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ORIGINAL ARTICLE

Different mutations in the *oafA* gene lead to loss of O5-antigen expression in *Salmonella enterica* serovar Typhimurium

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Abstract

Aims: To analyse genetic changes in the *oafA* gene explaining the loss of O5-antigen expression in *Salmonella* Typhimurium and *Salm.* 4,[5],12:i:-.

Methods and Results: The *oafA* gene in 52 O5-antigen-negative and 77 O5-antigen-positive *Salm.* Typhimurium ($N = 47$) and *Salm.* 4,[5],12:i:- (monophasic *Salm.* Typhimurium strains, $N = 82$) was investigated by a combination of PCR screening and DNA sequencing to identify mutations leading to the suppression of the O5-antigen. Various DNA sequence changes within the open reading frame (ORF) of *oafA* in O5-antigen-negative strains could be identified. In 77% of the O5-antigen-negative strains, a 7-bp deletion of a duplicated sequence within the functional *oafA* gene led to a frameshift in the ORF. In four strains, an IS*4* element and in two, an IS*1* element was inserted at different positions. Four other strains carried at different positions single base pair substitutions causing a premature stop codon. Finally, in two strains, a deletion of the *oafA* 3′end of undetermined size was responsible for the lack of O5-antigen expression. In none of the strains investigated, the complete ORF of *oafA* was deleted. Primers were designed and used to detect the most prominent variants.

Conclusions: O5-antigen-negative *Salm.* Typhimurium and *Salm.* 4,[5],12:i:- strains carry an *oafA* pseudogene caused by different genetic events indicating that there is a selection for *oafA* mutations leading to the loss of O5-antigen expression.

Significance and Impact of the Study: The loss of O5-antigen expression may be an example of a common evolutionary mechanism to escape host defence or to adapt to environmental changes. The data are the basis for the development of diagnostic PCR assays for the differentiation of O5-antigen-positive and O5-antigen-negative *Salm.* Typhimurium and its monophasic (*Salm.* 4,[5],12:i-) strains.

Introduction

After *Salmonella* Enteritidis, *Salmonella* Typhimurium is the second most common serotype in Europe causing 21·9% of all confirmed human salmonellosis cases in 2008 (Anonymous 2010) and the recent worldwide emergence of a multidrug-resistant monophasic *Salm.* Typhimurium (*Salm.* 4,[5],12:i:-) variant raises public health concerns (Hopkins *et al.* 2010). A number of large outbreaks were caused by *Salm.* Typhimurium and increasingly by *Salm.* 4,[5],12:i:- within the last years (Mossong *et al.* 2007; Ethelberg *et al.* 2008; Bone *et al.* 2010). Pig meat and products thereof are one of the most important vehicles causing *Salm.* Typhimurium outbreaks (Anonymous 2010). This observation is in line with the EU baseline survey on the prevalence of *Salmonella* in slaughter pigs conducted in 2006/2007 revealing that *Salm.* Typhimurium was the most frequently isolated serovar

from lymph nodes and *Salm*. 4,[5],12:i:- was the fourth most prevalent serovar in slaughter pigs (EFSA 2008).

According to the White–Kauffmann–Le Minor scheme, *Salm.* Typhimurium has the antigenic formula 1,4,[5],12:i:1,2. Factor 5 that is written in brackets may be present or absent in group O:1,4,12 without relation to phage conversion (Grimont and Weill 2007). The expression of the O5-antigen (factor 5) has formerly been used for the discrimination of *Salm.* Typhimurium strains (Rabsch *et al.* 2002; Hauser *et al.* 2009). The variant of *Salm.* Typhimurium not expressing the factor 5 has been named variant Copenhagen (Kauffmann 1934). The molecular basis for O5-antigen presence or absence is that the polymer of the lipopolysaccharide (LPS) of *Salmonella enterica* serovar Typhimurium (*Salm*. Typhimurium) consists of repeating units of galactose–rhamnose–mannose with abequose attached to the mannose residue (Hellerqvist *et al.* 1968; Reeves 1993). If the abequose residue is acetylated on the 2-hydroxyl group, then this confers the O5 serotype (Hellerqvist *et al.* 1968).

The LPS of gram-negative bacteria serves as a barrier between the cell and its environment. The O-antigen polymer of the LPS represents its immunodominant portion. Previously, it was shown that a monoclonal immunoglobulin A antibody directed against O5-antigen (Sal4) was sufficient to prevent *Salm*. Typhimurium adherence to and invasion in polarized cell monolayers in culture (Michetti *et al.* 1994) and acetylation affected the structural and immunological properties of *Salm*. Typhimurium LPS O-antigen (Slauch *et al.* 1995). However, the loss of acetylation has no effect on the virulence of *Salm*. Typhimurium (Michetti *et al.* 1992).

The O5-antigen is encoded by the *oafA* gene. The OafA protein belongs to a family of membrane-spanning proteins involved in the acetylation of exported carbohydrate moieties (Slauch *et al.* 1996). OafA is solely responsible for the acetylation reaction. The gene is located in *Salm*. Typhimurium upstream to STM 2231 and a putative peptidase (STM 2230) adjacent to the tRNA*proL* gene and downstream to a cluster of phage-related genes followed by the *sspH2* gene (McClelland *et al.* 2001; Hansen-Wester and Hensel 2002). The gene *sspH2* encodes an effector protein of the type III secretion system located on the *Salmonella* pathogenicity island 2 (SPI2) of serovar Typhimurium (Miao *et al.* 1999). In *Salm*. Typhi, an O5-antigen-negative serovar, the *oafA* gene and phage-related genes are not present (Hansen-Wester and Hensel 2002). Later, this region has been designated as *Salmonella* pathogenicity island 12 (SPI-12) following sequencing of the *Salmonella* Choleraesuis genome (Chiu *et al.* 2005).

In this study, we identified by a PCR strategy *oafA* gene variants within a set of *Salm*. Typhimurium and monophasic strains (*Salm*. 4,[5],12:i-) that were not able to express the O5-antigen. DNA sequencing of the *oafA* genes from selected strains revealed that various genetic changes of the *oafA* open reading frame (ORF) leaded to the loss of O5-antigen expression.

Materials and methods

Selection of *Salmonella* strains

Forty-seven *Salm*. Typhimurium strains isolated in Germany from porcine lymph nodes during an EU-Monitoring study in 2006/2007 on the prevalence of *Salmonella* in slaughter age pigs were selected (EFSA 2008). They were the representatives of various geographical origins in Germany as well as different seasons. Of these, 25 strains expressed the O5-antigen. Furthermore, 82 *Salm*. 4,[5],12:i:- strains isolated from porcine lymph nodes during the EU-Monitoring study in 2006/2007 and from pork as previously described (Hauser *et al.* 2010) were included in this study. Fifty-two of these monophasic strains were serologically O5-antigen positive.

Serotyping

All *Salmonella* strains were serotyped according to the White–Kauffmann–Le Minor scheme (Grimont and Weill 2007) by slide agglutination with O- and H-antigen-specific sera (Sifin Diagnostics, Berlin, Germany).

DNA sample preparation

DNA for PCRs was prepared by thermal cell lysis. Briefly, a 1-ml aliquot of an overnight culture of *Salmonella* cultured in Luria–Bertani broth at 37°C was centrifuged for 5 min at 10 000 ***g*** and 4°C. The supernatant was discarded and the cell pellet resuspended in 300 μl TE buffer (10 mmol l^{-1} Tris pH 8, 0·1 mmol l^{-1} EDTA). The resuspension was heated for 10 min at 95–100°C, then immediately cooled and again centrifuged at 14 000 ***g*** for 10 min. As template in PCR, a 1 : 10 diluted 5-μl aliquot of the supernatant containing DNA (*c.* DNA of 10^6 bacterial cells) was used.

Polymerase chain reaction

Primers were designed using the program ARRAY DESIGNER 4.0 (Premier Biosoft, Palo Alto, CA, USA). The annealing temperature for all primers was demanded to be 55 ± 2°C in the PCR with GC-content not <40%. Nucleotide sequences of all primers used in this study are shown in Table 1. The positions of primers within the *oafA* gene are indicated in Fig. 1. All PCRs were carried out in a GenAmp PCR System 9700 thermocycler

Table 1 Primer sequences used in this study

Designation	Primer sequence (5′ → 3′)	Start-position
P-439	ACGAAGCACTTAGCAAGAACG	290*
P-440	CAACAGCAACAACAATGAGGAC	700*
P-1034	CAGTGACCTTCTTTGATGTAG	−131*
P-1035	TTCTTTGGTGTAATTGTGTCTT	919*
P-1036	CATCAGAAAAGCTATACACATA	1911*
P-1039	GTATACATAGCATCTGTACGT	613*
P-1040	AAGATGACAGGCCGTATTGC	1340*
P-1041	ATTAATACCGTAGTAGGCTTGT	1421*
P-1072	AATGACTAATAAAGGATATAAAATAT	459*
P-IS4-1r	CCTGCTTACTCAAAATCTAT	4601446†
P-IS1-1r	TGTCATGCAGCTCCACCGAT	35511‡
P-1187	TGAAATCTGCTTTCTCACTTC	1824*
P-1188	AGGGAATGCTCTTTCGCCA	1670*

*Position according to Fig. 1.
†Position within IS*4* according to accession number CP001120.1 (IS*4*).
‡Position within IS*1* according to accession number AM991977.1 (IS*1*).

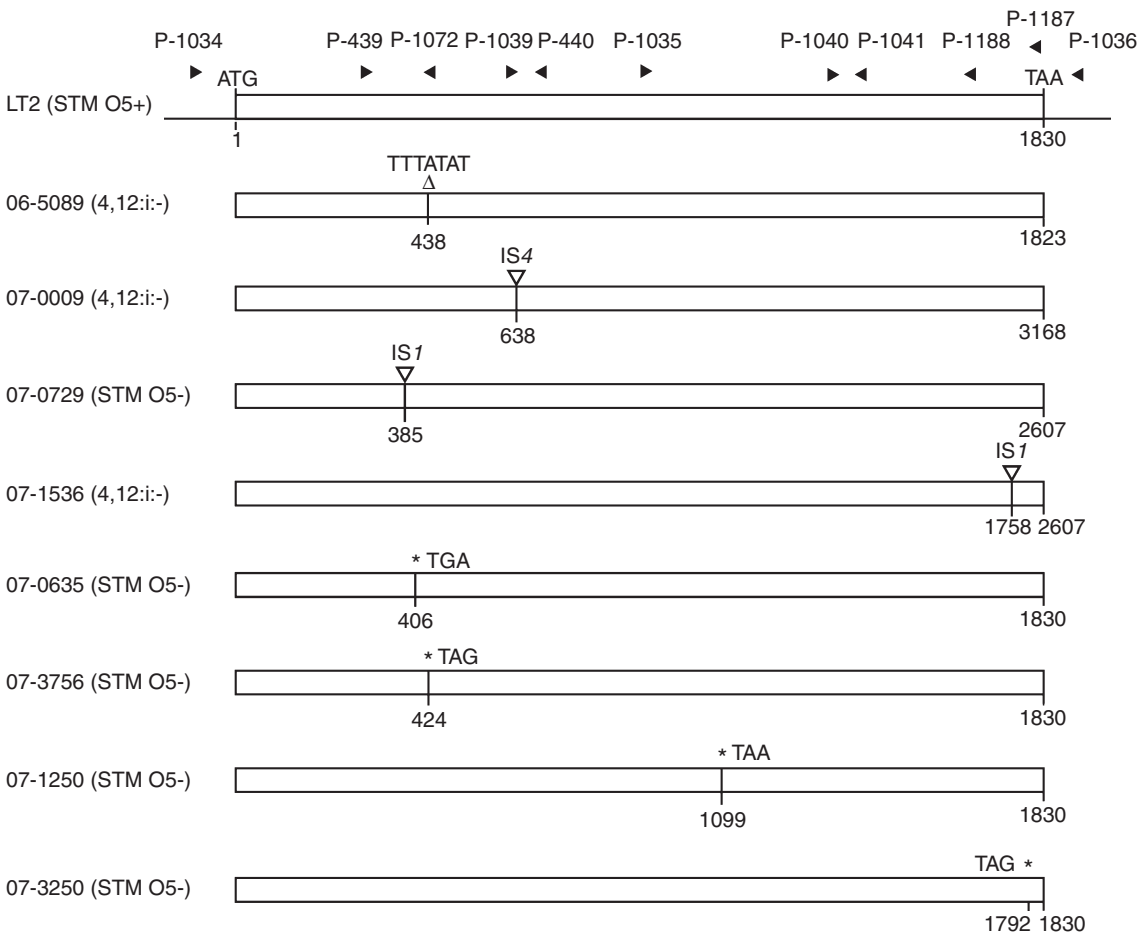

Figure 1 Overview of identified interruptions in *oafA*. Rectangle indicates schematically the *oafA* sequence of particular strain. Interruptions found are indicated by triangles (Insertion element IS*1* or IS*4*), * (stop codon) or Δ (7-bp deletion). On the left reference, strain no. is indicated. Regular *oafA* start- and stop codon is indicated by ATG or TAA within the strain LT2 (*Salmonella* Typhimurium) schematic drawing. Numbers below rectangles specify nucleotide position of *oafA* start and stop as well as position of interruption/mutation event. STM is the abbreviation for *Salm*. Typhimurium. O5- indicates O5-antigen-negative strains; O5+ indicates O5-antigen-positive strains.

(Applied Biosystems, Weiterstadt, Germany). A 25-μl PCR contained 0·4 μmol of the corresponding primers, 200 μmol l^{-1} of each dNTP (Roche Applied Science, Mannheim, Germany), 1× PCR buffer [20 mmol l^{-1} Tris–HCl (pH 8·4), 50 mmol l^{-1} KCl], 1·5 mmol l^{-1} $MgCl_2$, 1 U Platinum *Taq* polymerase (Invitrogen, Karlsruhe, Germany) and a 5-μl aliquot of the sample DNA. Reaction conditions for all PCRs were 95°C for 1 min, followed by 33 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. A final extension of 72°C for 4 min was employed. A 5-μl aliquot of a PCR product was loaded on a 1·5% agarose gel and electrophoresed at 6 V cm^{-1} for 60–90 min. After electrophoresis, the agarose gel was stained for 20 min in 0·5 μg ml^{-1} ethidium bromide solution and photographed under UV light.

DNA sequencing

The *oafA* ORF of strains 06-5089, 07-0009, 07-0729, 07-1536, 07-0635, 07-3756, 07-1250 and 07-3250 was completely amplified using primers P-1034 and P-1036. PCR conditions were used as described earlier. DNA sequencing was performed by AGOWA (Berlin, Germany) using the sequence primers shown in Table 1. For assembling and alignment of DNA sequences, Lasergene Software package version 7.2 (DNASTAR, Madison, WI, USA) was used.

Nucleotide sequence accession numbers

The *oafA* DNA sequences of strains 06-5089, 07-0009, 07-0729, 07-1536, 07-0635, 07-3756, 07-1250 and 07-3250 were entered into Genbank under accession numbers HM769323 – HM769330.

Results

A set of 47 *Salm*. Typhimurium and 82 *Salm*. 4,[5],12:i:- epidemiologically unrelated strains were investigated by PCR using primers P-439 and P-440 (Table 1) for the presence of the *oafA* gene. All 77 strains exhibiting serotypically the O5-antigen gave the expected PCR product size of *c.* 400 bp (Table 2). Forty-seven of 52 O5-antigen-negative strains tested had also a fragment of this size. Four other strains gave a PCR product of *c.* 1·6 kb and the remaining strain (07-0729) a product of *c.* 1·1 kb (Table 2).

Of each group of strains, one strain was randomly selected and the *oafA* ORF completely sequenced. Nucleotide sequences indicated that the *oafA* ORF of O5-negative strain 07-0729 was disrupted by an IS*1* element at position 385 (Fig. 1). In another strain (07-0009), *oafA* was disrupted by an IS*4* element at position 638, and in strain (06-5089), a 7-bp nucleotide sequence TTTATAT was deleted. This 7-bp nucleotide sequence is arranged as a tandem repeat within the functional *oafA* ORF.

Table 2 Genetic *oafA* variants, PCR screening results and frequency of variants found in *Salmonella* Typhimurium (n = 47) and *Salm*. 4,[5],12:i:- (n = 82)

Variation	O5-antigen	PCR size in kb (P-439/P-440)	No. of strains *Salm*. Typhimurium	No. of strains *Salm*. 4,[5], 12:i:-
–	+	0·4	25	52
Δ 7 bp	–	0·4	15	25
IS*4*	–	1·6	2	2
Δ 3′end	–	0·4	0	2
IS*1*	–	1·1	1	0
IS*1* reverse	–	0·4	0	1
TGG → TGA*	–	0·4	1	0
CAA → TAA*	–	0·4	1	0
TGG → TAG*	–	0·4	2	0

*Nucleotide exchange in amino acid encoding DNA sequence leading to premature stop codon.

Specific PCR primers were developed to identify the variations among the nonsequenced strains. All four strains that gave originally a 1·6-kb PCR product with primers P-439 and P-440 were positive for the IS*4* element inserted at position 638 in *oafA* (Fig. 1). The 7-bp deletion in *oafA* was recognized in 40 strains by PCR using primers P-439 and P-1072. With these primers, a 170-bp PCR product was detected if the tandem repeat was present. If a strain positive for *oafA* in PCR with primers P-439 and P-440 harboured the 7-bp deletion, no PCR product with primer P-439 and P-1072 was obtained.

For seven strains, obviously none of the *oafA* variations identified was responsible for the absence of the O5-antigen. Therefore, sequencing of the complete *oafA* gene of these strains was intended. A PCR product using primers P-1034 and P-1036 was obtained from five of the seven strains. One strain (07-1536) gave a larger PCR product of *c.* 2·7 kb instead of an expected one of 2 kb. Sequencing of those PCR products revealed additional variants. The *oafA* of strain 07-1536 was disrupted again by an inverted IS*1* element at position 1759 (Fig. 1). In four other strains, base pair substitutions at various positions lead to premature stop codons and therefore loss of OafA synthesis (Fig. 1). The two remaining strains (07-1566 and 07-4678) gave no PCR products with primers nearly located upstream or downstream of the *oafA* TAA stop codon. We were not successful to identify the reason for PCR failing. A PCR product has been obtained with reverse primer P-1188 but not with reverse primer P-1036 (Fig. 1). Probably, a deletion within these strains that begins somewhere prior to the regular *oafA* stop codon TAA and is stretched downstream causes the lack of O5-antigen expression.

Discussion

Since many decades, *Salm*. Typhimurium strains have been serologically differentiated by the presence or absence of the O5-antigen. However, the molecular basis for the phenotypic presence or absence has not been determined so far. In this study, we have extensively analysed the *oafA* locus in *Salm*. Typhimurium and its monophasic variant *Salm*. 4,[5],12:i:-. Surprisingly, not a common, but various DNA changes within the ORF of *oafA* could be identified leading to the abortion of OafA protein synthesis on the translational level. In most O5-antigen-negative strains (77%), a 7-bp deletion of a tandem duplicated sequence in the functional *oafA* gene caused a frameshift of the ORF. In four strains, an IS*4* element and in two, other strains an IS*1* element was inserted at different positions. In four other strains, single base pair substitutions caused a premature stop codon, in each strain at a different position. Finally, in two strains, probably a deletion of the *oafA* 3′end of unknown size was responsible for its O5-antigen-negative status. In none of the strains determined, the complete *oafA* locus was deleted like described for *Salm*. Typhi (Hansen-Wester and Hensel 2002) and other serovars. These variations indicate that there is a selection for *oafA* mutations leading to the loss of O5-antigen expression. Multiple independent events can lead to the interruption of the abequose acetylation in *Salm*. Typhimurium and in the monophasic variant *Salm*. 4,[5],12:i:-. This loss of function may be a common evolutionary mechanism through which immunological host defence can be escaped or *Salmonella* may adapt to other environmental changes. Generally, it has been hypothesized that loss of surface protein expression may be a mechanism of targeting the invading pathogen preferentially to particular tissues or host cells and avoiding the potential stimulation of non-specific inflammation (Thomson *et al.* 2008). OafA might be such an example, too. Although the strains determined in this study originated all from pig, it has been described that some O5-antigen-negative *Salm*. Typhimurium strains are preferentially associated with birds, especially pigeons (Rabsch *et al.* 2002). In this study, such isolates were not included, because they might have a specific clonal genetic background differing from swine isolates. However, the loss of acetylation has no direct effect on the virulence of *Salm*. Typhimurium in mice (Michetti *et al.* 1992) but affects the structural and immunological properties of the LPS O-antigen (Slauch *et al.* 1995).

Genome sequencing of various *Salmonella* serovars including *Salm*. Typhimurium has revealed that their genomes harbour many truncated genes or pseudogenes that carry frameshift mutations or premature stop codons. This functional gene loss leads to loss of function of pathways and can influence the ability of the pathogen to survive in the environment or under stressed conditions in the host. Especially, more pseudogenes in host-specific serovars such as *Salmonella* Gallinarum (poultry) or *Salm*. Typhi (human) were found than in ubiquitous serovars such as *Salm*. Enteritidis or *Salm*. Typhimurium (Thomson *et al.* 2008) supporting the hypothesis of adaptation to the host by gene loss.

In conclusion, the exhibition of the O5-antigen in *Salm*. Typhimurium and in the monophasic variant *Salm*. 4,[5],12:i:- can be interrupted because of alteration of the DNA sequence in the *oafA* gene caused by import of insertion elements, 7-bp deletion or point mutations leading to a premature stop codon. The observation of different reasons for the interruption indicates that there is a selection for *oafA* mutations leading to the loss of O5-antigen expression. Primers designed to detect the most prominent *oafA* variants might be the basis for further development of diagnostic PCR assays for the differentiation of O5-antigen-positive and O5-antigen-negative *Salmonella* strains.

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