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Association of a Regulatory Gene, *slyA* with a Mouse Virulence of *Salmonella* Serovar Choleraesuis

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***Abstract*: The influence of *slyA* gene, originally found in *Salmonella* serovar Typhimurium as a regulatory gene for the expression of virulence genes, on a mouse virulence of *S.* serovar Choleraesuis was investigated by using an *slyA*-defective mutant. The defective mutant was constructed by the insertion of a kanamycin-resistance gene (*aph*) into the cloned *slyA* gene, and the homologous recombination with the intact *slyA* gene on the chromosome. The mutant strain showed the LD_{50} value for BALB/c mouse approximately 10^5 higher than that of the parent strain. The increase of the LD_{50} value was the same order as that shown by the mutation of the *slyA* gene of *S.* serovar Typhimurium, although LD_{50} of the wild-type strain of *S.* serovar Choleraesuis was 40-fold higher than that of *S.* serovar Typhimurium. The time course of infection observed in the mice organs also proved the clear difference of the virulence between the parent and the mutant strains. These results suggested that the *slyA* gene product functions as a virulence-associated regulator also in *S.* serovar Choleraesuis.**

***Key words*: Virulence, *slyA*, *Salmonella* serovar Choleraesuis**

For the full expression of the *Salmonella* virulence, many classes of virulence genes such as *inv* genes (7), *pag* genes (10), and plasmid-encoded *spv* genes (9, 11) are required. Among them regulatory genes are of highest importance for the timely expression of the virulence genes to adapt to the environmental changes during the infection process. Besides *phoP/phoQ* (19) and *rpoS* (27), a new regulatory gene *slyA* was cloned by Libby et al. (15) from *Salmonella* serovar Typhimurium as a cytolysin-encoding gene, but later it was proved to be a gene encoding a transcriptional regulatory protein (5), which belongs to a family of regulators including EmrR (16), MarR (28), HprR (23), and PecS (21). This regulatory gene of *S.* serovar Typhimurium is required for cytotoxicity to M-cells (4), survival in macrophages (13), and resistance to oxidative stress (3). In contrast, *slyA* is not involved in invasion or colonization in the murine intestine, and therefore is not essential for the enteric salmonellosis (29).

The *slyA* gene is commonly shared among major pathogenic serovars of *Salmonella* (14, 15), and homologous genes have been found also in other members of *Enterobacteriaseae* such as *Escherichia coli* (17, 22), *Shigella* (17), and *Yersinia* (24). We have sequenced ORF and upstream region of *slyA* of several serovars of *Salmonella*, and found that all of the serovars so far examined use the sequence TTG as initiation codon of *slyA*, and the nucleotide sequences of this region were highly conserved (14). In our study of *Salmonella* virulence, *S.* serovar Choleraesuis has been mainly used (1, 11, 13, 18), because this serovar shows a high tendency to cause systemic salmonellosis to human and swine compared with *S.* serovar Typhimurium (2, 25), and the genes responsible for systemic salmonellosis are expected to be studied in simpler system by using this serovar. In this context the function of *slyA* in *S.* serovar Choleraesuis has been investigated in relation to our previous and ongoing studies on the pathogenesis of this serovar. In the present study, *slyA*-defective mutants of *S.* serovar

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Abbreviations: CFU, colony forming unit; LB broth, Luria-Bertani broth; LPS, lipopolysaccharide; ORF, open reading frame.

Table 1. Bacterial strains and plasmids

Strain and plasmid		Relevant characteristics	Reference
Strain			
E. coli DH5 α		F⁻ Φ80d*lac*ZΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*	Gibco/BRL
E. coli S17-1 *λpir*		RP4-2-Tc::Mu-Km::Tn*7* *λpir*	26
S. serovar Choleraesuis			
	RF-1	wild type, Rf^r, derived from a swine isolate	12
	KAD001	RF-1 *slyA*$_{SC}$::*aph*, Km^r	This study
S. serovar Typhimurium			
	SR-11	wild type, Nr^r	8
	KAD101	SR-11 *slyA*$_{ST}$::*aph*, Km^r	This study
Plasmid			
pUC119		cloning vector, Ap^r	Toyobo
pUC4K		Km cassette (*aph*), Ap^r,Km^r	Toyobo
pCVD442		suicide vector for homologous recombination, Ap^r	26
pKAD301		pUC119, *slyA*$_{SC}$	14
pKAD302		pUC119, *slyA*$_{SC}$::*aph*	This study
pKAD306		pCVD442, *slyA*$_{SC}$::*aph*	This study
pKAD308		pUC119, *slyA*$_{ST}$	14
pKAD309		pUC119, *slyA*$_{ST}$::*aph*	This study
pKAD313		pCVD442, *slyA*$_{ST}$::*aph*	This study

Choleraesuis and *S.* serovar Typhimurium were constructed and the decrease of virulence from these mutations were compared.

The *slyA*-defective mutants were constructed by the method of homologous recombination. The *slyA* genes of *S.* serovar Choleraesuis RF-1 and *S.* serovar Typhimurium SR-11 were amplified by PCR and ligated into pUC119 as described in a previous paper (14). The kanamycin-resistance cassette gene (*aph*) was prepared from pUC4K by *Bam*HI digestion, blunted, and inserted into *Rsa*I site of *slyA* gene to form pKAD302 and pKAD309. The mutated *slyA* genes (*slyA*::*aph*) were prepared from pKAD302 and pKAD309 by *Bam*HI and *Eco*RI digestions, blunted, and ligated into *Sma*I site of the suicide vector pCVD442 to form pKAD306 and pKAD313.

Conjugation and allelic exchange was performed using pKAD306 and pKAD313 by the conventional method (26), and putative transconjugants were designated KAD001 and KAD101. The bacterial strains and plasmids used or prepared in this study are summarized in Table 1. The chromosomal DNA preparations of these transconjugants were digested, and the insertion of the *aph* gene was examined by Southern hybridization. When the 40 bp (from 318 to 357) of *slyA* gene from *S.* serovar Typhimurium (15) was used as a DNA probe, increase of molecular size of the *slyA*-containing fragments was observed for both transconjugant strains, KAD001 and KAD101 as shown in Fig. 1. These results indicated that *slyA* genes of the parent strains were knocked out by the *aph* gene insertion. By the *slyA* mutation, colony morphology, polysaccharide length of

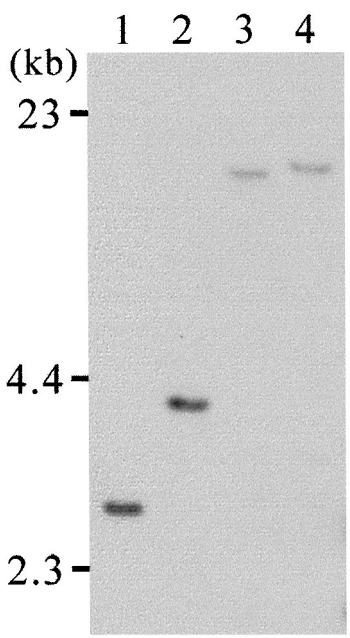

Fig. 1. Southern hybridization of wild-type and *slyA* mutant strains by the 40 bp DNA probe from *slyA* gene. Chromosomal DNA prepared from each strain was digested with *Not*I. Lanes 1, RF-1; 2, KAD001; 3, SR-11; 4, KAD101.

Table 2. Effect of the *slyA* mutation on *Salmonella* virulence

Strain	Genotype	LD_{50}(CFU/mouse)
S. serovar Choleraesuis		
RF-1	wild type	7.6×10^{2}
KAD001	$slyA_{SC}$::*aph*	5.7×10^{7}
S. serovar Typhimurium		
SR-11	wild type	1.9×10^{1}
KAD101	$slyA_{ST}$::*aph*	1.1×10^{6}

Salmonella strains grown overnight in LB broth were recovered by centrifugation and resuspended in sterile PBS (−) to approximately 5×10^{1} to10^{8} CFU/ml. The actual number of bacteria present was determined by plating on agar medium. BALB/c mice were challenged intraperitoneally (0.2 ml of bacterial suspension), and observed for 14 days.

LPS, O and H antigenicity, and growth rate in LB broth were found to be unchanged.

The parent (wild-type) strains and the *slyA*-defective strains were grown in LB broth and inoculated to BALB/c mice (female, 5 weeks old, CLEA Japan, Tokyo) intraperitoneally (5 mice/dose) according to the method described previously (20). The LD_{50}s of the strains were determined by the method of Beherns-Karber (6). All animal experiments were done in accordance with the Kitasato University Guideline for Animal Care and Experimentation. As shown in Table 2, LD_{50} of *S.* serovar Typhimurium was much lower than that of *S.* serovar Choleraesuis as reported previously (20). The LD_{50} values in this study are generally higher than those reported in the previous paper (20), but these differ-

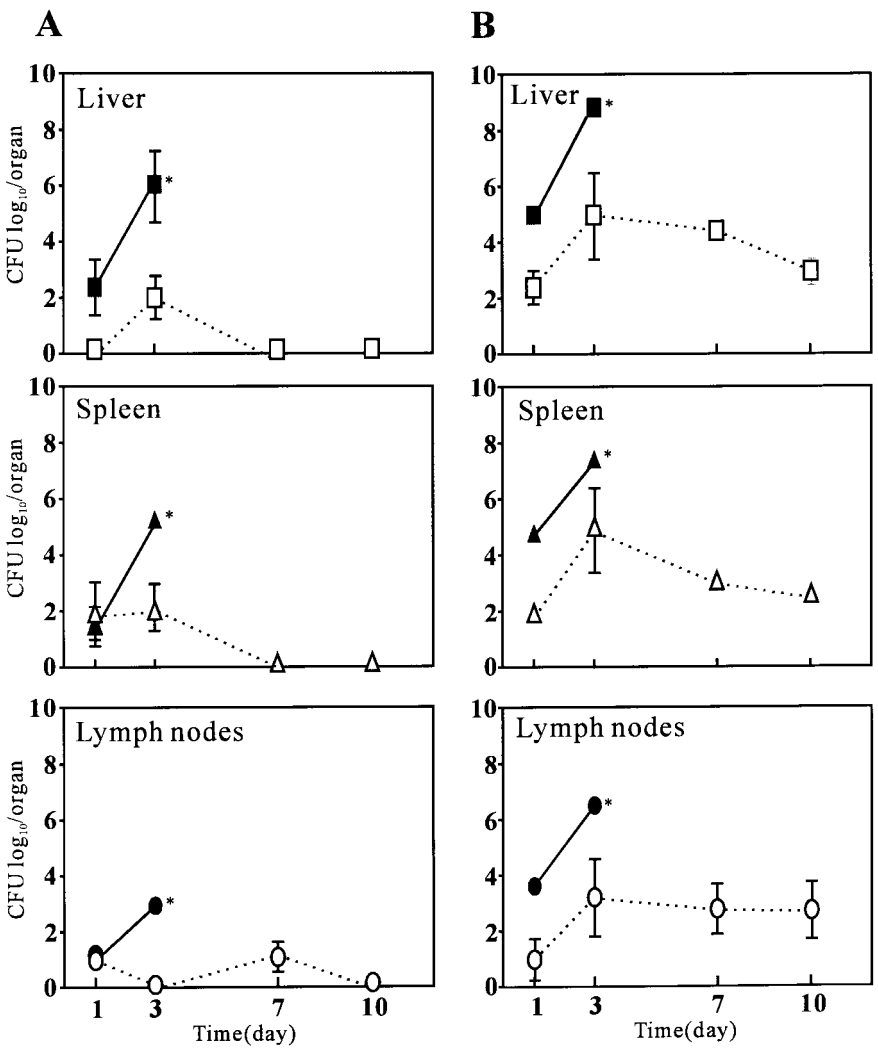

Fig. 2. Course of infection of wild-type and *slyA* mutant strains. A: *S.* serovar Choleraesuis KAD001 (dotted line) and RF-1(solid line). B: *S.* serovar Typhimurium KAD101 (dotted line) and SR-11(solid line). Mice were inoculated intraperitoneally with 1×10^{4} CFU/mouse, and sacrificed on the days indicated. Livers (□, ■), spleens (△, ▲) and intestinal lymph nodes (○, ●) were dissected and homogenized, and the number of bacteria in each organ was determined by plating homogenates onto LB agar. Asterisks indicate the death of mice.

ences might arise from the difference of mice age used in the experiments. By the mutation in *slyA*, LD_{50} of *S.* serovar Choleraesuis was increased in the order of 10^5, and the same degree of increase in LD_{50} was observed by the *slyA* mutation of *S.* serovar Typhimurium. These results suggested that SlyA protein of *S.* serovar Choleraesuis behaves in a manner similar to that of *S.* serovar Typhimurium, although other virulence factors and the potency of mouse virulence are different between these two serovars.

In the next experiment, the bacterial cell number in the organs of mice was measured after intraperitoneal infection of the *Salmonella* strains. The bacterial cell suspension in PBS(−) was inoculated intraperitoneally to BALB/c mice (female, 5 weeks old, CLEA Japan) at the dose of 10^4 CFU/mouse. Bacterial cell number (CFU) in the liver, spleen, and intestinal lymph nodes at day 1, 3, 7, and 10 was measured by plating on agar medium after the organs were homogenized by a stomacher (Japan Organo Co., Ltd., Tokyo). Five mice were used for one group, and the mean values of CFU were calculated. The results shown in Fig. 2 indicated that the numbers of wild-type strains of *S.* serovar Choleraesuis and *S.* serovar Typhimurium increased rapidly from day 1 to day 3. There was a clear difference between bacterial numbers of both serovars, but no difference of mice death between both serovars was observed because all mice died before day 7. In contrast, the mutant strains showed slow growth from day 1 to day 3 and the bacterial number decreased or kept constant after day 3. These results agreed with the values of LD_{50} shown in Table 2, and clearly indicated that the virulence of *S.* serovar Choleraesuis was much reduced by the *slyA* destruction in the same manner as for *S.* serovar Typhimurium.

In the present study we could demonstrate that in *S.* serovar Choleraesuis *slyA* gene is associated with mouse virulence. In *S.* serovar Typhimurium, *slyA* was reported to be required for survival in macrophages, and for systemic infection. The present results suggested that the *slyA* gene functions in *S.* serovar Choleraesuis as in *S.* serovar Typhimurium. As shown in our previous studies, *S.* serovar Choleraesuis harbors plasmid-encoded (11) as well as chromosomal virulence genes (H. Danbara et al., unpublished data). Together with the present results *S.* serovar Choleraesuis was proved to have a full set of functioning genes required for systemic infection, and therefore be a useful organism to study *Salmonella* pathogenesis.

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