This report should be submitted within 2 weeks after you return to Japan.

（Abroad・Domestic）Internship report form（Student） 2019/05/25

(Year/Month/Day)

Name	Kentaro Koide
Laboratory	Div. Bioresources, CZC
Year (Grade)	D4
Internship institution	University College Dublin, Ireland
Internship period	Internship period: 01/22/2019 - 05/19/2019 (Departure Date from Sapporo: 01/20/2019, Arrival Date in Sapporo: 05/22/2019)
Purpose	1. To identify a relationship between quinolone resistance and pathogenicity in *Salmonella*. The influence of DNA gyrase containing amino acid substitution causing quinolone resistance is going to be evaluated on the pathogenicity of *Salmonella* Typhimurium. 2. To gain experience of conducting research at an overseas university. Knowing the research environment abroad, and learning how researchers are approaching, I will use it for postgraduate career development and research. 3. To build a good collaborative relationship for future research. I will deepen my understanding by discussing with researchers in different fields and strengthen a relationship necessary for corroborative research in the future.

- The reason why you chose this institute

I have studied quinolone resistant nontyphoidal *Salmonella* by using protein engineering and bioinformatics techniques. I have examined the impact of amino acid substitution causing quinolone resistance on DNA gyrase, making me a strong candidate to participate in Dr. Tadhg Ó'Cróinín's research with relaxation of DNA supercoiling leading an increased pathogenesis. I am interested in how the amino acid substitutions on DNA gyrase exert influence on *Salmonella* Typhimurium pathogenesis. In addition, from January to March 2018, I had studied the supercoiling activity of DNA gyrase of *Campylobacter jejuni* with

Matthew V. X. Whelan, a PhD student in Dr. Tadhg Ó'Cróinín's lab. Through conversation with him, I saw the difference between not only our methods but also our focus, despite having studied the same enzyme. As he studied in our laboratory, I would like to gain a similar experience to his in his research laboratory. I wished to broad my understanding of his research and pave the way for future research collaboration through this research activity.

- Result of the activity （about 800 words、provide photos, tables and figures that clearly show the activities during the period）

Selection for quinolone resistant mutants

S. enterica serovar Typhimurium strains used in this study include the wild-type (WT) strain SL1344 and 4/74. Both strains are grown in Mueller Hinton (MH) agar at 37°C with aeration. For liquid cultures, *S.* Typhimurium strains were equalized to specific optical densities in MH broth and incubated under aerobic conditions at 37°C, shaking at 200 rpm.

SL1344 or 4/74 were cultured in MH broth with increasing concentrations of nalidixic acid (NAL) or ciprofloxacin (CIP). Of the three mutants selected for nalidixic acid, the mutant isolated from the lowest concentration of nalidixic acid was N1, the mutant that isolated from the middle concentration was N2, and the mutant that isolated from the highest concentration was N3. Three mutants selected in ciprofloxacin were assigned as C1, C2 and C3 in same way with nalidixic acid. Those were maintained using MH broth supplemented with 20% glycerol and stored at -80°C until used for the subsequent experiments.

Table 1. MIC and amino acid substitution of mutants used in this study

Strain	MIC (μg/ml)		Amino Acid Substitution	
	NAL	CIP	GyrA	ParC
S. Typhimurium SL1344	4	0.016	WT	WT
ST1344-N1	128	0.125	Ala119Glu	WT
ST1344-N2	256	0.125	Asp87Tyr	WT
ST1344-N3	>512	0.25	Asp87Tyr	WT
ST1344-C1	>512	4	Asp87Tyr	WT
ST1344-C2	>512	8	Asp87Tyr	WT
ST1344-C3	>512	32	Asp87Tyr	WT

S. Typhimurium 4/74	4	0.016	WT	WT
ST474-N1	256	0.125	Asp87Tyr	WT
ST474-N2	256	0.125	Asp87Tyr	WT
ST474-N3	>512	0.25	Asp87Tyr	WT
ST474-C1	>512	4	Asp87Tyr	WT
ST474-C2	>512	8	Asp87Tyr	WT
ST474-C3	>512	32	Asp87Tyr	WT

Quinolone susceptibility testing

Minimum inhibitory concentrations (MIC) of nalidixic acid and ciprofloxacin were determined by the broth dilution method. WT and mutant strains were tested for antimicrobial susceptibility in accordance with the procedure of the Clinical and Laboratory Standards Institution (CLSI). The result was summarized in Table 1. The guideline of 32 μg/ml nalidixic acid resistance and 4μg/ml ciprofloxacin was determined from the CLSI. Mutants N1, 2, 3, showed nalidixic acid resistance but no ciprofloxacin resistance. However, ciprofloxacin resistant mutants C1, 2, 3, were nalidixic acid resistant as well as ciprofloxacin. C3 of both strains showed the highest-level ciprofloxacin resistance, MIC of ciprofloxacin being 32μg/ml. This MIC was very high and has not been reported in clinical isolates of *Salmonella* Typhimurium.

Identification of amino acid substitution on GyrA and ParC

Base substitution on quinolone resistance region of *gyrA* was found in every mutant but *parC* of every mutant were wildtype. GyrA and ParC are subunits of DNA gyrase and TopoIV, respectively. Those are target enzymes of quinolone and amino acid substitution on GyrA and ParC are frequently reported in quinolone resistant *Salmonella*. The amino acid substitution of GyrA found in this study was only Ser83Tyr except that it was Ala119Glu in the lowest nalidixic acid resistant strain of SL1344. Ser83Tyr is one of the most frequently reported mutations in clinical isolates. On the other hand, ParC of every mutant was wild type. Topo IV is a relatively weaker target enzyme of quinolone than DNA gyrase in *Salmonella* and it is expected that strains with mutations in ParC would cause higher resistant to quinolones.

Measurement of bacterial growth and motility

Bacterial growth was evaluated by measuring the turbidity in bacterial culture. Twenty µl of overnight culture was transferred into 20ml of MH broth and incubated at 37°C shaking at 200rpm. Optical density (OD_{600}) was measured at 0, 3, 6, 9, 24 hours after incubation started. In motility assay, each strain was inoculated into the center of a 25ml motility agar plate (MH broth containing 0.3% agar) using a 200ul sterile pipette tip. Each plate was incubated upright for 9 and 24 hours, after which it was imaged and the halo diameter was measured.

In the result, strains with high MIC of ciprofloxacin showed much lower growth and motility than WT.

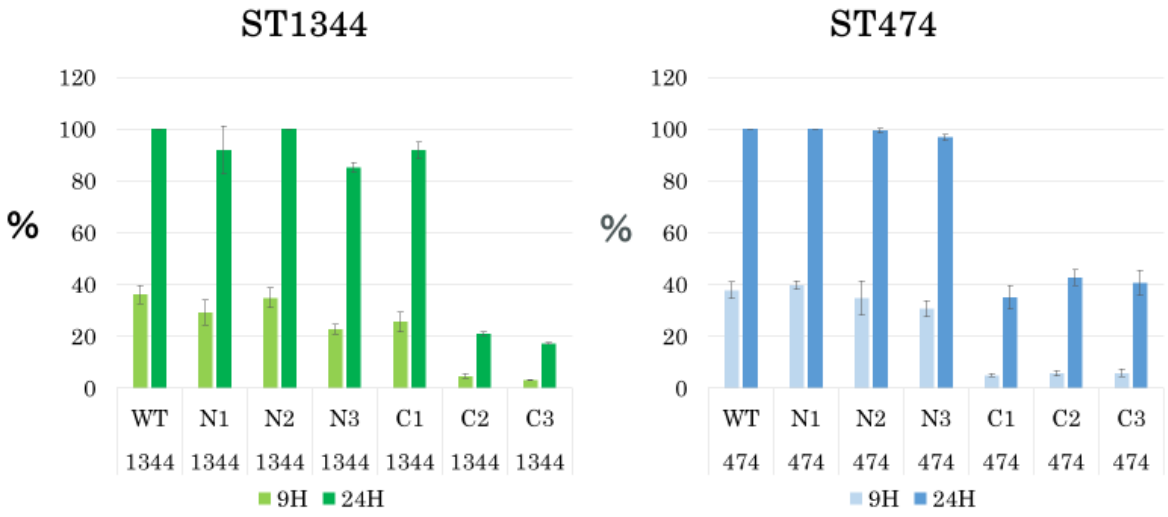

Figure 1. Motility by strains

Visualization of biofilm formation

Overnight cultures were equalized to an OD_{600} of 0.1 in MH broth and 200µl was seeded in triplicate into the wells of a 96 well plate. These were then incubated for 24h at 37°C. The metabolically active bacteria were then stained for 30 mins with PBS containing 40µg/ml 5-TAMRA-SE (5-Carboxytetramethylrhodamine, Succinimidyl Ester, single isomer, ThermoFisher Scientific) viable stain. Widefield imaging was carried out at 50x magnification using a Leica DMI600 epifluorescence system. Strains with high MIC of ciprofloxacin showed much lower ability to form biofilm.

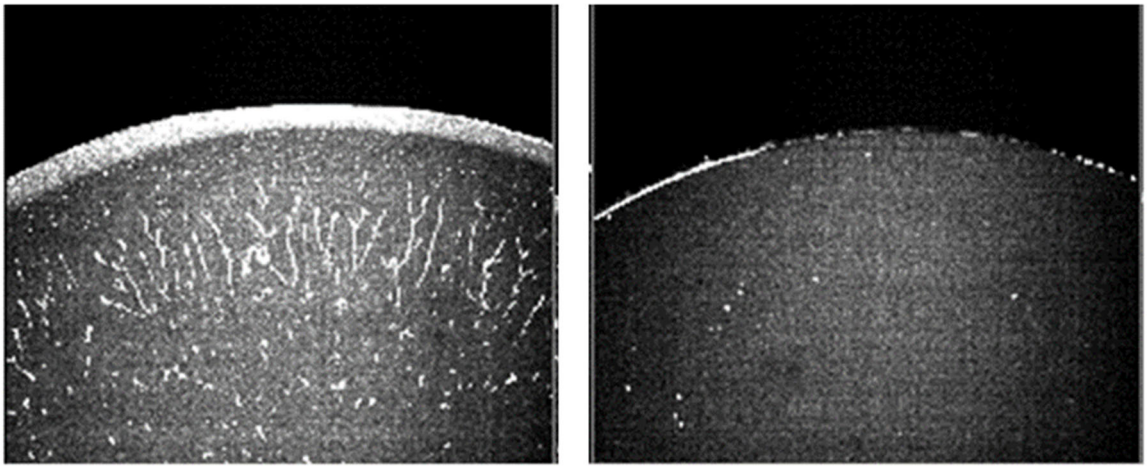

Figure 2. Biofilm images of *S.* Typhimurium 4/74 and ST474-C3

Invasion assay

Epithelial cells, $5x10^4/cm^2$ HT29, were seeded onto 12 well plates. Strains were then equalized to OD_{600} of 0.2 in McCoy's 5A media supplemented with 10% heat inactivated FBS and invaded on to the HT29 monolayer for 1h. After this, cells were washed 3 times with PBS and lysed with 1% Saponin in PBS for 15 min. Serial dilutions of the cell lysates were carried out using MH broth and plated on MH agar. All MH plates were incubated for 24 hours at 37°C followed by counting of colony forming units.

In order to quantify the internalized bacteria after a one-hour invasion, invasion assays were carried out as described previously with the following alterations. The media overlying the infected HT29 cells was changed to complete McCoy's 5A media supplemented with 200 µg/ml gentamycin sulphate and infected HT29 cells were incubated at 37°C for a further 3 hours. After this, cells were lysed with 1% Saponin in PBS and serial dilutions of the cell lysates were carried out.

Colony forming units of total association and internalization are summarized in Figure 3. Strains with high MIC of ciprofloxacin showed lower ability to adhere and invade the epithelial cells.

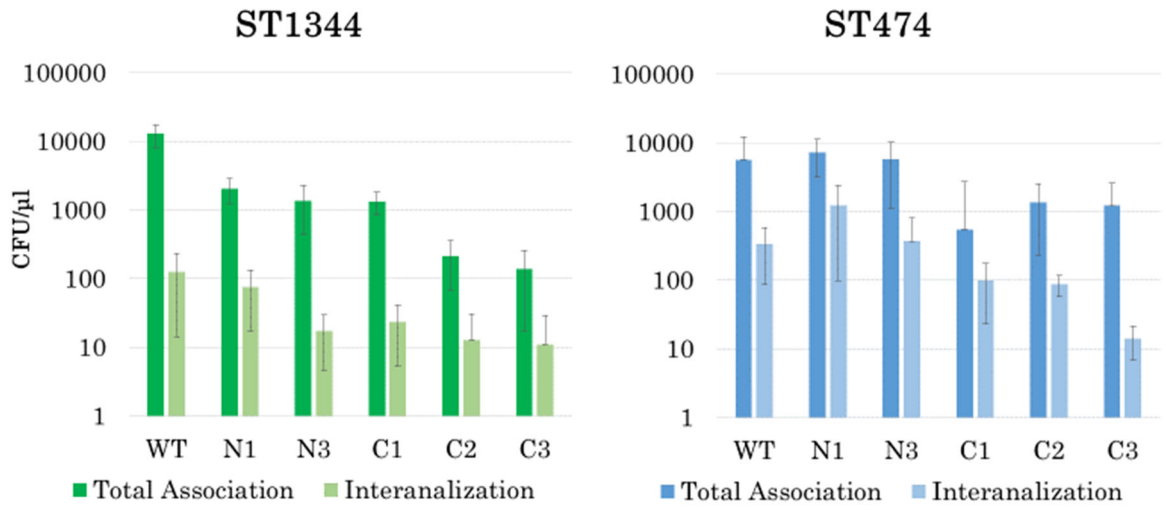

Figure 3. Colony forming unit by strains in invasion assay

- What do you think the positive impact of the activity will have on your further career path?

Discussions with researchers and graduate students in different fields at UCD deepened my understanding of antimicrobial resistant bacteria. Dr. Tadhg Ó'Cróinín's lab is specialized in basic bacteriology. Their suggestion and ideas were really interesting and productive for me. With a deep understanding of each other's research methods and background, it can be expected that future collaborative research can be performed more actively.

This was my first long-term overseas research activity. The research environment at UCD is different from CZC, and my ability to adapt to the environment has been trained well. Discussion and presentation with native English speakers were tough. However, I was confident that my understanding and consideration for my research was sufficient to carry out research activity abroad.

- Advice for your junior fellows

When choosing an institute for internship, you should consider what is important in your research and career path, rather than prioritizing famous research institutes and universities.

Approval of supervisor	Institution・Official title・Name

※1 Send the electronic file to the Leading School section, International Affairs Office
※2 Attach a copy certificate of the content of internship activity that is prepared by the counterpart at the internship institution (any form with a signature of the counterpart).
※3 The Steering Committee of the Leading Program will first confirm the content of this report and report will be forwarded to the Educational Affairs Committee for credits evaluation.

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