subtraction efficiency of subtracted DNA.
PCRs with *fliC* were performed on the unsubtracted (Lane 6–10) and subtracted (Lanes 1–5) DNA for 18 (Lanes 1 and 6), 21 (Lanes 2 and 7), 24 (Lanes 3 and 8), 27 (Lanes 4 and 9), and 30 cycles (Lanes 5 and 10), respectively. Lane M: DL 2000 marker (Takara).

product was detected in the subtracted DNA under the same PCR conditions, indicating that most of the same DNA fragments between the strains had already been subtracted (Fig. 2).

From the two subtracted libraries, 363 clones were analyzed using a PCR with Nested Primer I and Nested Primer 2R. After dot-blot screening, 40 clones were then sequenced and searched for homologically in GenBank, resulting in the identification of 20 DNA fragments (Table 1). BLASTX and BLASTN analyses of these fragments revealed a high homology with DNA sequences in other *Salmonella* serovars, such as *S.* Dublin, *S.* Typhimurium,

Table 1. Results of homology search with subtracted DNA fragments.

Fragment name	Fragment size (bp)	Homology with BLAST[a]	e-value[b] identity(%)	Accession No
PG3[c]	666	Putative cytoplasmic protein of *S.* Typhimurium LT2	4e-43 100%	NP461940.1
PG41[c]	546	Putative outer membrane protein of *S.* Typhimurium LT2	2e-68 96%	NP461943.1
PG12[c]	530	Phage ORF30 protein of *S.* Typhimurium DT104	1e-45 100%	YP006385.1
PG23[c]	789	Phage tailspike protein of *S.* Typhimurium DT104	1e-54 100%	YP006420.1
PG32[c]	863	Phage superinfection exclusion protein of *S.* Typhimurium DT104	2e-57 100%	YP006372.1
PG86[c]	1781	Phage Xis protein of *S.* Typhimurium DT104	1e-131 100%	YP006364.1
PE5[d]	523	Sequence of *S.* Dublin plasmid pOU1115[e]	0.0 100%	DQ115388.1
PE23[d]	726	Conjugative transfer assembly and aggregate stability protein of *S.* Dublin strain OU7025 plasmid pOU1113	2e-88 100%	YP271771.1
PE28[d]	743	Sequence of *S.* Paratyphi A str. ATCC 9150[e]	0.0 97%	CP000026.1
PE75[d]	941	Rhs-family protein of *S. typhi* str. CT18	3e-32 73%	NP454898.1
PE88[d]	834	Rhs-family protein of *S. typhi* str. CT18	7e-09 57%	NP454898.1
PG210[c]	454	Putative IpaJ protein encoded by plasmid pSFD10 of *S.* Choleraesuis	7e-46 100%	NP203135.1
PE31[d]	458	Putative IpaJ protein encoded by plasmid pSFD10 of *S.* Choleraesuis	4e-47 100%	NP203135.1
PE44[d]	455	Putative IpaJ protein encoded by plasmid pSFD10 of *S.* Choleraesuis	9e-84 100%	NP203135.1
PG215[c]	640	MobA prtotein encoded by plasmid pSFD10 of *S.* Choleraesuis	8e-42 88%	NP203138.1
PG220[c]	689	MobA protein encoded by plasmid pSFD10 of *S.* Choleraesuis	e-132 100%	NP203138.1
PE30[d]	782	MobA protein encoded by plasmid pSFD10 of *S.* Choleraesuis	2e-150 100%	NP203138.1
PG92[c]	378	Colicin Y encoded by plasmid pCol-let of *E. coli*[e]	e-134 93%	AF197335.1
PE81[d]	756	Hypothetical protein pPCP04 encoded by plasmid pPCP1 of *Yersinia pestis* biovar Medievalis str. 91001	1e-28 66%	NP995570.1
PE42[d]	606	Hypothetical protein SPAB 03319 encoded by *Salmonella enterica* subsp. *enterica* serovar Paratyphi B str. SPB7	0.0 99%	YP001589511.1

[a]Sequences with the highest homology to the fragment are listed. The homology is often not for the entire length of the fragment.
[b]As the homology of the sequences increases, the e-value approaches 0.
[c]Fragments found in the subtracted library between *S.* Pullorum and *S.* Gallinarum
[d]Fragments found in the subtracted library between *S.* Pullorum and *S.* Enteritidis.
[e]Homology listed is based on nucleotide sequences (BLASTN). All other homologies listed are based on (BLASTX).

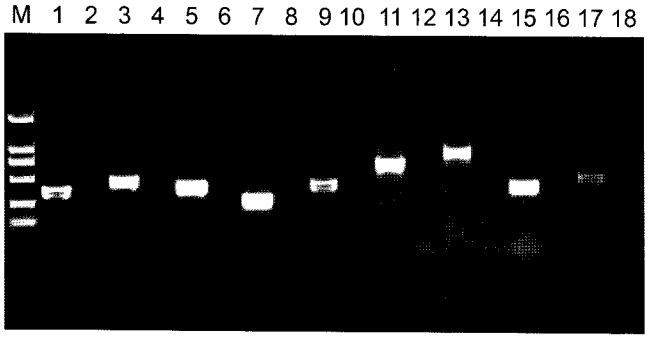

Fig. 3. Identification of positive clones using PCR analysis.
The PCR-amplified fragments were PG3 (Lanes 1 and 2), PG41 (Lanes 3 and 4), PG23 (Lanes 5 and 6), PG92 (Lanes 7 and 8), PE5 (Lanes 9 and 10), PE23 (Lanes 11 and 12), PE75 (Lanes 13 and 14), PE31 (Lanes 15 and 16), and PE42 (Lanes 17 and 18). Lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17 show the PCR-amplified fragments when using the genomic DNA of strain C79-13 as the template. Lanes 2, 4, 6, and 8 show the PCR-amplified fragments when using the genomic DNA of SG9 as the template. Lanes 10, 12, 14, 16, and 18 show the PCR-amplified fragments when using *S.* Enteritidis CMCC(B) 50041 DNA as the template.

and *S.* Choleraesuis, although only two fragments were similar to DNA sequences in other Gram-negative bacteria.

The identified sequences were divided into three types: phage sequences, plasmid sequences, and sequences with an unknown function (Table 1). Four fragments exhibited a high homology to corresponding sequences in prophage ST104 of *S.* Typhimurium DT104. Ten fragments exhibited a homology to parts of virulence plasmids, including virulence plasmids pOU1115 and pOU1113 of *S.* Dublin, plasmid pSFD10 of *S.* Choleraesuis, plasmid pCol-let of *E. coli*, and plasmid pPCP1 of *Yersinia pestis*. Finally, 6 fragments had unknown functions, as they exhibited homology to putative genes or low level homology to known sequences. Two of these fragments showed a similarity to the Rhs family protein of *S.* Typhi.

For further identification of the positive clones, the fragments from the tester genomic DNA of strain C79-13, were amplified using a PCR with specific primer pairs, yet the fragments from the driver genomic DNA of SG9 or *S.* Enteritidis CMCC(B) 50041 could not be amplified (Fig. 3).

DISCUSSION

SSH is one of the most simple and powerful methods for isolating specific DNA fragments in different bacteria [1]. In the present study, *S.* Gallinarum and *S.* Enteritidis, which are closely related to *S.* Pullorum, were chosen as the drivers to screen specific DNA fragments in *S.* Pullorum. From the two subtracted libraries, 20 fragments were obtained after a BLAST analysis on GenBank, including some important virulence-related genes encoding the IpaJ protein, colicinY, tailspike protein, excisionase, and Rhs protein.

In *Shigella*, the expression of a core set of virulence genes results in the invasion of host colonic epithelial cells and dysenteric syndrome. Some of these virulence genes, including the invasion plasmid genes, are carried by a 230 kb invasion plasmid. The *ipaJ* gene is an invasion plasmid antigen gene of *Shigella*. The IpaJ protein expressed *in vitro* reacts with convalescent sera in a Western blot analysis, confirming its identification as a *Shigella* immunogen, which is also related to the intercellular dissemination of *Shigella* [7]. In the present study, 3 fragments showed a high degree of similarity to the *ipaJ* in pSFD10, which was isolated from the vaccine strain *S.* Choleraesuis C500 in China [22]. The putative IpaJ protein in *S.* Choleraesuis has 49% homology to the IpaJ protein secreted by the type III secretion apparatus of *Shigella flexneri* [7]. Interestingly, the IpaJ protein of *S.* Pullorum also reacted with convalescent sera from a young bird (unpublished data), indicating that the protein is an immunogen of *S.* Pullorum. In addition, the present authors also isolated pSFD10 from *S.* Pullorum, and a PCR analysis showed that the *ipaJ* gene existed in all the *S.* pullorum isolates in our laboratory from the 1960s to 2006, yet not in all the *S.* Choleraesuis strains, except for *S.* Choleraesuis C500. Thus, it is speculated that there may have been a horizontal transfer between *S.* Pullorum and *S.* Choleraesuis C500. However, the mutation of the *ipaJ* gene in *S.* Pullorum showed a lower invasion ability of HD-11 avian macrophages, implying that the gene may instead participate in the pathogenetic process of the agent.

Colicins are antibacterial proteins and their interaction with susceptible bacterial cells occurs in three steps: attachment to a specific receptor in the outer membrane, translocation through the cell envelope, and lethal action against the cell target [6, 12]. Colicins can be divided into two types: pore-former and nuclease colicins. Colicin Y is a pore-former colicin that kills bacterial cells by creating channels in the cytoplasmic membrane [25]. In this study, the PG92 fragment exhibited high homology to Colicin Y encoded by plasmid pCol-let of *E. coli*, indicating that this type of plasmid may exist in *S.* Pullorum.

Four fragments exhibited homology to prophage ST104 of *S.* Typhimurium DT104, which is a multidrug-resistant strain that has emerged and spread in many countries [15, 29]. The tailspike protein (TSP) of *S.* Typhimurium phage P22 is part of the apparatus used by the phage to bind to and hydrolyze the O antigen in *Salmonella* lipopolysaccharide [27]. After hydrolyzing the rhamnosyl–galactose linkages in the O antigen, the phage DNA is then injected into the cell for the purpose of infection [19]. The gp9 protein of prophage ST104 is similar to the TSP of phage P22 [28]. The Xis protein in *E. coli* can suppress aberrant recombination and promote HK022 excising recombination [14]. Therefore, these four sequences imply that a bacteriophage carrying these virulence genes can integrate into the bacteria chromosome and induce horizontal gene transfer.

The plasmid pPCP1 in *Yersinia pestis* encodes the plasminogen activator Pla, which is required for systemic spread after subcutaneous injection into a mammalian host [3]. A sequence analysis has shown that ColE1 is the origin of replication for pPCP1 [24]. In this study, PE81 exhibited homology to the hypothetical protein pPCP04 encoded by pPCP1, and this protein is closely related to the sequence in *S.* Typhimurium [8]. Thus, although *Salmonella* species are clearly distinct from *Yersinia* species as regards 16S rRNA and other sequenced loci, the high sequence identity of potentially mobile elements implies a horizontal genetic exchange between them [24]. Here, the similarity between PE81 and pPCP04 showed there may have been a horizontal DNA transfer between *Yersinia* and *Salmonella.*

There are two types of pSDV (virulence plasmid of *S.* Dublin): pOU1113 (pSDVu) and pOU1115 (pSDVr); both are approximately 80 kb in size [11]. Wallis and Paulin [30] previously reported that pSDV probably mediates the persistence of *S.* Dublin at systemic sites in cattle. Based on an analysis of the gene content in different *Salmonella* virulence plasmids, pSDV and pSPV (virulence plasmid of *S.* Pullorum) have been identified as Group I, containing the minor fimbria genes *faeH* and *faeI* without the serum resistance gene *rsk* [9]. In this study, PE5 and PE23 exhibited high homology to the sequences in pSDV, implying that pSDV and pSPV are closely related. Moreover, PE23 showed homology to the *traG* gene in pSDV. TraG is the coupling protein of TraJ, which is involved in DNA translocation [16]. Although the sequences of the *oriT-traM-traJ-traY-traA-traL* regions in pSPV have already been reported [10], this is the first report of the *traG* gene in *S.* Pullorum.

PE75 and PE81 both exhibited homology to the *Rhs* (recombinant hot spot) element of *S.* Typhi, and *Rhs* elements have also been found in other *Salmonella enterica* serotypes. *Rhs* elements are complex genetic composites that have already been well studied in *E. coli* [18, 31]. Although the biological function of *Rhs* elements remains unclear, they are not present in all *E. coli* isolates, yet are widespread in natural *E. coli* populations [18]. The predicted core protein sequence is similar to the sequence of a *Bacillus subtilis* wall-associated protein, and thus the product is expected to be a cell surface protein with a binding function that provides the cell with an advantage in a specific habitat [17]. However, the function of the *Rhs* element in *Salmonella* still needs to be elucidated.

Although some sequences showed homology to certain hypothetical proteins or putative proteins, their function was unknown. For example, fragment PE42 exhibited 99% homology to the hypothetical protein SPAB_0339 of *S.* Paratyphi *B,* which was first reported in July 2007. Therefore, the function of these genes remains to be identified.

In this study, two *Salmonella enterica* serotypes closely related to *S.* Pullorum were used as the source of driver DNA in SSH. As a result, some fragments specific to *S.* Pullorum were obtained that can be used as molecular markers to differentiate *S.* Pullorum from other *Salmonella* serotypes and to further our current understanding of the pathogenesis of the pathogen.

Acknowledgments

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