Szent-István University
Doctoral School of Veterinary Sciences

Construction of *Salmonella* Enteritidis strains for early protection of poultry: the use of new transposon mutagenesis systems on the virulence plasmid and on the flagellar genes.

PhD Thesis

By: Ariel IMRE

Veterinary Medical Research Institute
of the Hungarian Academy of Sciences

2007

Szent-István University
Doctoral School of Veterinary Sciences

Leader of the PhD work:

Prof. Dr. Béla Nagy, member of the Hungarian Academy of Sciences
Veterinary Medical Research Institute of the Hungarian Academy of Sciences
Budapest

Consultant:

Dr. Ferenc Olasz, leader of Environmental Biotechnology Unit
Institute of Genetics, Agricultural Biotechnology Center
Gödöllő

Introduction

One of the preventive measures against salmonellosis of farm animals is the vaccination, which has about 20 years of history. Although the experimental data related to the so far developed and marketed vaccines are generally favourable, there are still several aspects for further development of vaccines against salmonellosis of poultry. One aspect is efficacy which more and more requires the use of live oral attenuated vaccines in contrast to the inactivated vaccines. The strains used for vaccines should also meet several criteria of safety: they should be innocuous for the host and for the environment, and they should be easily distinguishable from the wild strains. With these requirements in mind we aimed to construct a *Salmonella* Enteritidis strain with a negative protein marker and with decreased virulence but still having the potential to provide early protection to poultry against *Salmonella*.

First we aimed to differentiate the potential vaccine strain from the wild strains by providing a serologically detectable marker on the strain. The most straightforward solution for that was the elimination of a surface protein (with good antigenicity) from the given strain. In case of *S*. Enteritidis, the flagellin proteins seemed to be the most suitable for the above purpose, partly because it's good antigenecity and consequent serological activity, and partly because the lack of flagellae (the non motile phenotype) can be easily detected. We planned to meet the above objectives by a new method of directed transposition.

As the serovar specific plasmid plays an important role in the propagation and survival of *Salmonella* in the host, we planned to decrease the virulence of our strain by elimination of its serovar specific virulence plasmid. The plasmid contains several groups of genes governing virulence related traits. Among them the most important is the *spv* (*Salmonella* plasmid virulence) region, assisting in bacterial survival in the granulocytes and macrophages. Further important gene groups are the *pef* (plasmid encoded fimbriae) operon, mediating adhesion to the intestinal cells, and the *rck* (resistance to complement killing). The function of these genes may be important in the infection process. Therefore elimination of the serovar specific virulence plasmid is regarded an essential step in the development of live oral salmonella vaccines. In the last phase of our work we have tested the residual *in vitro* and *in vivo* virulence, and the early protective capacity of the mutants of *S*. Enteritidis 11 strain which were produced by the above mentioned transposon based molecular genetic methods.

Aims

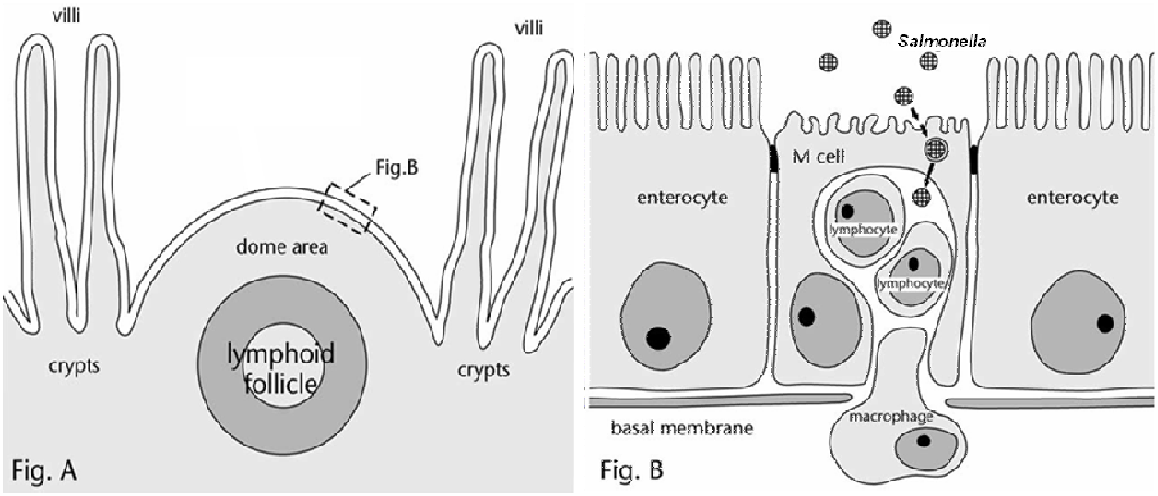
These studies aimed to work out and test different new transposon mutagenesis systems for markering and for reduction of virulence of *Salmonella* Enteritidis strains with the long term goal of potential vaccine development. Details of these aims were as follows:

1. PCR mapping of flagellin production and phase variation systems of *S*. Enteritidis and of some other serovars to decide about genetic intervention sites.

2. Development of a site directed transposon mutagenesis system and testing its function in order to produce nonflagellated (markered) potential future vaccine strains.

3. Reduction of virulence by elimination of the serovar specific virulence plasmid of the nonflagellated (markered) *S*. Enteritidis mutant.

4. Testing residual virulence and early protective activity of the resulting potential live oral *S*. Enteritidis vaccine strain.



Schematic representation of the *Salmonella* uptake through intestinal Peyer's patches (Fig. A), characterised by the presence of specialised antigensampling M cells (Fig. B) (Adapted from Jepson and Clark 2001.)

Description of the experimental work

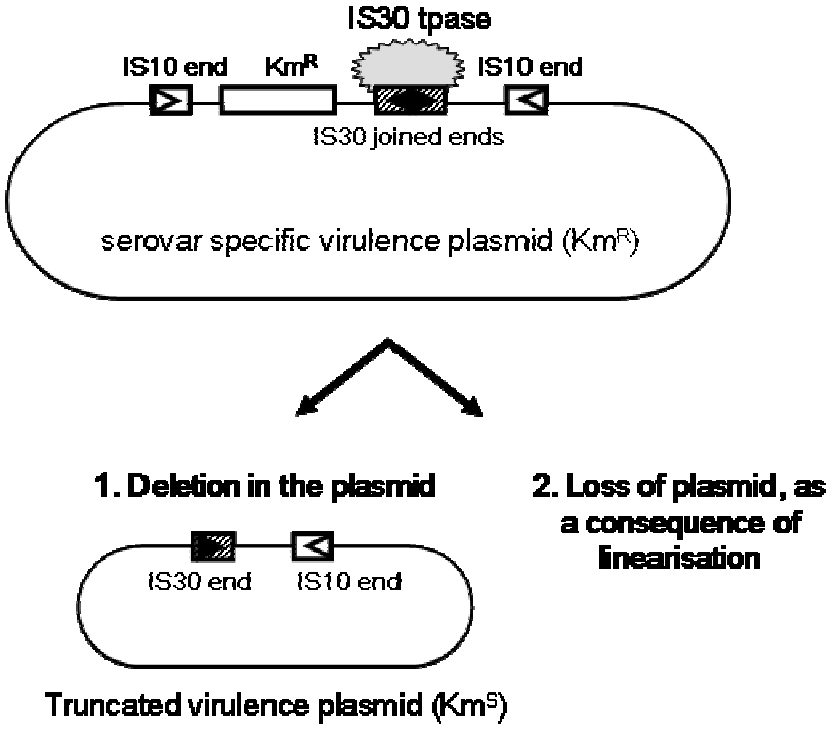
During these studies we aimed to work out and test *S*. Enteritidis strains that could serve as long term potential vaccine strains for early protection of day old chicks, and would posses appropriate serological marker. A further aim was that the strains should colonize the intestine effectively and be appropriately reduced in their virulence.

Blocking of the *S*. Enteritidis flagellin production system seemed to be the most logical way for markering the candidate strains. This required the constrtuction of appropriate PCR mapping system for the most important genetic elements of the flagellar phase variation. The use this PCR system on a large number of *S*. Enteritidis strains confirmed that the reason for the characteristic H1 monophasic character of *S*. Enteritidis is that this serovar is always lacking the *fljB* gene responsible for the H2 phase. Besides, the whole phase variation system (*hin, hixL, hixR*) is also missing. *S*. Enteritidis possess however, the *fliC* flagellin gene and its operator sequence resulting a continuous H1 flagellin production.

Using this PCR system and the informations gained, we aimed to target the mutation of *fliC* gene. With this aim in mind we have worked out a site directed transposition system which would focus transposition events onto the flagellar genes. The essence of the system was to introduce a recombinant plasmid into the *S*. Enteritidis 11 strain containing the fusion of the *fljA* flagellin repressor gene to the IS*30* transposon gene, from which the derived fusion protein (IS30-FljA) would produce mutations in the flagellin genes thereby blocking their functions. The above system was constructed based the assumption that the FljA component of the fusion transposase – as a DNA binding protein - would concentrate insertions with high frequency in the flagellin genes, more closely in the operator region of *fliC* gene. The system constructed on the above basis has worked well and produced mutations resulting non motile phenotypes, although the mutations did not occure in the *fliC* gene but in the neighbouring *fliD* flagellar gene. The produced non flagellated mutants proved to be stably non motile, and possessed a negative marker (the missing flagellae). Therefore they were appointed for the further experiments.

The next objective was to remove the highly stable serovar specific virulence plasmid of the nonflagellar mutants of *S*. Enteritidis 11 strain. First the classical ethidium bromide treatment

was applied, but in vain. This was probably due to the high stability of the plasmid. Therefore a new working hypothesis and a suitable two component (IS*10* – IS*30*) transposition model has been worked out. The first component of this system contained a reactive $(IS30)_2$ joined ends on a transposon with *IS10* IRs (inverted repeats) at both ends. This first component was inserted – among others - into the serovar specific virulence plasmid of the *S*. Enteritidis 11, and was further reacted by the second component (active IS30 transposase on a carrier plasmid). The working hypothesis was that such an introduced IS30 transposase would activate the IR*30* joined ends of the construct and utilize this site as a starting point for deletions and further genetic rearrangements occasionally leading to the complete loss of the serovar specific virulence plasmid (See Figure). Indeed, in case of the selected non motile mutant (*S*. Enteritidis 2102) of the strains *S*. Enteritidis 11 the system worked very efficiently: 50% of the resulting mutants have completely lost their virulence plasmid. A further advantage of this system was also to result several mutants with detectable partial deletions on their virulence plasmid. Consequently we have produced several mutants that contained no or partially deleted virulence plasmid.



Transposon-based plasmid elimination. After the virulence plasmid was targeted, IS30 transposase provided from an inducible plasmid can mediate deletion formation (step 1) or loss of the whole plasmid (step 2) utilizing the highly reactive joined IS*30* ends.

In further studies the question was raised about possible changes in the *in vitro* and *in vivo* virulence of the above (non flagellated and plasmidless) mutants, in terms of cell-, and organ-invasion as well as of intestinal colonization. The mutants produced above (designated as "nonmotile" and "plasmidless-nonmotile") have been tested for virulence under *in vitro* (cell culture) and *in vivo* (day old SPF chick oral infection) models in comparison with the parent *S*. Enteritidis 11 strain. Both mutants proved to have essentially reduced virulence under both conditions: reduced cell invasion (*in vitro*), and reduced organ (liver and spleen) invasion (*in vivo*), which proved to be dose dependent. However, there was no reduction in caecal colonization by these mutants in comparison to the parent strain, which was regarded as a favourable result, considering the requirements for efficiency of a potential live oral vaccine. On the other hand, reduced organ invasion indicated a reduced pathogenecity of the mutants.

One of the "plasmidless-nonmotile" mutants (SEΔ155) has been tested by oral application in the day old chicken infection model for its innocoucity and for early protective activity against the highly virulent *S*. Enteritidis 147 challenge strain, in comparison to those attributes of the parental *S*. Enteritidis 11 strain. The early protective activity against organ invasion and caecal colonization – resulted by the single oral application of the mutant - proved to be similar to that induced by the parent strain and was demonstrable during the first 4 weeks of life.

In conclusion, the aims of these studies have been achieved: mutants of *S*. Enteritidis have been constructed with a negative marker and with reduced organ invasion, but with retained intestinal colonization. These mutants provided an early protection of day old chicks against the wild *S*. Enteritidis challenge strain which was similar to that provided by the parent strain. The newly worked out transposon based methods (insertional paralysis of the flagellin system and partial or complete plasmid deletion) have been reported for the first time as part of these PhD Thesis.

Theses of the PhD work

The studies reported here, aimed to work out and test different new transposon mutagenesis systems for markering and for reduction of virulence of *Salmonella* Enteritidis strains with the long term goal of potential vaccine development. During these studies the following results with international relevance have been achieved.

1. A PCR mapping system has been developed and successfully applied to map essential genes of flagellin production-, and phase variation systems of *S*. Enteritidis and of some other biphasic and monophasic serovars. The use this PCR system confirmed that the reason for monophasic character of *S*. Enteritidis is that this serovar is lacking the *fljB* gene responsible for the H2 phase, and possess *fliC* gene and its operator sequence resulting H1 production.

2. In order to provide a molecular (negative flagellar) marker for our potential vaccine strains, a directed transposon mutagenesis system has been developed. The essence of the system was a fusion of the *fljA* flagellin repressor gene to the IS*30* transposase gene with the follow up testing of the fusion protein (IS30-FljA) in the *S*. Enteritidis 11 strain leading to several non-flagellated (negatively markered) mutants.

3. The highly stable serovar specific virulence plasmid of the nonflagellar mutant of *S*. Enteritidis 11, has been removed by using a two component (IS*10*-IS*30*) transposon system designed for that purpose. Using this system a 50 % efficiency of the complete virulence plasmid removal has been attained in the resulting mutants.

4. The mutants produced above (designated as “nonmotile” and “plasmidless-nonmotile”) have been tested for virulence under *in vitr*o (cell culture) and *in vivo* (day old chick oral infection) models. Both mutants proved to have essentially reduced virulence under both conditions: reduced cell invasion (*in vitro*), and reduced organ invasion (*in vivo*). However, there was no reduction in caecal colonization by these mutants, as compared to the parental *S*. Enteritidis 11 strain.

5. Testing one of the “plasmidless-nonmotile” mutants (SE Δ155) by oral application for its early protective activity against the highly virulent *S*. Enteritidis 147 challenge strain, resulted an effective early protection which was similar to that provided by the parent strain and was demonstrable during the first 4 weeks of life.

Publications

Full length research papers

1. Nógrády N., **Imre A.**, Rychlik I., Barrow P.A., Nagy B. (2003): Growth and colonization suppression of *Salmonella enterica* serovar Hadar *in vitro* and *in vivo*. FEMS Microbiology Letters 218:127-133.

2. Nógrády N., **Imre A.**, Rychlik I., Barrow P.A., Nagy B. (2003): Genes responsible for anaerobic fumarate and arginine metabolism are involved in growth suppression of *Salmonella enterica* serovar Typhimurium *in vitro*, without influencing colonisation inhibition in the chicken *in vivo*. Veterinary Microbiology 97:191-199.

3. **Imre A**., Olasz F., Nagy B. (2005): Development of a PCR system for characterisation of *Salmonella* flagellin genes. Acta Veterinaria Hungarica 53:163-172

4. **Imre A**., Olasz F., Kiss J., Nagy B. (2006): A novel transposon-based method for elimination of large bacterial plasmids. Plasmid 55: 235-241.

Conference Proceedings and Abstracts

1. Nógrády N., **Imre A**., Rychlik I., Barrow P. A., Nagy B. (2002): Lack of serovar specificity in quorum-sensing growth inhibition by *Salmonella* Hadar. Colin, P., Clément G., I3S International Symposium on Salmonella and Salmonellosis Proceedings, p221-222.

2. Nógrády N., **Imre A**., Nagy B. (2000) Growth inhibition studies on *Salmonella* Typhimurium under strict anaerobic conditions. 1st Joint Meeting of Slovenian Society for Microbiology and of Hungarian Society for Microbiology., Keszthely, B-13.

3. Nógrády N., **Imre A**., Nagy B. (2001) Interbacterial inhibitor signals in *Salmonella* populations (In hungarian: Interbakteriális gátló szignálok *Salmonella* populációkban)

Hungarian Society on Zoonozes, Szent-Iványi Binder Days, Tihany, Book of abstracts p141-143.,

4. **Imre A**., Nógrády N., Rychlik I., Barrow P. A., Nagy B. (2002): Characterization of *Salmonella* Hadar 18 transposon mutants regarding on their growth inhibition and virulence properties (In hungarian: *Salmonella* Hadar 18 transzpozon-mutánsok jellemzése növekedésgátlási és virulencia tulajdonságok tekintetében) Congress of the Hungarian Society for Microbiology, Balatonfüred Book of abstracts, Presentation B-17.

5. **Imre A**., Olasz F., Nagy B. (2003): Comparative studies on genes involved in flagella production in different *Salmonella* serovars. Abstract Book FEMS Congress of European Microbiologists, Ljubjana, Slovenia, p455 (p13-8).

6. **Imre A**., Olasz F., Nagy B. (2003): Terms of genetic markering on *Salmonella* vaccine candidate strains (In hungarian: A genetikai markerezés feltételei *Salmonella* vakcina jelölt törzseken) Hungarian Society on Zoonozes, Szent-Iványi Binder Days, Eger Book of abstracts p74-77.

7. **Imre A**., Olasz F., Nagy B. (2003): PCR-mapping of selected *Salmonella* genotypes and serotypes for their flagellar systems. Abstracts of 14th Intern.Congr., Hung. Soc. Microbiol., Balatonfüred, B37.

8. **Imre A**., Olasz F., Nagy B. (2004): Directed mutagenesis of *Salmonella* Enteritidis strains and geno- and phenotypical examination of the selected mutants (In Hungarian: *Salmonella* Enteritidis törzsek célzott transzpozon –mutagenezise s a mutánsok geno- és fenotípusának vizsgálata) Congress of the Hungarian Society for Microbiology, Keszthely Book of abstracts p. 50.

9. **Imre A**., Olasz F., Nagy B. (2004): Production of *Salmonella* Enteritidis flagellin mutants using directed mutagenesis (In hungarian: *Salmonella* Enteritidis flagellin mutánsok előállítása irányított mutagenezissel) Hungarian Society for Biochemistry 9th Congress of Molecular Biology Section, Sopron, Book of abstracts, GP2, p. 99.

10. **Imre A**., Olasz F., Nagy B. (2006): Use of IS30 elements in gene targeting system for *Salmonella*. 58. Tagung der Deutschen Gesellschaft für hygiene und Mikrobiologie, Würzburg, International Journal of Medical Microbiology 296S3 Supplement 42 September 2006 p.87.

Acknowledgements

My first thanks are due to my subject leader, **Béla Nagy** for making this work possible in the research group of "Enteric Bacteriology and Foodborne Zoonoses", and for helping me with his experiences and knowledge. I am also greatly indebted to thanks to **Ferenc Olasz** (Biotechnology Center Gödöllő) for introducing me into the world of molecular genetics and for his great support by his knowledge and ideas.

I also thank to all coworkers of our research group: **Márta Puruczki, István Tóth, Péter Zsolt Fekete** and **Anna Malik** for helping me in this PhD work in many different ways. My special thanks are due to **Ama Szmolka** and to **Klara Rasky** for their essential help in the in vitro invasion/gene expression studies, as well as in the application of the ELISA system for measuring anti flagellar antibodies in chicks.

I further thank to **Móni Szabó, János Kiss, Zita Nagy** and to the whole Transposon Group of the Agricultural Biotechnology Center, Gödöllő).

I am also thankful to **Noémi Nógrády** for her initial support and advises.

Finally I am grateful to the Director and to the coworkers of the Veterinary Medical Research Institute of the Hungarian Academy of Sciences for their help and for their work.

This work was supported by the NKFP 4/040/2001 (with a subcontract to CEVA Phylaxia), and by the EU FP6 2002-FOOD-1 Proposal No. 505523 (SUPASALVAC) projectcs.