2. Molecular genetics and genomics

### 2.01 Characterization of NikR, a nickel-responsive pleiotropic autoregulator of Helicobacter pylori

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**Helicobacter pylori** genome contains a gene (HP1338) that encodes a nickel-dependent regulator homologous to the Escherichia coli nickel-responsive regulator NikR. Due to limited identity with E. coli NikR, **H. pylori** nikR was only able to partially complement a nikR deficient E. coli strain, and displayed a pleiotropic metal-dependent regulatory function. A non-polar isogenic mutant deleted for the nikR gene was constructed, and native and maltose binding protein-fused recombinant NikR proteins were purified. The NikR protein was shown to be essential for **H. pylori** resistance to high nickel (>300 µM) and cobalt (>2µM) ion concentrations in vitro, but was not essential for the colonization of mouse gastric mucosa. Using a differential gene expression analysis, we screened a DNA macroarray for genes that were differentially expressed in parental and nikR deficient **H. pylori** strains grown under either normal or nickel overload (250 µM) conditions. We showed that **H. pylori** NikR can mediate directly or indirectly the regulation of a series of nickel-activated or -repressed genes. The transcriptome data were further confirmed for 10 of these genes in slot blot DNA/RNA hybridization experiments from RNA prepared from three independent bacterial cultures. Based on gel shift and β-galactosidase assays with the intergenic region between nikR and the exB/exD divergent operon, we provide evidence that NikR is an autoregulator which binds to the intergenic region, and we suggest that it might also control the exB/exD/tonB operon that provides energy for ferric iron uptake systems. Thus, as previously suggested for Fur in **H. pylori**, NikR appears to act as a global regulator for the metabolism of the divalent cations within a highly complex regulated network.

### 2.02 Complete interaction map of Helicobacter pylori Cag pathogenicity island


The Cag pathogenicity island (PAI) of Helicobacter pylori is present in approximately 50% of strains and is associated with increased severity of disease. Comparative analysis of the genes encoded on the PAI has identified a potential type IV secretion system (TFSS). Several reports have shown that the TFSS is essential for translocation of CagA into the host cell. The yeast-two hybrid system has been used effectively to identify protein-protein interactions which are essential for the assembly and function of TFSSs. The aim of this study was to identify pair protein interactions between the different Cag-encoding polypeptides.

Each gene within the cag PAI, with the exception of cag 527, was cloned into both the prey and bait vectors for the yeast two-hybrid system. Each of the prey and bait combination was then introduced into the selective yeast strain P169 which harbours nutritional deficiencies which facilitates maintenance of the plasmids and identification of protein interactions. Semi-quantitative analysis of the intensity of interaction was identified using the non-selective β-galactosidase assay.

This study has confirmed known homoprotein interactions, which was discovered on the basis of a structural analysis, between cag 523, thus validating the technique. Several novel interactions have been identified including the hetero-dimeric cag 523/cag 530 and homo-dimeric cag 526/cag 526 interactions. Cag 523, 530 and 526 proteins have no known sequence homology to proteins in the database and to date little is known about their role in the context of the TFSS. The identification of protein-protein interactions within the Cag PAI may elucidate novel structural and effector proteins of the TFSS of Helicobacter pylori.

### 2.04 A revised annotation of the Helicobacter pylori genomes


With the genomic era, huge amounts of information are generated. Therefore, biologists are increasingly demanding structured, exhaustive and comparative databases. The PyloriGene database (http://genolist.pasteur.fr/PyloriGene) was developed to answer to such challenges by integrating and connecting the information generated by the sequencing of two distinct strains of Helicobacter pylori, a human gastric pathogen. This pointed to the need for a general annotation consensus of both strains as their physical and functional annotation differed significantly in some cases. A revised functional classification was created in order to accommodate the existing data on both sequenced genomes and the possibility of classifying coding sequences (CDS) into several functional categories were used to harmonize CDS classification. Revision of the annotation of the two complete genomes in light of new data allowed us to reduce the percentage of hypothetical proteins from around 40% to 31%. This also resulted in a reassignment of functions or new functional assignments for a total of 108 CDS, representing around 7% of the total CDS. Interestingly, only around 5% of the total CDS with an assigned function were based on work done on **H. pylori**. Finally, comparison of the two genomes revealed a significant amount of CDS size variation among corresponding (orthologues) CDS. Although a majority of these size variations were due to natural polymorphism, interestingly, as much as 113 CDS pairs differed in size due to different start codon assignment, a recurrent problem of genomes physical annotations.

### 2.05 Identification of a locus associated with cell integrity in Helicobacter pylori by transposon shuttle random mutagenesis

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To identify genes involved in adhesion, we generated 1500 insertion mutants of a Helicobacter pylori strain by transposon shuttle mutagenesis. All mutant strains were screened for their adhesion ability with cultured Helicobacter pylori mutants of a wild-type strain by transposon shuttle mutagenesis. Six mutants exhibited 10-fold decrease of recovered adhered bacteria number than **H. pylori** wild-type strain after co-incubated with SC-M1 cell for 30 minutes. DNA sequencing revealed that the interrupted loci of these six mutants are the same. The sequence of this gene does not have homologues with sequenced **H. pylori** 26695 and J99 strains. This novel open reading frame contains 1491 base pairs. Light microscopic observations revealed that these mutant cells were self-aggregated frequently. This mutant also revealed cell elongation under electron microscopic examination. Complementation of this locus turned the morphology into the same as wild type. Therefore, this novel ORF may be involved in **H. pylori** structure integrity.

### 2.06 Geographic genomics of Helicobacter pylori: Clues to population-specific markers from analysis of single nucleotide polymorphisms in ahpC, atpA and ure sequences of isolates from African and Asian countries


Objectives: Application of multilocus sequence typing shows that recombination is so frequent within **H. pylori** that each isolate appears to possess unique sequences for each housekeeping gene analysed. Even so, there is evidence that geographic separation may have resulted in distinct populations on different continents. Our aim was to explore the existence of remnant pockets of conservation by analysis of sequences of three loci in antral isolates from dyspeptic patients in geographically separated populations.

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**Image**: DNA macroarray for genes that were differentially expressed. Gene expression analysis. DNA/RNA hybridization experiments. **Figure**: Complete interaction map of Helicobacter pylori Cag pathogenicity island. **Diagram**: A revised annotation of the Helicobacter pylori genomes. **Map**: Identification of a locus associated with cell integrity in Helicobacter pylori by transposon shuttle random mutagenesis. **Graph**: Geographic genomics of Helicobacter pylori: Clues to population-specific markers from analysis of single nucleotide polymorphisms.
Methods: Nucleotide sequences of *albC*, *urel* and *atpA* internal fragments of about 500 bp were determined or obtained from public databases for isolates from Nigerians (8) Japanese (4), and Chinese (4). The two sets of sequences were compared and contrasted with those in our database for 124 additional strains from England and 11 other countries. Phylogenetic analyses were performed and trees constructed with BioEdit and TREECON, and analysis of single/multiple nucleotide polymorphisms (SNPs) was performed with DNAsp.

Results: Analysis of *urel* sequences showed a high degree of intra-set relatedness for isolates from the different countries although each set was well separated in the general phylogenetic tree. Similar associations were evident from analysis of *albC* data whereas linkages were more diverse on *atpA* data. Interestingly the Nigerian and Japanese/Chinese sets each had distinctive but unique amino acid sequences derived from *albC*. This observation prompted analysis of individual polymorphisms which showed two population specific polymorphisms at *a*t positions 125 and 139. Twelve other SNPs that distinguished the two sets were present in isolates from the UK and other countries.

Discussion: We found evidence of population-specific polymorphisms in *albC* in isolates from remote countries. Although the frequency of these markers needs to be determined in a larger number of isolates, the findings are suggestive that conserved remnants exist despite genomic disruption due to frequent recombination and mutation.

2.07 Single basepair substitutions in the 16S rRNA genes mediate tetracycline resistance of *Helicobacter pylori*

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Introduction: Most *Helicobacter pylori* strains are susceptible to tetracycline, a commonly used antibiotic for the eradication of *H. pylori*. In the past two years the incidence of tetracycline resistance in *H. pylori* has significantly increased. Tetracycline resistance in *H. pylori* is mediated by a triple base pair substitution (AGA926-928→TTC) in both 16S rRNA genes.

Aim: To determine whether all three base pair substitutions are required for tetracycline resistance in *H. pylori*, or whether single base pair substitutions would suffice.

Methods: All base pair substitutions or combinations thereof found at base pair position 926-928 of the tetracycline-resistant (TetR) strains were transferred to the tetracycline-sensitive (TetS) *H. pylori* strain 28695. The effect of these 16S rRNA base pair substitutions on level of tetracycline resistance, growth rate, and stability of tetracycline resistance were determined.

Results: Substitution of only one or two base pairs was already sufficient to confer tetracycline resistance. However, these mutations displayed lower MIC values and reduced growth rate. The original triple base pair mutant had the highest MIC value and growth rate. All mutations were stable in the presence or absence of tetracycline.

Conclusions: In *H. pylori*, the substitution of one or two base pairs at position 926-928 in the 16S rRNA gene is sufficient to confer tetracycline resistance. The higher growth rates and MIC of tetracycline may explain the preference for this triple base pair substitution. This preferential TTC mutation opens possibilities for the development of molecular tests for tetracycline resistance in *H. pylori*.

2.08 Localization of *Helicobacter pylori* Heat Shock Protein 20

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*Helicobacter pylori* Heat Shock Protein 20 (HSP20) was identified through ORF prediction in *H. pylori* genome sequences. It is a homologue of HslV protein in *E. coli*, which was found to be a peptidase that participates in the degradation of a cell division inhibitor, SulA. In order to characterize HSP20, the gene (hp20) was cloned and inserted into expression vector pET11b fused with His-tag. The recombinant protein (HSP20) was expressed by IPTG induction in *E. coli* BL21 and purified by affinity chromatography. Antibody was raised against HSP20 in rabbits. Protein A sepharose purified anti-HSP20 antibody was applied as a probe to determine the localization of HSP20 in *H. pylori* cells by immuno-gold labeling. Simultaneously, HSP20 was also used as HSP20 antigen to test against sera obtained from patients with different gastroduodenal diseases. The results from Western Blotting and immuno-gold labeled transmission electron microscopy show that HSP20 is localized on cell surface of *H. pylori* and mainly expressed in spiral-shaped *H. pylori*. ELISA shows both *H. pylori* infected and non-infected sera were seropositive to HSP20.

The cross-reaction could be due to earlier exposure of patients to HP20 homologues from other bacteria, e.g. HspD of *E. coli*. However, the mechanism has yet to be determined. Could it be that HSP20 emulates HSP60 as a molecular mimicry agent? The surface localization of HSP20 on Helicobacter pylori appears to lead the way to understanding *Helicobacter pylori*- host interaction.

2.09 Association of interleukin-1 RNA polymorphism and gastric cancer in Taiwan

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Aim: To evaluate the genetic associations of IL-1B and IL-1RN polymorphisms with gastric cancer in Taiwan.

Methods: Genomic DNA from 98 unrelated Taiwanese patients with gastric adenocarcinoma and 100 ethically matched healthy controls was typed for polymorphisms at positions -31, -511, and +3954 in the IL-1B gene, and the variable number of tandem repeats polymorphisms in intron 2 of the IL-1RN gene.

Results: The allele frequencies of IL-1RN+2 in gastric cancer cases were significantly higher than those in healthy controls (9% vs. 4%, p = 0.024). The allele frequencies of IL-1B -31, -511 and IL-1B +3954 did not differ. A significant association was found between IL-1RN+2 carriers and the risk of developing intestinal type gastric carcinoma, with an odds ratio (OR) of 2.8 (95% CI: 1.0-7.6, p = 0.014). A trend towards an increased risk for the development of diffuse type gastric carcinoma in IL-1 RN+2 carriers was also observed (OR= 2.4; 95% CI: 0.8-7.1; p = 0.130). No significant relationship was noted in patients with atypical carcinoma. Additionally, a synergistic association between blood type A and IL-1 RN+2 carriers existed (OR= 4.8; 95% CI: 1.0-23.5).

Conclusions: Genetic polymorphism of IL-1RN+2 and blood type A is associated with the development of gastric cancer, especially intestinal type gastric cancer in Taiwan.

2.10 Potential involvement of alkylhydroperoxide reductase (AhpC) in metronidazole activity in UK isolates of *Helicobacter pylori* - development of resistance is unlikely to be due to mutational alteration of the *ahpC* gene

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Objectives: Metronidazole (Mtz) resistance in *H. pylori* reportedly occurs by mutational inactivation of various oxidase and reductase genes, including *rdxA*. Recently oxidase and nitroreductase activity that may be partially attributable to alkylhydroperoxide reductase (AhpC) was reported to be higher in Mtz resistant (R) than Mtz sensitive (S) strains. In contrast, proteomic-based evidence suggested that, when grown on Mtz-containing media, compensatory upregulation of *ahpC* gene expression occurs in Mtz-R strains with mutated *rdxA* genes. This study aimed to examine *ahpC* sequences in Mtz-S and Mtz-R strains of known *rdxA* sequence, to identify any genotypic polymorphisms that could lead to functional inactivation, or to enhanced expression, of the gene and account for Mtz resistotype.

Methods: 30 strains comprising paired isolates from 11 dyspeptic patients that were either Mtz-S (3) or mixed Mtz-S/R (8) before therapy and Mtz-R post-therapy were examined. *ahpC* genes were sequenced for comparison with corresponding *rdxA* sequences.

Results: *ahpC* sequences varied by up to 5% between patient sets, but matched strain sequences were identical, regardless of Mtz phenotype. Various mutations were identified in *rdxA* of Mtz-R strains (post-treatment) that were absent in matched Mtz-S strains (pre-treatment). However, *rdxA* sequences from pre-treatment Mtz-R and Mtz-S subpopulations were identical in 6/8 cases.

Discussion: Although AhpC may be involved in Mtz metabolism and resistotype development, as proposed, no mutational differences were observed in the strain sets of differing Mtz phenotypes in this study. Regulation of *ahpC* expression by mutational modification is therefore unlikely, and *rdxA* mutations were not linked to *ahpC* sequence alterations. Mutations in *rdxA* were observed in strain sets but may not always be essential for Mtz resistance acquisition. Further examination of *ahpC* expression at the transcription and translational level may elucidate its role in Mtz action and resistance in *H. pylori*, either alone or combined with mutated *rdxA*. 
Simultaneous colonization with multiple quacispecies and multiple strains of Helicobacter pylori in different regions of the human stomach

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The aim of this study was to document simultaneous colonization by quacispecies versus multiple strains in a single host. Eight H. pylori infected adult patients were studied and biopsies from antrum, corpus, fundus and incisura were taken, and inoculated in selective media. From the primary growth of each biopsy site, six to seven single colonies were isolated and propagated for DNA isolation. For macrodiversity studies, RAPD fingerprinting was done to DNA from all H. pylori isolates. For microdiversity studies, PCR-typing for vacA alleles, and for detection of cagA, picB, and cag PAI were done to DNA samples. We studied a mean of 28 colonies per patient. H. pylori colonies isolated from the four stomach regions of seven patients presented the same RAPD pattern, suggesting colonization with a single strain. In contrast, colonies from the eighth patient had three different RAPD patterns suggesting infection with multiple strains. Among the seven patients with a single RAPD pattern, in three cases all isolates were cagA and picB positives. However more than one vacA allele was identified among isolates from the four stomach regions. In three other cases with the same RAPD pattern, all isolates had the same vacA allele, s2m2. However, isolates from each patient differed in the presence of cagA, picB and cag PAI. In the eighth patient colonized with three different RAPD patterns; isolates within each RAPD pattern presented different vacA alleles and differed in the presence of cagA, picB and cag PAI. Thus, six patients where colonized by a single strain which has evolved into multiple quacispecies. One case was colonized by multiple strains and within each strain; the presence of multiple quacispecies was documented. This study demonstrates that a single host may be colonized simultaneously by multiple strains and multiple quacispecies.

HP1361 is essential for DNA transformation in Helicobacter pylori

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Using bioinformatic database search we found HP ORF1361 was homologous to Bacillus subtilis transformation related gene. We prepared a knockout mutant strain for this gene. The knock-out mutant of HP ORF1361 was obtained by inserting a kanamycin cassette into this gene using transposon shuttle mutagenesis. Transformation experiments were performed with 2 donor DNAs (a chloramphenicol acetyltransferase (CAT) cassette inserted into the gene H. pylori; HP ORF0691) and clarithromycin resistant 23S rRNA genes (A to G mutation at 2143). Both natural transformation and electroporation revealed that transformation frequency were totally abolished (transformation frequency was less than 10⁻⁷) in the HP ORF1361 knock-out mutant. Transformation frequency decreased to 3 x 10⁻⁵ in wild type transformed with a shuttle vector pHel2 (Heuermann, D. and Hass, R., Mol. Gen. Genet. 257: 519-528, 1998). Complememntation with shuttle vector pHel2 carrying HP ORF1361 restored electroporation efficiency to 4.21 x 10⁻⁷ and natural competence ability to 3.62 x 10⁻⁷. The results suggest that HP ORF1361 is essential for natural competence and electroporation of H. pylori.

Frameshift mutations in frxA: A frequent occurrence that may be unrelated to metronidazole resistance in UK isolates of Helicobacter pylori

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Objectives: Previous transformation studies have suggested that in addition to the NADPH nitroreductase (encoded by rdxA), NADPH flavin oxidoreductase (encoded by frxA) may be involved in the metronidazole (Mtz) mechanism of action and in the development of Mtz resistance in H. pylori. Mutations in frxA genes, including those that cause frameshift, reportedly contribute to the Mtz-R phenotype. We examined the contribution of frxA alteration to Mtz resistance in a strain set where mutations in rdxA had not provided an explanation for the Mtz-R phenotype in all cases.

Methods: frxA was examined in paired Mtz sensitive (S) and resistant (R) isolates, recovered from 11 dyspeptic patients before and after therapy, eight of which had mixed Mtz/S-R strain populations pre-therapy. Following PCR amplification, frxA sequence was determined from each of the 30 isolates. These were translated and amino acid sequences aligned with 23 frxA sequences currently deposited in Genbank.

Results: Multiple alignment of frxA genes demonstrated that at least one strain from 10/11 patient sets had a frameshift mutation and 6/10 occurred at position 18. In 4/11 patient sets, frameshifts were observed in the Mtz-R strains only, whereas in 6/11 patients, frameshifts were observed in all strains in each patient set, including the Mtz-S strains.

Discussion: Frameshift mutations in frxA have been reported in previous studies, but only in Mtz-R strains. Our study demonstrates that such mutations also occur in Mtz-S strains and are thus unlikely to contribute to the Mtz-R phenotype. Altered FrxA amino acid sequence occurred relatively early at position 18 in many of the strains examined would functionally inactivate the protein, suggesting that this is a non-essential enzyme. Further investigation of frxA and other candidate genes in a larger study population will be essential to improve understanding of their role in Mtz resistance.

Clinical role of CYP2C19 in the management of failed Helicobacter pylori (Hp) infected patients

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Background: Hp infected patients not responding to conventional thera- pie H. pylori, were compared to CYP2C19 polymorphism, a higher amount of PPI is metabolized being not more available for acting on gastric H⁺/K⁺ ATPase.

Aim: to assess the eradication rate in non responders EM CYP2C19 Hp+ve patients (who previously received at least two treatments) using a triple therapy with high dose of PPI.

Methods: 42 non-responders Hp+ve patients (17 male/25 female; mean age 53.2 yrs, SD 13.3), who were EM CYP2C19 previously determined by RFLP), underwent endoscopy with histology, RUT and culture. 28 isolates were also available for susceptibility testing by E-test. Isolates were considered Clarithromycin (C1a) resistant if the MIC was ≥1mg/ml. All patients received daily Omeprazole 120 mg, Cla 1.5 gr and Amoxicillin 1 gr. for 7 days, 4-6 weeks after stopping treatment, all patients underwent 15C-UBT.

Results: 14 out of 42 patients were eradicated (Eradication rate 33.3% [95% C.I.: 21% to 48.4%]). No difference in the eradication rate was found between male and female (54.5% and 32.2% respectively, p >0.5). 27 out of 48 isolates were C1a-resistant. The eradication rate in this group was 8/27 (29.6% [95% C.I.: 15.9% to 48.5%]).

Conclusions: Our data showed an interesting eradication rate in patients EM CYP2C19 previously not responding to other treatments, even in presence of C1a-resistant strains. Therefore, for these patients may be clinically useful to evaluate the CYP2C19 status in order to administer a high PPI dosage’s triple therapy.

Association of genotypes of Helicobacter pylori with gastrin and pepsinogen in duodenal ulcer patients

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Purpose: Helicobacter pylori is the major cause of duodenal ulcers and H. pylori is responsible for the abnormalities in the regulatory control of gastric function, such as hypergastrinemia and hyperpepsinogenemia. To investigate H. pylori isolates from DU patients whether the genotype of the infecting isolates could be correlated with hypergastrinemia or hyperpepsinogenemia, we performed the following study.

Methods: Fifty-seven DU patients with H. pylori infection were enrolled in this study. Genomic DNA was amplified by PCR for different genes, including vacA (s1a,s1b, s2, m1, m1T, m2), cagA (A3, C, F, N, T), ORF13, IS605, iceA (A1, A2), and picB.

2.12 HP1361 is essential for DNA transformation in Helicobacter pylori
Results: The dominant genotype in isolates from our DU patients was cagA+/s1a+/m2+/iceA+/picB+. Gastrin or pepsinogen levels do not correlate with the presence of these genes. No specific genotypes of *H. pylori* was found to play a role in gastrin or pepsinogen release.

Conclusion: No significant differences in the presence of the candidate virulence genes vacA, cagA, iceA, or picB were detected in isolates from DU patients with hypergastrinemia or hyperpepsinogenemia.

2.16 The crystal structures of *Helicobacter pylori* cysteine rich proteins B and C reveal a novel fold for penicillin binding proteins

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Aim: The genomes of *Helicobacter pylori* strains J99 and 26695 have been completely sequenced, but functional and three-dimensional structural information is available for less than one third of all open reading frames. We investigated the functions and three-dimensional structures of two members from a family of cysteine-rich hypothetical proteins (Hcp), which are known to be unique to *Helicobacter pylori* and *Campylobacter jejuni*.

Methods: The plasmids harboring the genes encoding for the Hcps were obtained by the American Tissue and Culture Collection. The genes were amplified by polymerase chain reaction, inserted into pTFT74 expression vectors and transformed into Escherichia coli BL21(DE3) cells.

The expressed Hcps were purified and refolded by immobilizing the solubilized inclusion bodies on a nickel nitritotriacetic acid-agarose (QIAGEN) column. The hydrolysis of antibiotics by Hcps was monitored by following the absorption variation resulting from the opening of the β-lactam ring.

Crystals of the two proteins HcpB and HcpC were grown in a PEG solution using the vapour diffusion method. The HcpB structure was solved by a classical MAD phasing experiment and was used to solve the structure of HcpC by molecular replacement.

Results and Discussion: The structures of the *Helicobacter* cysteine rich proteins (Hcp) B and C possess modular architectures consisting of α/α motifs that are crosslinked by disulfide bridges. The Hcp-repeat is similar to the tetracopeptide repeat (TPR), which is a frequently found domain involved in protein-protein interactions. In contrast to the TPR, the Hcp-repeat is 36 amino acids long.

Up to now, biochemical experiments showed that four out of six Hcp are capable of binding and hydrolyzing penicillin and cephalosporanic acid derivatives. Since the Hcp fold is distinct from the fold of other known penicillin binding proteins, the Hcp proteins belong to a new family of penicillin binding proteins.