A system for monitoring mutation-in-progress : a study of adaptive mutation to Ciprofloxacin resistance in Staphylococcus aureus

Sutandar, Andiyanto

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**NANYANG
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UNIVERSITY**

**A SYSTEM FOR MONITORING MUTATION-IN-
PROGRESS: A STUDY OF ADAPTIVE MUTATION
TO CIPROFLOXACIN RESISTANCE IN
*STAPHYLOCOCCUS AUREUS***

ANDIYANTO SUTANDAR

SCHOOL OF MECHANICAL & AEROSPACE ENGINEERING

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**A System for Monitoring Mutation-in-Progress:
A Study of Adaptive Mutation to Ciprofloxacin
Resistance in *Staphylococcus aureus***

Andiyanto Sutandar

School of Mechanical & Aerospace Engineering

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ABSTRACT

In nature, evolution progresses to assure the survival of the strongest of a species. With each mutation, those characteristics that best assure the survival of the species are propagated through successive, on-going mutations. As there is no known method to monitor such mutation-in-progress, the objective of this research was to devise a technique to monitor the progression of mutation.

Pathogens are known to survive (mutate to gain resistance) in an antibiotic-containing environment. One such pathogen, *staphylococcus aureus* is known to cause diseases ranging from superficial dermatological lesions to systemic and life threatening infections such as endocarditis. One particular strain of *staphylococcus aureus* - methicillin resistant *Staphylococcus aureus* (MRSA) - is known to be resistant to almost all antibiotics.

A pilot study established that it is feasible to monitor the growth of *staphylococcus aureus* using overnight cultures as well as direct culture of nasal swab through visible spectroscopy. Using such spectroscopic technique, it was possible to discriminate between methicillin susceptible and resistant strains of *Staphylococcus aureus*. Based on these results, a novel system coined the System for Monitoring Mutation-in-Progress (SMMIP) was devised. SMMIP allows nutrients to be replenished and extracted to allow for long term tracking. In this study, the use of SMMIP to monitor the mutation-in-progress is demonstrated through the adaptation of ciprofloxacin resistance, a fluoroquinolone antibiotic, in *Staphylococcus aureus*.

SMMIP was successful in automatically monitoring the growth of methicillin susceptible *Staphylococcus aureus* (MSSA) ATCC 29213 in Mannitol Salt Broth (MSB) containing ciprofloxacin. Resistant mutants in MSB containing 0.5 µg/ml of ciprofloxacin emerged on Days 6, 4, and 7 from samples containing initial inocula of 6.0×10^6 CFU/ml, 1.3×10^7 CFU/ml, and 1.3×10^8 CFU/ml respectively. The ciprofloxacin mutants were detected by monitoring wavelength shifts of the acidogenic profiles of the growth medium. The SMMIP measures the growth of

mutants on the same day that an increase of viable count was observed. It also requires fewer resources than conventional standard plate count and Population Analysis Profile (PAP) techniques. Sequencing of the quinolone resistant determining region (QRDR) gene sections are then performed to further confirm the existence of mutants.

The Scanning Electron Microscope (SEM) images of the bacteria show the exploded state of *Staphylococcus aureus*. The release of the cell content correlated well to the alkalization and clearing of the growth medium seen by the spectroscopic readings. The scanning microscopy images also confirm the presence of viable bacteria with normal morphological shape.

SMMIP can be utilized to study the interaction between various antibiotics and bacteria provided that there is an inducible colour change in the sample. With the built-in method to replenish and extract the nutrient, simulations of more diverse drug profiles can be carried out.

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TABLE OF CONTENT

ABSTRACT.....	i
ACKNOWLEDGEMENT	iii
TABLE OF CONTENT	iv
LIST OF FIGURES	xi
LIST OF TABLES.....	xix
Chapter 1 INTRODUCTION.....	1
1.1. Objectives.....	5
1.2. Scope	5
Chapter 2 LITERATURE REVIEW.....	6
2.1. Mutation and Antibiotic Resistance	6
2.1.1. Mutation and Mechanisms of Antibiotic Resistance.....	6
2.1.2. Development of Antibiotic Resistance.....	7
2.2. Development of Antibiotic Resistance in <i>Staphylococcus aureus</i>	9
2.2.1. Potential of Antibiotic Resistance of <i>Staphylococcus aureus</i>	9
2.2.2. Danger of the Antibiotic Resistant Mutants of <i>Staphylococcus aureus</i>	12
2.3. Quinolone Resistance.....	13
2.3.1. Activity of Quinolone	13
2.3.2. Development of Ciprofloxacin Resistance	15
2.3.2.1. Ciprofloxacin Resistance by Alteration of Target Sites	16
2.3.2.2. Ciprofloxacin Resistance by Reduction of Antibiotic Concentration inside Bacteria	18
2.4. Methods Used to Monitor Mutation-in-Progress	20
2.4.1. Phenotypic Methods Used to Monitor Mutation-in-Progress	20
2.4.2. Molecular Methods Used to Detect Mutation.....	22
2.4.3. Advantage of Phenotypic Methods in Monitoring Mutation-in-Progress.....	23
2.5. Feasibility of Spectroscopy as a Monitoring Method	24
2.5.1. Basic of Spectroscopy	24

2.5.2.	Feasibility of Vibrational Spectroscopy as a Monitoring Method	25
2.5.3.	Feasibility of Fluorescence Spectroscopy as a Monitoring Method	26
2.5.4.	Feasibility of Nuclear Magnetic Resonance (NMR) Spectroscopy as a Monitoring Method	27
2.5.5.	Feasibility of UV-Visible Spectroscopy as a Monitoring Method	29
2.6.	Mathematical Model of the Emergence of Antibiotic Resistance	30
2.6.1.	General Growth Model	31
2.6.2.	Mathematical Model of Growth in the Absence of Antibiotic	34
2.6.3.	Mathematical Model of Growth in the Presence of Antibiotic	37
2.6.4.	Mathematical Model of the Emergence of Antibiotic Resistance	41
Chapter 3	MATERIALS PREPARATION	47
3.1.	Preparation of Antibiotic Stock Solution	48
3.2.	Preparation of Growth Media	48
3.2.1.	Preparation of Agar Media	48
3.2.2.	Preparation of Agar Slants	49
3.2.3.	Preparation of Broth Media	50
3.2.4.	Preparation of Glycerol-Containing Broth Media	50
3.2.5.	Preparation of Antibiotic-Containing Agar and Broth Media	51
3.3.	Preparation of Culture Stock Solution	52
3.4.	Preparation of Working Culture	52
Chapter 4	SYSTEM FOR MONITORING MUTATION-IN-PROGRESS	54
4.1.	Initial Optical Set-up	54
4.2.	Schematic of the System for Monitoring the Mutation-in-Progress (SMMIP)	55
4.3.	Electrical Circuitry of the System for Monitoring Mutation-in-Progress (SMMIP)	59
4.3.1.	LED Control Circuit	59
4.3.2.	Shaker Control Circuit	60

4.3.3.	Temperature Control Circuit	61
Chapter 5	EXPERIMENTAL PROCEDURES.....	62
5.1.	Procedures of Standard Plate Count Technique.....	62
5.2.	Procedures of Latex Agglutination Test.....	63
5.3.	Procedures of Scanning Electron Microscopy (SEM) Imaging of <i>Staphylococcus aureus</i>	63
5.4.	Procedures of the Detection of Antibiotic Resistance	64
5.4.1.	Procedures of Agar Diffusion Susceptibility Test.....	64
5.4.2.	Procedures of Minimal Inhibitory Concentration (MIC) Dilution Test.....	65
5.4.3.	Procedures of Population Analysis Profile (PAP) Test.....	65
5.5.	Procedures of the Media and System Characterization Studies	66
5.5.1.	Procedures of the Experiments to Study the Change of Medium pH during Bacterial Growth.....	66
5.5.2.	Procedures of the Experiments to Determine the Light Source .	67
5.5.3.	Procedures of the Experiments to Study the Effect of pH Change on Recorded Spectra.....	68
5.5.4.	Procedures of the Experiments to Study the Stability of Broth .	68
5.5.5.	Procedures of the Experiments to Study the Temperature Stability of the Controlled Environmental Chamber.....	69
5.6.	Procedures of Growth Monitoring.....	70
5.6.1.	Procedures of the Experiments to Develop a Growth Curve of <i>Staphylococcus aureus</i> in Mannitol Salt Broth (MSB)	70
5.6.2.	Procedures of the Experiments to Study the Possibility of Direct Clinical Identification	70
5.6.3.	Procedures of the Experiments to Study the Possibility of Oxacillin Resistant Strain Identification by Monitoring the Growth	71
5.6.4.	Procedures of the Experiments to Determine Effect of Temperature to Growth	73
5.6.5.	Procedures of the Experiments to Study the Contribution of the Acidity and Turbidity of the Media to the Spectral Reading	74

5.7.	Procedures of the Studies on the Emergence of Antibiotic Resistance....	75
5.7.1.	Procedures of Monitoring the Emergence of Antibiotic Resistance.....	75
5.7.2.	Procedures of Experiments to Determine the Stability of the Ciprofloxacin Resistant Mutants.....	78
5.7.3.	Procedures of the Experiment to Characterize the Mutants at Molecular Level.....	78
Chapter 6	MEDIA AND SYSTEM CHARACTERIZATION STUDIES.	81
6.1.	Acidification of MSB during Bacterial Growth.....	81
6.2.	Determination of the Appropriate Light Source	85
6.2.1.	Tungsten Halogen Lamp	85
6.2.2.	Light Emitting Diodes (LED).....	89
6.3.	Effect of Temperature Change on Measured pH	91
6.4.	Effect of pH Change on Recorded Spectra	93
6.5.	Stability of Broth.....	97
6.5.1.	Effect of Incubation on Spectral Reading of Blank Broth Containing No Antibiotics.....	97
6.5.2.	Effect of Incubation on Spectral Reading of Blank Broth Containing Antibiotics.....	99
6.5.3.	Effect of Non Viable Bacteria on Spectral Reading of Blank Broth.....	101
6.6.	Temperature Stability of the Controlled Environmental Chamber (CEC)	102
6.7.	Summary of the Characterization Studies	103
Chapter 7	GROWTH OF <i>STAPHYLOCOCCUS AUREUS</i> IN MANNITOL SALT BROTH (MSB)	105
7.1.	Establishing the Growth Curve of <i>Staphylococcus aureus</i> in MSB.....	105
7.2.	Growth Monitoring and Detection of <i>Staphylococcus aureus</i> from Clinical Samples.....	108
7.3.	Use of Growth Monitoring to Detect Oxacillin Resistance in <i>Staphylococcus aureus</i>	114
7.4.	Effect of Different Incubation Temperature to Bacterial Growth	123

7.5. Separating Contribution of Acidity and Turbidity to the Spectral Plots	127
7.6. Summary of the Growth of <i>Staphylococcus aureus</i> in MSB.....	132
Chapter 8 MONITORING OF THE EMERGENCE OF ANTIBIOTIC RESISTANCE	135
8.1. Ultrastructural Changes of <i>Staphylococcus aureus</i> at Exposure to Ciprofloxacin	135
8.2. SMMIP in Monitoring the Emergence of Antibiotic Resistance	138
8.3. Comparative Conventional Methods to Monitor the Emergence of Antibiotic Resistance.....	150
8.4. Superiority of SMMIP Compared to the Conventional Methods in Monitoring the Emergence of Antibiotic Resistance	156
8.5. Characterization of Ciprofloxacin Resistant Mutants.....	157
8.5.1. Stability of the Ciprofloxacin Resistant Mutants Produced by SMMIP.....	157
8.5.2. Molecular Characterization of the Ciprofloxacin Resistant Mutants.....	161
8.6. Summary of the Study on the Emergence of Antibiotic Resistance	165
Chapter 9 MODELING OF THE EMERGENCE OF ANTIBIOTIC RESISTANCE	167
9.1. Modeling the Wavelength Shift Data of the Emergence of Antibiotic Resistance as Monitored by the SMMIP	167
9.1.1. Model Wv-1 to Explain the Wavelength Shift Data Produced by SMMIP.....	168
9.1.1.1. The Adjustment Function of Model Wv-1.....	169
9.1.1.2. The Limiting Function of Model Wv-1.....	170
9.1.1.3. The Inhibition Function of Model Wv-1.....	171
9.1.1.4. The General Formula of Model Wv-1.....	171
9.1.2. Model Wv-2 to Explain the Wavelength Shift Data Produced by SMMIP.....	173
9.1.2.1. The Adjustment Function of Model Wv-2.....	174
9.1.2.2. The Limiting Function of Model Wv-2.....	174
9.1.2.3. The Modification Function of Model Wv-2.....	174

9.1.2.4.	The Inhibition Function of Model Wv-2.....	175
9.1.2.5.	The General Formula of Model Wv-2.....	175
9.1.3.	The Fitting of the Normalized Wavelength Shift with the Models	177
9.1.3.1.	The Fitting of the Normalized Wavelength Shift with Model Wv-1	179
9.1.3.2.	The Fitting of the Normalized Wavelength Shift with Model Wv-2.....	187
9.1.3.3.	The Fitting of Weibull Function to the Normalized Wavelength Shift Data.....	193
9.1.3.4.	The Fitting of Logistic Function to the Normalized Wavelength Shift Data.....	199
9.1.3.5.	The Fitting of Gompertz Function to the Normalized Wavelength Shift Data.....	204
9.1.4.	Comparison of Model Wv-2 and Other Models for the Fitting of the Wavelength Shift Data.....	211
9.2.	Modeling the Intensity Ratio Data of the Emergence of Antibiotic Resistance as Monitored by the SMMIP	215
9.2.1.	Model IR-1 to Explain the Normalized IR Data Produced by SMMIP	215
9.2.2.	Model IR-2 to Explain the Normalized IR Data Produced by SMMIP	216
9.2.2.1.	First Component of Model IR-2	217
9.2.2.2.	Second Component of Model IR-2	220
9.2.2.3.	General Form of Model IR-2.....	223
9.2.3.	The Fitting of the Normalized IR Data with the Models.....	223
9.2.3.1.	The Fitting of Normalized IR with Model IR-1	224
9.2.3.2.	The Fitting of Normalized IR with Model IR-2.....	229
9.2.4.	Comparison of Model IR-1 and Model IR-2 for the Fitting of the Intensity Ratio Data	238
Chapter 10	CONCLUSIONS	240
Chapter 11	FUTURE WORK	245

Chapter 12	REFERENCES	247
APPENDIX A		275
APPENDIX B		276
APPENDIX C-1		277
APPENDIX C-2		280
APPENDIX C-3		283
APPENDIX D		291
APPENDIX E-1		292
APPENDIX E-2		293
APPENDIX E-3		294
APPENDIX F		295
APPENDIX G		296
APPENDIX H-1		297
APPENDIX H-2		298
APPENDIX H-3		299
APPENDIX H-4		300
APPENDIX H-5		301
APPENDIX H-6		302
APPENDIX H-7		303
APPENDIX H-8		304
APPENDIX H-9		305
APPENDIX H-10		306
GLOSSARY		307

LIST OF FIGURES

Figure 1. Scanning Electron Microscopy (SEM) of <i>Staphylococcus aureus</i>	10
Figure 2. Schematic circular diagrams of the MRSA252 and MSSA476 chromosomes [105]	11
Figure 3. Core structure of antibiotics from the class of quinolone [61]	14
Figure 4. Structure of Ciprofloxacin [121]	16
Figure 5. Growth trend obtained using standard plate count during incubation of MRSA 8043 and MRSA 8043L0-1 in levofloxacin containing broth [52]	21
Figure 6. Electromagnetic spectrum and its effect on atom and molecule [149]	25
Figure 7. Range of visible spectrum [148].....	29
Figure 8. Typical bacterial growth curve in a batch culture	32
Figure 9. An agar slant is positioned for cooling.....	49
Figure 10. Optical set-up used in characterization works	54
Figure 11. Schematic of the circuit for the LED used in the initial optical set-up ..	55
Figure 12. Schematic of the System for Monitoring Mutation-in-Progress (SMMIP)	56
Figure 13. State of the light source used in SMMIP.....	60
Figure 14. State of the shaker used in SMMIP	60
Figure 15. Cuvette designed for use with SMMIP	75
Figure 16. Experimental protocol used to monitor the emergence of antibiotic resistance using SMMIP	77
Figure 17. Temperature and pH development of MSB inoculated with 7.1×10^7 CFU/ml of MSSA ATCC 29213 and incubated in air at 36 °C	81
Figure 18. Temperature and pH development of MSB incubated in air at 36 °C and inoculated with (A) 4.0×10^6 CFU/ml of MSSA ATCC 29213, (B) 3.6 $\times 10^6$ CFU/ml of MRSA ATCC 43300.....	83
Figure 19. Temperature and pH development of MSB inoculated with 9.7×10^5 CFU/ml of MRSA ATCC 43300 and incubated in air at 36 °C.....	85
Figure 20. Spectral reading of blank MSB incubated for 720 minutes in air at 36 °C under illumination by tungsten halogen lamp.....	86

Figure 21. Intensity ratio (IR) of blank MSB under illumination by tungsten halogen lamp	87
Figure 22. Intensity ratio (IR) plot of a blank MSB by setting 60 th minute as the reference time.....	89
Figure 23. Spectral reading of LED with emission peaked at 575 nm	90
Figure 24. Intensity ratio of LED with emission peak at 575 nm for the first 10 minutes of activity	91
Figure 25. Effect of the temperature of the broth on the measured pH.....	92
Figure 26. Effect of the pH of the MSB solution on the peak wavelength of the spectral reading	93
Figure 27. Spectral readings of MSB with different pH value in room temperature	94
Figure 28. Intensity ratio plot of the effect of pH change on the spectral reading with reference to MSB at pH 7.23 (T = ~ 23 °C)	94
Figure 29. Normalized wavelength shift of MSB with different pH measured under room temperature.....	95
Figure 30. Development of intensity ratio (IR) at 557.47 nm of blank MSB under pH adjustment using 0.1 M HCl.....	96
Figure 31. Intensity ratio of blank MSB with LED as light source	97
Figure 32. Normalized wavelength shift of blank broth containing no antibiotics under incubation in air at 36 °C.....	98
Figure 33. Normalized wavelength shift experienced by blank MSBOxa-12	100
Figure 34. Normalized wavelength shift experienced by blank broth of MSBCip-1.25	100
Figure 35. Normalized wavelength shift experienced by MSBOxa-4 spiked with non viable MSSA ATCC 29213 and MRSA ATCC 43300.....	101
Figure 36. Normalized wavelength shift experienced by MSBOxa-12 spiked with non viable MSSA ATCC 29213 and MRSA ATCC 43300.....	102
Figure 37. Temperature profile of the Controlled Environmental Chamber (CEC) set at 36 °C	103
Figure 38. Changes of the spectral readings of MSB inoculated with MSSA ATCC 29213	105

Figure 39. Growth curve of <i>Staphylococcus aureus</i> MSSA ATCC 29213 and MRSA 43300 under incubation in air at 36 °C based on the normalized wavelength shift of MSB.....	106
Figure 40. Viable count development during the growth in MSB of <i>Staphylococcus aureus</i> MSSA ATCC 29213 and MRSA 43300 under incubation in air at 36 °C.....	107
Figure 41. Spectral reading of MSB spiked with 3.5×10^6 CFU/ml of <i>Staphylococcus aureus</i>	109
Figure 42. Intensity ratio of MSB spiked with 3.5×10^6 CFU/ml of <i>Staphylococcus aureus</i>	109
Figure 43. Normalized wavelength shift experienced by MSB spiked with <i>Staphylococcus aureus</i> obtained from nasal swab.....	110
Figure 44. Intensity ratio at 557.47 nm of MSB spiked with <i>Staphylococcus aureus</i> obtained from nasal swab	111
Figure 45. Intensity ratio at 600 nm of MSB spiked with <i>Staphylococcus aureus</i> obtained from nasal swab	112
Figure 46. Normalized wavelength shift of sample from nasal swab of healthy volunteers	113
Figure 47. Normalized wavelength shift experienced by MSBOxa-4 spiked with MSSA ATCC 29213.....	115
Figure 48. Normalized intensity ratio at 600 nm experienced by MSBOxa-4 spiked with MSSA ATCC 29213	115
Figure 49. Normalized wavelength shift experienced by MSBOxa-4 spiked with MRSA ATCC 43300	116
Figure 50. Normalized intensity ratio at 600 nm experienced by MSBOxa-4 spiked with MRSA ATCC 43300.....	116
Figure 51. Normalized wavelength shift of MSSA ATCC 29213 with similar inoculum during growth in MSB with different oxacillin concentrations	119
Figure 52. Normalized intensity ratio at 600 nm of MSSA ATCC 29213 with similar inoculum during growth in MSB with different oxacillin concentrations	119

Figure 53. Normalized wavelength shift of MRSA ATCC 43300 with similar inoculum during growth in MSB with different oxacillin concentrations	120
Figure 54. Normalized intensity ratio of MRSA ATCC 43300 with similar inoculum during growth in MSB with different oxacillin concentrations	120
Figure 55. Normalized intensity index changes compared to normalized wavelength shift for MSBOxa-4	123
Figure 56. Normalized wavelength shift of MSB inoculated with various initial inoculum sizes of MSSA ATCC 29213 and incubated under two different temperatures	124
Figure 57. Normalized intensity ratio at 600 nm of MSB inoculated with various initial inoculum sizes of MSSA ATCC 29213 and incubated under two different temperatures	125
Figure 58. Normalized wavelength shift of MSB inoculated with various initial inoculum sizes of MRSA ATCC 43300 and incubated under two different temperatures	125
Figure 59. Normalized intensity ratio at 600 nm of MSB inoculated with various initial inoculum sizes of MRSA ATCC 43300 and incubated under two different temperatures	126
Figure 60. Plot of the change of intensity ratio (IR) experienced by MSBw/oPR spiked with 2.7×10^8 CFU/ml of MSSA ATCC 29213	127
Figure 61. Development of intensity ratio (IR) of MSB without phenol red spiked with 2.7×10^8 CFU/ml of MSSA ATCC 29213 at various wavelengths	128
Figure 62. Development of IR_{600}^- of samples with similar initial inoculum of MSSA and MRSA	129
Figure 63. (A) Comparison of IR_{600}^- and normalized wavelength shift experienced by MSB; (B) Comparison between normalized IR at 561.01 nm experienced by MSB and IR_{600}^-	130
Figure 64. IR_{600}^- plot when the broths were inoculated with MSSA ATCC 29213	131

Figure 65. Development of IR at 561.01 nm with respect to colour change in MSB	132
Figure 66. Scanning Electron Microscopy (SEM) pictures of <i>Staphylococcus aureus</i> after 24 hours incubation in MSBCip-0.5	135
Figure 67. Scanning Electron Microscopy (SEM) pictures of <i>Staphylococcus aureus</i> with normal and filamentous morphology after 24 hours incubation in MSBCip-0.5	136
Figure 68. Scanning Electron Microscopy (SEM) pictures of <i>Staphylococcus aureus</i> after 24 hours incubation in MSBCip-2	137
Figure 69. Development of normalized wavelength shift of MSB containing increasing concentration of ciprofloxacin which was spiked with MSSA ATCC 29213	140
Figure 70. Development of normalized intensity ratio (IR) at 600 nm for MSB containing increasing concentration of ciprofloxacin which was spiked with MSSA ATCC 29213	142
Figure 71. Development of normalized wavelength shift for MSBCip-0.5 which was spiked with MSSA ATCC 29213	143
Figure 72. Development of the amount of normalized wavelength shift for some selected incubation time point over five incubation days for Sample 1	145
Figure 73. Development of the amount of normalized wavelength shift for some selected incubation time point over five incubation days for Sample 2	146
Figure 74. Development of the amount of normalized wavelength shift for some selected incubation time point over five incubation days for Sample 3	146
Figure 75. Development of normalized intensity ratio (IR) at 600 nm for MSBCip-0.5 which was spiked with MSSA ATCC 29213	148
Figure 76. Development of the viable count of MSSA ATCC 29213 during incubation in MSBCip-0.5	150

Figure 77. Population Analysis Profile (PAP) of the bacterial population found in MSBCip-0.5 which was initially inoculated with 1.3×10^8 CFU/ml of MSSA ATCC 29213 (Sample 3).....	153
Figure 78. Population Analysis Profile (PAP) of the bacterial population found in MSBip-0.5 which is initially inoculated with 1.3×10^7 CFU/ml of MSSA ATCC 29213 (Sample 2).....	154
Figure 79. Population Analysis Profile (PAP) of the bacterial population found in MSBip-0.5 which is initially inoculated with 6.0×10^6 CFU/ml of MSSA ATCC 29213 (Sample 1).....	155
Figure 80. Population Analysis Profiles (PAPs) of some mutants produced	160
Figure 81. Multiple sequence alignment of the nucleotides of the mutants, the parent strain, and the database-derived sequence which showed mutation in the <i>grlB</i> gene of mutants A1F, B1D, and B1E.....	162
Figure 82. Multiple sequence alignment of the GrlB protein of the mutants, the parent strain, and the database-derived sequence which showed mutation in the GrlB protein of mutants A1F, B1D, and B1E	163
Figure 83. Multiple sequence alignment of the nucleotides of the mutants, the parent strain, and the database-derived sequence which showed mutation in the <i>grlB</i> gene section of mutants C1E.....	163
Figure 84. Multiple sequence alignment of the nucleotides of the mutants, the parent strain, and the database-derived sequence which showed mutation in the GrlB protein of mutants C1E.....	164
Figure 85. Curve fitting of Model Wv-1 on magnitude of the wavelength shift of Sample 1	180
Figure 86. Curve fitting of Model Wv-1 on magnitude of the wavelength shift of Sample 2.....	181
Figure 87. Curve fitting of Model Wv-1 on magnitude of the wavelength shift of Sample 3.....	182
Figure 88. Curve fitting of Model Wv-2 on the magnitude of wavelength shift of Sample 1.....	188
Figure 89. Curve fitting of Model Wv-2 on the magnitude of wavelength shift of Sample 2.....	189

Figure 90. Curve fitting of Model Wv-2 on the magnitude of wavelength shift of Sample 3	190
Figure 91. Curve fitting of Weibull function on the magnitude of wavelength shift of Sample 1	196
Figure 92. Curve fitting of Weibull function on the magnitude of wavelength shift of Sample 2	197
Figure 93. Curve fitting of Weibull function on the magnitude of wavelength shift of Sample 3	198
Figure 94. Curve fitting of Logistic function on the magnitude of wavelength shift of Sample 1	200
Figure 95. Curve fitting of Logistic function on the magnitude of wavelength shift of Sample 2	201
Figure 96. Curve fitting of Logistic function on the magnitude of wavelength shift of Sample 3	202
Figure 97. Curve fitting of Gompertz function on the magnitude of wavelength shift of Sample 1	206
Figure 98. Curve fitting of Gompertz function on the magnitude of wavelength shift of Sample 2	207
Figure 99. Curve fitting of Gompertz function on the magnitude of wavelength shift of Sample 3	208
Figure 100. Development of wavelength shift data near the emergence of mutant for Sample 2 and Sample 3	214
Figure 101. Curve fitting of Model IR-1 on the normalized intensity ratio of Sample 1	225
Figure 102. Curve fitting of Model IR-1 on the normalized intensity ratio of Sample 2	226
Figure 103. Curve fitting of Model IR-1 on the normalized intensity ratio of Sample 3	227
Figure 104. Curve fitting of Model IR-2 on the normalized intensity ratio of Sample 1	234
Figure 105. Curve fitting of Model IR-2 on the normalized intensity ratio of Sample 2	235

Figure 106. Curve fitting of Model IR-2 on the normalized intensity ratio of Sample 3	236
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LIST OF TABLES

Table 1. Mechanism of resistance for several antibiotic classes	7
Table 2. Mutations in the <i>grrA</i> gene of single step mutant of <i>Staphylococcus aureus</i> strains with higher ciprofloxacin resistance than the parent strain (The complete name of the amino acid abbreviation can be found in Appendix G).....	17
Table 3. Quasi chemical growth model [179].....	36
Table 4. Primers used to perform PCR on QRDR and norA promoter region [207]	79
Table 5. A summary of the data obtained from pH development tests at 36 °C	84
Table 6. Comparison of the time required to indicate the presence of mutants.....	149
Table 7. Comparison of the expected MICs of some mutants with the MICs of the mutants after seven passages on antibiotic-free agar.....	159
Table 8. Parameters and AIC _C obtained when Model Wv-1 was fitted the magnitude of wavelength shift of Sample 1	183
Table 9. Parameters and AIC _C obtained when Model Wv-1 was fitted the magnitude of wavelength shift of Sample 2.....	183
Table 10. Parameters and AIC _C obtained when Model Wv-1 was fitted the magnitude of wavelength shift of Sample 3	184
Table 11. Parameters and AIC _C obtained when Model Wv-2 was fitted the magnitude of wavelength shift of Sample 1	191
Table 12. Parameters and AIC _C obtained when Model Wv-2 was fitted the magnitude of wavelength shift of Sample 2	191
Table 13. Parameters and AIC _C obtained when Model Wv-2 was fitted to the magnitude of wavelength shift of Sample 3	192
Table 14. Parameters and AIC _C obtained when Weibull function was fitted the wavelength shift of Sample 1	194
Table 15. Parameters and AIC _C obtained when Weibull function was fitted the wavelength shift of Sample 2	194
Table 16. Parameters and AIC _C obtained when Weibull function was fitted to the wavelength shift of Sample 3	195

Table 17. Parameters and AIC_C obtained when Logistic function was fitted to the magnitude of wavelength shift of Sample 1	203
Table 18. Parameters and AIC_C obtained when Logistic function was fitted to the magnitude of wavelength shift of Sample 2	203
Table 19. Parameters and AIC_C obtained when Logistic function was fitted to the magnitude of wavelength shift of Sample 3	204
Table 20. Parameters and AIC_C obtained when Gompertz function was fitted to the magnitude of wavelength shift of Sample 1	209
Table 21. Parameters and AIC_C obtained when Gompertz function was fitted to the magnitude of wavelength shift of Sample 2	210
Table 22. Parameters and AIC_C obtained when Gompertz function was fitted to the magnitude of wavelength shift of Sample 3	210
Table 23. Comparison of the AIC_C obtained from the fitting of the wavelength shift data of Sample 1 with Model Wv-1, Model Wv-2, Weibull function, Logistic function, and Gompertz function	211
Table 24. Comparison of the AIC_C obtained from the fitting of the wavelength shift data of Sample 2 with Model Wv-1, Model Wv-2, Weibull function, Logistic function, and Gompertz function	211
Table 25. Comparison of the AIC_C obtained from the fitting of the wavelength shift data of Sample 3 with Model Wv-1, Model Wv-2, Weibull function, Logistic function, and Gompertz function	212
Table 26. Evidence ratio of Model Wv-2 with respect to other models in describing the wavelength shift data of Sample 1	212
Table 27. Evidence ratio of Model Wv-2 with respect to other models in describing the wavelength shift data of Sample 2	212
Table 28. Evidence ratio of Model Wv-2 with respect to other models in describing the wavelength shift data of Sample 3	213
Table 29. Parameters and AIC_C obtained when Model IR-1 was fitted to the normalized IR of Sample 1	228
Table 30. Parameters and AIC_C obtained when Model IR-1 was fitted to the normalized IR of Sample 2	228

Table 31. Parameters and AIC_C obtained when Model IR-1 was fitted to the normalized IR of Sample 3	229
Table 32. List of t_0 used in fitting normalized IR data with Model IR-2.....	230
Table 33. Parameters of $f(t)$ when the function was fitted to Sample 1	230
Table 34. Parameters of $g(t)$ when the function was fitted to Sample 1	230
Table 35. Parameters of $f(t)$ when the function was fitted to Sample 2	231
Table 36. Parameters of $g(t)$ when the function was fitted to Sample 2	231
Table 37. Parameters of $f(t)$ when the function was fitted to Sample 3	232
Table 38. Parameters of $g(t)$ when the function was fitted to Sample 3	232
Table 39. Comparison of the AIC_C obtained from the fitting of the intensity ratio data of Sample 1 with Model IR-1 and Model IR-2.....	238
Table 40. Comparison of the AIC_C obtained from the fitting of the intensity ratio data of Sample 2 with Model IR-1 and Model IR-2.....	238
Table 41. Comparison of the AIC_C obtained from the fitting of the intensity ratio data of Sample 3 with Model IR-1 and Model IR-2.....	238
Table 42. Evidence ratio of Model IR-2 with respect to Model IR-1 in describing the intensity ratio data of Sample 2 and Sample 3.....	239

Chapter 1

INTRODUCTION

Mutation is defined as an inheritable change in the base sequence of the nucleic acid comprising the genome of an organism [1]. Therefore, a mutant possesses a different genotype from its parental strain although not necessarily a different phenotype. A mutant gains a competitive advantage over its parent strain through expressed mutations. Yet, it must be noted that the usefulness of an expressed mutation is relative to the growth environment.

Different environments provide different selective pressure to the organisms living within it. Sub-populations of mutated organisms with a better chance of survival may emerge. In this report, a study on the mutation in progress is elaborated. The author would like to define “mutation-in-progress” as a mutational process where the mutant is not yet stable and will most likely experience further mutations. For example, a newly emerged antibiotic resistant mutant during growth in an antibiotic-containing environment may undergo further mutation to have a higher possibility of survival. The population is then said to undergo a “mutation-in-progress”. The author devised a technique to monitor such mutation especially in terms of the emergence of antibiotic resistance.

The study on the emergence of antibiotic resistant pathogens is chosen since mutant strains are exhausting the known treatment modalities. Antibiotics resistance can develop from a spontaneous mutation in one or more genes, an acquisition of resistance genes or plasmids from other strain or species, and directed mutations due to the increase in the selective pressure [2, 3]. Abuse of antibiotics especially encourages the evolution of bacteria toward resistance [4, 5]. Many organizations have already been established all over the world to address the threat of antibiotic resistant pathogens [6, 7].

Pathogens such as *Staphylococcus aureus*, are a classic example on how overuse of antibiotics and therefore, the resulting mutations, wreak havoc in the healthcare

scene. The overuse of penicillin and methicillin quickly causes the emergence of their respective resistance in *Staphylococcus aureus* [8, 9]. A particular strain called methicillin resistant *Staphylococcus aureus* (MRSA) is noted as a common cause of nosocomial as well as community infections worldwide. The strain is resistant to multiple classes of antibiotics and is known to gain antibiotic resistance easily. Huge amount of resources have been poured out to delve into the evolutionary history, screening, and detection of MRSA over the years [8-35].

As MRSA becomes more commonly found as the cause of infections, vancomycin (an antibiotic belonging to the glycopeptides class) is used as a cornerstone of therapy for serious MRSA infections. However, at the time of writing (January 2007), vancomycin resistant strains of *Staphylococcus aureus*, or the so called Vancomycin Resistant *Staphylococcus aureus* (VRSA) strains, had been isolated [36-38]. There had even been few cases of infections caused by linezolid resistant *Staphylococcus aureus* [39]. It was particularly worrying since linezolid is one of the very few drugs shown to possess bactericidal activity against MRSA and even VRSA in an *in vitro* study [40]. The same turn of event could be observed for daptomycin, the only approved antibiotic from the novel cyclic lipopeptides class [41]. Although some antibiotics were shown not to induce resistance *in vitro* easily, cases where the resistance occurred in a course of time were quite widely known [41-43].

It is clear that *Staphylococcus aureus* is acquiring greater amount of antibiotic resistance, from penicillin resistance to linezolid resistance, and is set to stay put as a troublemaker in the healthcare scene. Unfortunately, *Staphylococcus aureus* is not the only bacterial species that contributes its antibiotic resistant strains to the problem. The emergence of multiple-resistant pathogens is also noted in many other bacterial species, e.g. *Enterococcus faecium* [44], *Acinetobacter baumannii* [45], *Pseudomonas aeruginosa* [46], *Haemophilus influenzae* [47], and *Mycobacterium tuberculosis* [47]. Due to the concern for the emergence of antibiotic resistance, researchers have been looking into conditions that encouraged such development. By understanding the relationship between antibiotics and emergence of resistance,

it is possible to optimize dosage regimens, to determine non-mutant selective drug combinations, and to establish the best choice of drugs for treatment. Hence, further onslaught of multiple-resistant pathogens can be prevented.

The study on the emergence of resistance in a bacterial species will provide a more complete picture if it is performed in a real physiological (*in vivo*) environment rather than in a laboratory (*in vitro*) set up. However, it is not possible to perform such a study on humans due to the inherent ethical issues and tremendous risks involved. The *in vivo* study was usually performed in an animal model of infection. Nonetheless, the study on the animal model was cumbersome.

A study on an animal model normally requires a licence from an ethics council. The decision to grant the licence was made based on several factors including the qualifications of the personnel involved, the assessment of the facilities, and the appropriateness and rationality for the research [48]. There were inherently long and tedious procedural works required for a study using an animal model. Furthermore, it might not be possible for a poorly equipped and staffed microbiology laboratory to conduct such a study. The extreme difficulty to replicate human pharmacokinetics in animal model was also acknowledged [49].

In vitro study is more readily performed than *in vivo* study. *In vitro* study involves simulation of growth environment in test tubes, beakers, or bioreactors [7, 50-60]. No licence is needed for such study unless it deals with some potentially dangerous substances or organisms. The study can generally be conducted in any conventional microbiological laboratory. Further improvement to simplify *in vitro* study can be easily performed due to its more mechanistic nature. As shown later in this study, an *in vitro* study can be modified such that the involvement of personnel is minimal.

Regardless of whether the study is performed *in vitro* or *in vivo*, the emergence of resistant mutants is normally determined through the development of viable bacterial count [50, 51, 56]. While the bacterium in question was exposed to an antibiotic-containing environment, the change in the number of living bacteria was

monitored by standard plate count technique. The plate counting technique is a tedious and resource intensive activity. Furthermore, for monitoring purposes, plate count was normally performed at frequent intervals, which could be conducted at under one hour intermissions [51, 53]. When mutations were suspected, genetic tests on the mutants may or may not be performed.

There is currently no *in vitro* system for the study of mutation in progress. Various studies on the emergence of antibiotic resistance utilized non-dedicated systems such as bioreactors [50, 51]. The studies used regular plate count method to determine the change of viable bacterial number. Minimal inhibitory concentration (MIC) of the sample was then determined at the later stage to confirm the suspected emergence of antibiotic resistant mutants. There were also some studies that conducted the mutational study using cultures grown in test tubes and performing regular plate count by withdrawing samples from the test tubes [7, 54-56, 58, 59, 61].

It was then decided to embark on a study to devise a system and technique to monitor mutation in progress, especially in terms of monitoring the emergence of antibiotic resistance. The use of visible spectroscopy in the monitoring work was investigated. The monitoring property of visible spectroscopy had been previously studied widely in microbiology, especially in the measurement of turbidity and biochemical property [62-64]. Spectroscopy method has the inherent ability to sense minute intensity change over a wide range of wavelengths. Therefore, it is expected that slight changes in the growth medium pertaining to the emergence of more resistant mutants can be monitored in real-time.

1.1. Objectives

The project was set in order to achieve the following objectives:

1. To investigate the possibility of using visible spectroscopy in monitoring growth performance
2. To devise a system capable of performing real-time monitoring of mutation-in-progress
3. To investigate the strengths and weaknesses of such system in performing real-time monitoring of mutation-in-progress
4. To propose mathematical formulations based on the results for prediction purposes
5. To compare the novel system with conventional laboratory standards of testing

1.2. Scope

To show that the SMMIP (System for Monitoring Mutation-in-Progress) could be used to monitor mutation-in-progress in terms of antibiotic resistance, and perform a study on the emergence of ciprofloxacin resistance on *Staphylococcus aureus* as a model organism.

Chapter 2

LITERATURE REVIEW

2.1. Mutation and Antibiotic Resistance

2.1.1. Mutation and Mechanisms of Antibiotic Resistance

The mutation relevant to this study was the mutation conferring expressed antibiotic resistance. Such mutants thrived in the presence of normally inhibiting or even killing antibiotic concentration. Many bacteria possessed a wealth of natural resistance to currently marketed antibiotic and drugs yet to be marketed [1]. Natural resistance referred to intrinsic resistance of bacteria to an antibiotic even before the bacterial strain is ever exposed to the antimicrobial agent. Examples of such natural resistance were the resistance of *Kluyvera ascorbata* and *Kluyvera cryocrescens* to several macrolides, streptogramins, glycopeptides, rifampicin, fusidic acid, linezolid, penicillin G, lincosamides, oxacillin, and amoxicillin [65]. Another example was the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides, tetracycline, and erythromycin by the virtue of the operation of broad-spectrum drugs efflux pumps [66]. Fortunately, the natural resistance did not extend to all classes of antibiotics, e.g. *Kluyvera* infections could still be treated with tetracycline [65].

Bacteria could, however, augment its arsenal of antibiotic resistance by acquiring more antibiotic resistance through mutations or obtaining plasmids. Mutations occurred in the chromosomal genetic element while plasmid was an extrachromosomal genetic element inherited from parent or transferred to the bacteria from another cell, strain, or even species [1, 42, 67, 68]. A list of generally encountered mechanisms of antibiotic resistance in bacteria is summarized in Table 1. The mechanisms normally prevented the antibiotics from affecting their target or increased the amount of target.

Table 1. Mechanism of resistance for several antibiotic classes

No.	Mechanism of Resistance	Antibiotic Class	Reference
1.	Changes in cell permeability	Glycopeptide, Aminoglycoside, Chloramphenicol, Isoniazid, Sulfonamide, Tetracycline	[68-73]
2.	Active efflux of antibiotic from bacterial cell	Macrolide, Azalide, Lincosamide, Group B Streptogramin, Tetracycline	[69, 73-75]
3.	Mutation at target sites	Penicillin, Macrolide, Lincosamide, Azalide, Group B Streptogramin, Fluoroquinolone, Oxazolidinone	[69, 73, 74, 76-78]
4.	Enzymatic modification or degradation of the antibiotic	β -Lactam, Glycylcycline, Tetracycline	[69, 73, 75, 79]
5.	Bypass of metabolic pathway	Sulfonamide, Trimethoprim	[69, 73, 80]
6.	Overproduction of target sites	Glycopeptides, β -Lactam, Isoniazid, Globomycin, Folate biosynthesis inhibitor, Ethionamide, Ethambutol, Diazoburine, Canavanine, Sulfonamide, Trimethoprim	[73, 81, 82]

Infections caused by pathogens with multiple-antibiotic-resistance were difficult and complex to treat. This led to an increase in treatment cost. Furthermore, large pharmaceutical companies had terminated their anti-infective research programs due to the slow return-on-investment (ROI) of new drugs [47]. Such termination of research programs slowed down the discovery of new drugs and consequently further diminished the already limited pool of investigative drugs to be available in the future for treatment of infections due to multiply-resistant “superbugs”.

2.1.2. Development of Antibiotic Resistance

Overuse and abuse of antibiotics was implicated with the provision of selective pressure which favors the existence of more resistant mutant strains. Therefore, there existed a vicious cycle involving drugs and their associated resistances [83-85]. As more types of antibiotic are rolled out to the market and more advanced technologies were used to study antibiotic resistances, new type of antibiotic resistance was expected to surface. The resistance might possibly emerge faster if

antibiotics uses are not controlled. Consequently, it was suggested that educating antibiotic prescribers and community on the risk of careless and unnecessary use antibiotics would lessen the selective pressure [86, 87].

The emergence of resistant mutants under antibiotics pressure was categorized as an adaptive mutation. Adaptive mutations occurred in non-dividing or slowly dividing cells, which were undergoing a selection for a particular phenotype. The mutations seemed to produce only specific mutants, which were capable of growing under the selective environment [88, 89]. The long-term incubation of a particular bacterium in the presence of an antibiotic provided the selective pressure for the bacterium to mutate and acquire capability to grow in such environment, for example in gaining antibiotic resistance.

Pseudomonas aeruginosa, one of the opportunistic pathogen involved in nosocomial infections, was shown to possess higher mutation rate per cell per day when it was subjected to long-term incubation pressure in the presence of tetracycline [90]. Interaction of *Vibrio cholerae* with β -lactams was also shown to produce high amount of resistant cells [91]. Last but not least, quinolone was known to induce SOS mutagenic response and consequently increase the rate of the emergence of resistance in *Escherichia coli* and *Staphylococcus aureus* [61, 88, 89].

Zhao, X. and Drlica, K. proposed that there was a mutant selection window (MSW) where the selection of antibiotic-resistant mutant bacteria occurred [92]. The MSW extended from the minimum inhibitory concentration (MIC) of susceptible cells to the MIC of the least susceptible, single-step bacterial mutants (the mutant prevention concentration [MPC]) in existence. It was proposed that an exposure of a bacterium to its MSW of a particular drug would enrich the resistant mutant subpopulations. On the other hand, an exposure of the same bacterium to a drug concentration above its MSW would inhibit the mutant selection [93]. Some studies confirmed the existence of MSW of some antimicrobial agents with *Streptococcus pneumoniae*, *Escherichia coli*, *Haemophilus influenzae*, *Mycobacterium smegmatis*, and *Staphylococcus aureus* [58, 94-96]. Unfortunately, the work by Drlica, K.'s

group stopped short of linking the MSW with adaptive mutation. In addition, the concept on MSW was shown mainly with antibiotics from quinolone class [58, 88, 93, 95].

Since it was clear that bacteria may acquire resistance when they were under exposure to some types of antibiotics, it was necessary to understand the mutational chance of the bacterium towards the current and future antibiotics. It would therefore be beneficial if the mutational progress can be monitored. It might then be possible to determine the dosage regimen to provide minimum or null the selective pressure for the selection of resistant mutants. It might even allow for prediction of possible newly emerging pathogens.

2.2. Development of Antibiotic Resistance in *Staphylococcus aureus*

2.2.1. Potential of Antibiotic Resistance of *Staphylococcus aureus*

One of the bacteria well known for its capability to acquire resistance was *Staphylococcus aureus*. Overuse of penicillin in the 1940s was linked to the development of penicillin resistance in *Staphylococcus aureus* within a short time. Methicillin was then introduced to treat infections by penicillin resistant strains in 1959. Shortly after, in 1961, resistance of *Staphylococcus aureus* to methicillin was reported [8, 9]. As of the time of writing (January 2007), strains of *Staphylococcus aureus* intermediately or highly resistant to vancomycin, a drug used to treat methicillin resistant *Staphylococcus aureus* (MRSA) infections, have been reported.

Most of the clinical isolates found to have intermediate resistance to vancomycin possessed thickened cell wall and was termed vancomycin intermediate *Staphylococcus aureus* (VISA) [97, 98]. Such VISA commonly possessed vancomycin minimal inhibitory concentration (MIC) of 8 or 16 µg/ml [99]. Patients infected by such isolates failed their vancomycin therapies. The truly vancomycin resistant strain, VRSA, obtained *vanA* gene encoding vancomycin resistance from vancomycin resistant enterococci either with [36, 100] or without [37] exposure to

vancomycin. Vancomycin MIC of VRSA was often as high as 64 µg/ml, up to 8 times higher than the MIC of VISA [99]. Considering its ability to acquire resistance relatively more easily than other bacteria, *Staphylococcus aureus* was dubbed as a “superbug” with a super genome [101, 102]. The term “superbug” was used more specifically to refer to MRSA whose reign as a dangerous and hard-to-eradicate infectious agent was well known [103].

Figure 1. Scanning Electron Microscopy (SEM) of *Staphylococcus aureus*

MRSA has the tendency to be multi-resistant to many types of antibiotics. It was known as one of the common causes of serious nosocomial infections and had manifested in serious community acquired infections as well [28]. *Staphylococcus aureus*, in general, is increasingly resistant to almost all types of antibiotics. Parts of Europe, USA, and Japan had recorded that 40-60% of *Staphylococcus aureus* isolated from all hospitals were MRSA [101]. In UK alone, in the year of 2002, the cost of nosocomial infections reached £1 billion per annum with MRSA as the main cause of concern [104].

Figure 2 shows the genomic sequences of two clinical strains of *Staphylococcus aureus*. It can be seen that MRSA and MSSA genome are collinear. Yet, the so called “core” genome is interrupted with some region of differences. MRSA252 has far more genomic islands compared to MSSA476 [105]. Many genomic islands in bacterial chromosome were known to be involved in human pathogenesis. Those particular genomic islands were called pathogenicity islands (PI) [106]. Genomic islands (Gislands) were formerly mobile genetic elements occurring as a distinct

unit on the core chromosome. Gislands were associated with tRNA loci whose 3' regions were frequently identical to the attachment sites of bacteriophages and therefore providing hot spots for the insertion of foreign DNA. It was known as well that genomic islands encode many different functions dependent on the environmental context where the bacteria possessing the genomes were isolated [107].

Figure 2. Schematic circular diagrams of the MRSA252 and MSSA476 chromosomes [105]

The presence of horizontally acquired genomic region in terms of genomic islands were also seen in *Staphylococcus aureus* strains Mu50, MW2, and N315 [106]. The presence of those exogenous genes encoding resistance and virulence factor in addition to a number of transposons and integrated plasmid showed the dynamicity of the genome. Therefore, there was clearly a possibility that *Staphylococcus aureus* could obtain more mobile genetic elements as it was exposed to more and more selective pressures.

2.2.2. Danger of the Antibiotic Resistant Mutants of *Staphylococcus aureus*

The potential danger of *Staphylococcus aureus* infections and therefore its mutated strain was very real since for human, anterior nares were the major reservoir of *Staphylococcus aureus* [31, 34, 108-110]. Kluytmans *et al.* reviewed a list of publications that linked the carriage of *Staphylococcus aureus* to the development of infections in various populations. The review mentioned that *Staphylococcus aureus* could be isolated consistently in the nares [108]. Carriage of *Staphylococcus aureus* in vestibulum nasi (anterior nares or nostrils of the nose) was very prevalent due to the relative absence of human defenses and/ or due to the capability of *Staphylococcus aureus* to defend against local antibacterial defenses [108].

Approximately 20% of humans were intranasally persistent carriers while another 60% of individuals are intermittently colonized. The rest of the populations were not colonized [109]. The higher rates of carriage happened in groups like dialysis patients, diabetics, parenteral drug users, and HIV-positive patients [21, 109]. It was suggested that despite not being the main factors, repetitive injection increased the *Staphylococcus aureus* colonization rate [111]. It was not clear on how the frequency of puncture wounds affect the intranasal colonization. Frequent antibiotic usages may also increase the colonization rate since antibiotic eliminates the normal flora and allows resistant microorganisms to flourish. Tetracycline resistant *Staphylococcus aureus* was generally found to colonize patients who were given tetracycline. However, it is acknowledged that determinants of *Staphylococcus aureus* carriage rate are complex. In infants, the host genetic factors were suggested as of overriding importance [112].

It was known, however, that the colonization in neonatal intensive care unit could even reach 70% before clinical infections indicated a problem [32]. Nevertheless, once the epidermal and mucosal barriers of the healthy host were compromised, the colonizing *Staphylococcus aureus* could get into the body. Once the bacterium was inside the body, it was capable of causing diseases from superficial wounds to deadly bacteremia. In bacteremia, the bacterium frequently penetrated to other

tissues (bones, joints, lungs, central nervous system, etc.) from the bloodstream [29, 110].

Infections due to *Staphylococcus aureus* were quite dangerous due to the many virulence factors possessed by the microorganism [30]. If the *Staphylococcus aureus* strains causing the infections were genetically mutated, for example MRSA strains, the danger was heightened due to the need for a careful choice of antibiotic used for treatment. More and more cases of MRSA colonization were being discovered in the community such as nursing homes and even general population who had no history of hospitalization. The strains acquired more compact resistance genes which provided them with better fitness to compete with the susceptible strains commonly found in the settings [8, 31, 113, 114].

Therefore, considering the prevalence *Staphylococcus aureus* in the population in general and its capability in acquiring drug resistance, it would be beneficial to understand its development of antibiotic resistance. Furthermore, it was known that there was a large potential of overuse and abuse of antibiotics in the population [86, 87]. Consequently, the pressure that the population unknowingly applied to the bacteria increased the possibility of the emergence of new antibiotic resistance.

2.3. Quinolone Resistance

2.3.1. Activity of Quinolone

As mentioned in Section 2.1., antibiotics provide selective pressure for the selection of resistant mutants. In the study of the emergence of antibiotic resistance reported here, quinolone was the antibiotic class used. Quinolone is a synthetic antibiotic accidentally isolated as a by-product during the synthesis of the antimalarial compound chloroquine in 1962 [115]. Antibiotics from quinolone class possess core structure as shown in Figure 3. The modifications to the X and R_x arms provide a whole possible range of quinolones. The modifications alter the

antimicrobial activity, pharmacokinetics, and metabolic properties of the quinolones compound.

Figure 3. Core structure of antibiotics from the class of quinolone [61]

The discovery of nalidixic acid as the first of the quinolone class spurred the synthesis of other quinolones used commercially today. Nalidixic acid as the original or so called first-generation quinolone is not active against aerobic, gram-positive bacteria and anaerobic bacteria. Key changes made to the core structure created further generations of quinolone. Arguably, the most important change was the addition of fluorine atom at the position of C-6 [115]. The change resulted in the use of the term fluoroquinolone to denote non first-generation quinolones. The addition of the fluorine atom increased DNA gyrase inhibitory activity, facilitated drug penetration into the bacterial cell, and provided activity against staphylococci.

Newer generations of fluoroquinolones was then synthesized with further modifications to provide greater potency and activity. Nevertheless, the presence of fluorine had been related to genotoxicity and central nervous defects. Optimization of all substituents had consequently allowed the removal of fluorine atom from R₆. The removal heralded the rise of fourth generation quinolones, termed des-fluoroquinolones [61, 115].

Despite the claim of genotoxicity, fluoroquinolones are still widely used and are mainly active against gram-negative bacteria and a variety of gram-positive. They were generally used in urinary tract infections [116]. The clinical effectiveness had also been shown in treatment from uncomplicated skin and soft tissue infections to nosocomial pneumonia [115]. Furthermore, their excellent bioavailability of between 80-95% and good distribution in body fluids and tissues allowed their use in infections that previously could only be treated parenterally [73].

Antibiotics from the quinolone class, in general, targets type II topoisomerases, which consist of topoisomerase II (DNA gyrase) and topoisomerase IV. Type II topoisomerases are key enzymes involved in DNA replication. Both DNA gyrase and topoisomerase IV are tetrameric structures comprising of 2 subunit pairs. The pairs belonging to topoisomerase II (DNA gyrase) are called GyrA (2 monomers) and GyrB (2 monomers), which are encoded by *gyrA* and *gyrB* genes respectively. Topoisomerase IV also has A and B subunits, which are encoded by the *griA* and *griB* genes respectively in *Staphylococcus aureus* [115]. GyrA is similar in structure to GriA while GyrB is similar in structure to GriB.

DNA gyrase is responsible for introducing negative supercoils into DNA during DNA packing and for relieving torsional stress existing ahead of transcription and replication complexes. Topoisomerase IV on the other hand is involved in the separation of interlocked DNA strands to allow segregation of daughter chromosomes to daughter cells. During the cleaving process, an enzyme reaction intermediate is formed. Quinolone acts on type II topoisomerase by stabilizing the enzyme reaction intermediate. In the DNA-enzyme complex, both DNA strands are cleaved and covalently linked to the breakage-reunion subunits. The stabilization of the complex prevents the completion of DNA synthesis and a series of events leading to cell death ensues. The complete mechanism of the lethal action was not completely known yet [61, 115, 117-119]. Yet, some antibiotics belonging to the quinolone class were known to select for more resistant mutants possessing as few as single- or two-step mutation from the populations of quinolone sensitive *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, and *Streptococcus pneumoniae* [7, 93, 94, 120].

2.3.2. Development of Ciprofloxacin Resistance

Ciprofloxacin was one of the early fluoroquinolones used clinically and was praised for its activity including against MRSA. Figure 4 shows how ciprofloxacin resulted from the modification of R₁, R₆, and R₇ position of the core quinolone structure presented in Figure 3. As said in the previous section, the addition of fluorine atom

at the position of C-6 or R₆ was the main change that classified ciprofloxacin into the fluoroquinolone class [115].

Figure 4. Structure of Ciprofloxacin [121]

At the time of introduction, ciprofloxacin was considered as an alternative therapy to treat infections caused by MRSA since it was shown to have good activities against both methicillin susceptible and methicillin resistance *Staphylococcus aureus* (MSSA and MRSA respectively) [122, 123]. Nevertheless, ciprofloxacin was not normally prescribed for MRSA infections since it was not the front line drug for therapy of the infection. The main activity of ciprofloxacin is against gram negative cocci. Yet, it was one of the most frequently prescribed fluoroquinolones in the United States in 2005 [115].

Ciprofloxacin was at times used for topical treatment of necrotizing otitis externa and otitis media. It was also prescribed for the topical treatment of acute bacterial conjunctivitis. Otitis externa, otitis media, and bacterial conjunctivitis are normally caused by *Staphylococcus aureus* [124, 125]. Unfortunately, many reports of ciprofloxacin resistant variant of clinical MRSA isolates were reported in the early 1990s [126]. There are two mechanisms which convey ciprofloxacin resistance to *Staphylococcus aureus*:

1. Alteration of target sites
2. Reduction of antibiotic concentrations inside bacteria

2.3.2.1. Ciprofloxacin Resistance by Alteration of Target Sites

The first mechanism, which occurs by alteration in target sites, results from mutations in the genes encoding DNA gyrase and topoisomerase IV [118, 127].

While the main target of fluoroquinolone in gram-negative organisms is DNA gyrase, the main target of fluoroquinolone in *Staphylococcus aureus* is DNA topoisomerase IV [128, 129]. Therefore, first-step mutations conferring reduced ciprofloxacin susceptibility in *Staphylococcus aureus* occur commonly in the main drug target, that is topoisomerase IV [61, 128]. Quinolone-resistance-determining-region (QRDR) is a conserved region in many bacteria that determines quinolone resistance. The QRDR regions include GyrA and GyrB subunits of DNA gyrase; and GrlA and GrlB subunits of DNA topoisomerase IV [118].

A single amino acid change in GrlA subunit is sufficient to reduce the ciprofloxacin susceptibility of the *Staphylococcus aureus* strain to exceed clinical laboratory breakpoints [61, 118, 128]. The single mutation in QRDR generally reduces the susceptibility by 4 to 8-fold while the second mutation at other site would raise the resistance level [61]. Nonetheless, a single change in GrlA might reduce the susceptibility of the strain to ciprofloxacin but not necessarily to other more potent fluoroquinolones [118]. Some of the common single step mutation in the QRDR region of *grlA* gene, which increased the ciprofloxacin MIC of the mutants, is listed in Table 2.

Table 2. Mutations in the *grlA* gene of single step mutant of *Staphylococcus aureus* strains with higher ciprofloxacin resistance than the parent strain (The complete name of the amino acid abbreviation can be found in Appendix G)

Codon	Amino Acid Change	Ciprofloxacin MIC ($\mu\text{g/ml}$) ^a	Reference
80	Ser -> Phe	3.13-12.5*	[119]
80	Ser -> Tyr	8**	[130]
80	Ser -> Ile	1-2	[131]
84	Glu -> Lys	1.56	[132]

^aCiprofloxacin MIC was determined in the presence of efflux pump inhibitor except (*) where only part of the strains were tested with the inhibitor and (**) where none of the strain were tested with the inhibitor

Mutations in *gyrA* gene are silent (not affecting the encoded protein) in the absence of mutations in *grlA* gene [130, 133]. However, the alteration of GyrA protein that occurs after mutations had taken place in *grlA* gene usually increases the level of

ciprofloxacin resistance [130]. On the other hand, there were only a few reports on first step mutation in GrlB subunit that produced ciprofloxacin resistance and no report was found on first step mutation of *gyrB* gene which was related to quinolone resistance in *Staphylococcus aureus*. A mutation at codon 470 of *grlB* gene from asparagine to aspartate was responsible for an increase in the MIC of ciprofloxacin (2 µg/ml). It was hypothesized that mutations in *grlB* gene reduced the catalytic efficiency of topoisomerase IV. Consequently, the amount of drug-enzyme-DNA complex was reduced and fewer events leading to cell death were initiated. However, the GrlB single step mutant was hypersusceptible to coumarin group of antibiotics (novobiocin and coumermycin). The reason for the hypersusceptibility is still unknown [133].

2.3.2.2. Ciprofloxacin Resistance by Reduction of Antibiotic Concentration inside Bacteria

The second mechanism leading to reduced susceptibility of fluoroquinolone in general involves alteration of access to target sites via reduction of intrabacterial concentrations [61, 118, 127, 128]. Such mechanism also frequently reduces the susceptibility of the strain to other antibiotics classes. Therefore, the alteration may create a multi drug resistant (MDR) strain [118].

Increased efflux of antibiotic molecules from the bacterial cell provides the cell with sub-optimal exposure to the antibiotic. Sub-optimal exposure favors the selection of more resistant subpopulations [134]. In some cases, the efflux pump mediated resistance reaches clinical significance [135]. Most of the time, however, such resistance is normally at a low level since it does not prevent entry of antibiotics completely. Therefore, a clinically significant resistance is often accompanied by mutations at other genes. The efflux pump can be encoded by chromosomal gene or plasmids. Since the expression of such efflux pumps reduces the entry of antibiotics in general, it was suggested that such a pump could be the reason for the intrinsic resistance observed in many bacteria (Section 2.1.1) especially if the pump was expressed constitutively [61].

Some strains of *Staphylococcus aureus* were found with an increased expression of an efflux pump called NorA and consequently possessed a low level ciprofloxacin resistance [118]. In general, such efflux pump mechanism had also been found in *Streptococcus pneumoniae* [136], *Pseudomonas aeruginosa* [66], *Stenotrophomonas maltophilia* [137], *Salmonella enterica* [138], and *Escherichia coli* [139]. Many other bacteria were found with multiple putative efflux pumps. The presence of such pumps was detrimental to the effectiveness of antibiotic in therapy [140].

Though mutation in *norA* gene was quite commonly found in *Staphylococcus aureus*, their effect on the ciprofloxacin resistance was quite minor compared to the effect conferred by mutations in type II topoisomerases. NorA-contributed ciprofloxacin resistance had been reported to exist at the absence of any mutations in the QRDR sites [141]. The existence of such resistant strain was found even in clinical strains [131]. It was suspected that there might be other efflux pumps not inhibited by reserpine, which was used to inhibit NorA protein, existing in *Staphylococcus aureus*. Those putative efflux pumps might have contributed to the diverse quinolone resistance level [142].

Fluoroquinolone itself have been noted time and again to be a mutagenic agent capable of producing an adaptive mutation or a stress-induced mutagenesis in *Staphylococcus aureus* [88]. The presence of ciprofloxacin or antibiotics in general puts the bacteria under stress, which might increase their mutation rates. Therefore, bacteria under infective process should have higher mutation rate *in vivo* compared to what determined in *in vitro* studies [89]. The selection of ciprofloxacin in inducing an adaptive mutation is such that the resulting mutant would likely be still susceptible to other antibiotic classes.

The mutant selection capability of ciprofloxacin is well known. Many studies showed at times the emergence of ciprofloxacin *Staphylococcus aureus* mutants after exposing the bacteria to the drug for as little as one day [54]. Therefore,

ciprofloxacin is an ideal choice of antibiotic for use in the study of a system devised for monitoring mutation-in-progress.

2.4. Methods Used to Monitor Mutation-in-Progress

2.4.1. Phenotypic Methods Used to Monitor Mutation-in-Progress

Currently, there is only one method to monitor “mutation-in-progress”. The method involves monitoring growth trend by performing standard plate count, which is a phenotypic-based method. Surprisingly though, no effort has been made to devise a dedicated system to study mutation in progress. Ba *et. al.* described a system for simulation of pharmacokinetics and pharmacodynamic [50]. Their system was capable of simulating some complex drug kinetics although accessories required was rather complex. Yet, despite the use of such system, the study relied on standard plate count technique to monitor the trend of the viable bacterial number.

At the time of writing (January 2007), the emergence of resistance was normally monitored through the change in growth curve of the bacteria [50-53, 56, 93, 137]. Although these studies utilized many different growth conditions and vessels, all of them relied on standard plate count technique to determine the emergence of resistance. Under antibiotic exposure, an increase of bacterial count was used as an indication of the presence of resistant mutants. A typical graph showing the development of the bacterial viable count is shown in Figure 5.

Figure 5. Growth trend obtained using standard plate count during incubation of MRSA 8043 and MRSA 8043L0-1 in levofloxacin containing broth [52]

As shown in Figure 5, the increasing growth trend obtained using standard plate count was a clear indicator of the existence of mutants capable of proliferating in the antibiotic-containing medium. However, the plate count technique required a lot of human intervention and many resources. Therefore, this technique to monitor the emergence of antibiotic resistant was not feasible for laboratory without extensive resources and well-trained personnel.

Studies on the emergence of resistance were also preferably done on broth medium instead of on agar. It is easier to manipulate the pharmacokinetic and pharmacodynamic profiles of a broth medium rather than with an agar medium. Agar medium usually has a fixed antibiotic concentration and therefore has limited ability to simulate drug concentration profiles.

Monitoring mutation by examining physiological changes shown in the medium allowed monitoring of expressed mutations. Since not all mutations endowed the bacterium with resistance to antibiotics, only mutations involving continuous

expressed resistance or inducible resistance received attention from scientists. Such resistance could well be detected by a phenotypic method which relied heavily on the monitoring of physiological changes.

2.4.2. Molecular Methods Used to Detect Mutation

No publication was found on the topic of monitoring mutation-in-progress using molecular methods. The high cost of molecular methods was perhaps the discouraging factor. Moreover, it is not yet possible to monitor changes on the entire genome at the same time. However, when a specific region was known to be involved in the resistance during the monitoring process, a microarray could be employed to study the resistance.

Tang *et. al.* [143] showed that their microarray was capable of detecting point mutations on a specific allele conferring drug resistance in *Mycobacterium tuberculosis*. There were some molecular assays, which were capable of detecting some known mutations conveying antibiotic resistance, sold commercially [144, 145]. Yet, no effort had been attempted to use a microarray to screen the hybridization of the whole genome. Furthermore, a microarray was normally employed as a confirmatory measure but not as a monitoring tool since the array required bacterium already grown for some time in either broth or agar plates. An array was only useful when the oligonucleotide sequences used were known to exist in the bacterium studied.

The high cost of manufacturing an array precluded the use of such an array in a monitoring effort. To really monitor using molecular method, a device, perhaps a microarray, capable of monitoring point mutations in the whole genome would be needed. Monitoring by sequencing the total genome of the bacterium would also serve the same purpose. However, both processes were definitely very costly and more importantly, they were not yet feasible with the technology at the time of writing (January 2007).

By monitoring changes in the nucleotide sequence, mutation can be detected but the phenotypic changes may be unknown. Studies on the evolution of ciprofloxacin resistance in *Staphylococcus aureus* strains have encountered silent mutations [51, 52, 119, 128]. Consequently, such mutations that do not impart any phenotype changes can be detected as false positive during molecular studies using polymerase chain reaction (PCR) [146].

2.4.3. Advantage of Phenotypic Methods in Monitoring Mutation-in-Progress

One clear advantage of monitoring the phenotypic changes to determine drug resistance is in the detection of inducible mutations. There were some cases of inducible mutations, for example inducible clindamycin resistance in *Staphylococcus aureus* strain. The strain modified its ribosome when it was exposed to clindamycin and thus became clindamycin resistant [147]. Such resistance would only be detectable when physiological changes involving clindamycin resistance was monitored based on the phenotypic expression. In addition, novel antibiotic resistances could be determined and monitored via the physiological changes as long as the resistances were expressed.

A monitoring study implies a rather frequent data acquisition. Therefore, the high cost of molecular assays and the requirement for skilled personnel discourages frequent use of molecular methods. Therefore, it is preferable to have an automatic, easy-to-use and relatively less resource intensive phenotypic system, which is capable of monitoring such mutations in progress. The automation of the system reduces human intervention and amount of materials needed. The system can be easily employed to monitor mutation in progress that took weeks and even months.

2.5. Feasibility of Spectroscopy as a Monitoring Method

2.5.1. Basic of Spectroscopy

Spectroscopy is a study of molecular structure and dynamics through absorption, emission, and scattering of electromagnetic radiation. In absorption process, electrons on ground state are excited to higher state by passing a radiation field through the molecules. The energy transferred from the radiation field to the electron should be the same as the difference in the energy between the excited and the ground state, as described by the following equation [148].

$$E_1 - E_2 = h \nu \quad (1)$$

Where,

E_1 = energy excited state (J)

E_2 = energy at ground state (J)

h = Planck constant (6.626×10^{-34} Js)

ν = frequency of the vibration (Hz)

The whole range of electromagnetic radiation has been utilized in spectroscopic method. Each radiation corresponds to certain process undergone by the atoms in the molecules subjected to the radiation (Figure 6). Some bacteria related phenomena have already been studied successfully with spectroscopy. Some of them will be reviewed in the subsequent subsections.

Figure 6. Electromagnetic spectrum and its effect on atom and molecule [149]

2.5.2. Feasibility of Vibrational Spectroscopy as a Monitoring Method

Among all spectroscopic methods, vibrational spectroscopy (either infrared absorption spectroscopy or Raman spectroscopy) is relatively more widely used since it is capable of producing fingerprint type of spectra. The unique spectra of compounds under scrutiny by vibrational spectroscopy are produced according to the molecular structure of the compounds [148, 150]. Efforts to use infrared absorption spectroscopy in the identification of bacteria and bacterial related compounds were started in 1950s [151-153].

Though infrared spectrum was taken in real-time, infrared spectroscopy could not be easily implemented to monitor mutation-in-progress. The detail and fingerprinting nature of the spectra demanded a highly pure sample. On the other hand, samples used in mutational studies contain mixture of mutants and the parent strain. There was also a need of major statistical analysis on infrared spectra. The spectra were normally compared with provided databases for identification or classification purpose. Raman spectroscopy faced the same challenges as well [154-156].

It might be feasible to perform a monitoring study using vibrational spectroscopy technique on samples grown on solid media if there was a system capable of taking the spectra of numerous small size colonies with highly accurate spatial positioning.

To use such a system, the large number of bacteria on the plate had to be spread out with a good spatial distribution and setting. It was normally difficult to spread the bacteria evenly since there are normally up to 300 colonies that needed to be distributed.

2.5.3. Feasibility of Fluorescence Spectroscopy as a Monitoring Method

Fluorescence spectroscopy was another spectroscopic method commonly used in the studies with bacterial sample. Fluorescence spectroscopy derives its signal from the emission of photon by excited electrons when it relaxes back to the ground electronic state after absorption of photon during its ground state. Normally used for quantitative measurement of fluorescent molecules in liquid sample, fluorescence detection was particularly superior from absorption measurement due to its zero background detection. The excitation signal was normally filtered off to prevent it from reaching the detector as a background noise [148].

The intrinsic fluorescence signal (without help from external fluorescence probe) from bacteria was normally obtained at the wavelength characteristic of the amino acid tryptophan whose excitation was normally at 270 or 290 nm [157, 158]. Spectra obtained by exciting aromatic amino acids and nucleic acids, NADH, and FAD at 250 nm, 316 nm, and 380 nm respectively had also been used for bacterial identification purpose [159, 160]. Spectra corresponding to one or more excitations were then used by itself or together as a database for pattern recognition by statistical analysis.

Since the conventional set-up was quite bulky, Werkhaven *et. al.* used an optical fiber attached to a spectrofluorometer to perform identification of 3 bacterial species in otitis media [161, 162]. Using the limited library generated from seven cases, they showed that the optical fiber set up actually managed to perform well in species identification. Despite all those studies on the potential of using fluorescence spectroscopy to identify bacteria, the identification was at species level only.

Mutated pathogens differ in strain level from their parent. Even though there were efforts to classify up to the level of strains, these studies were usually performed with pure cultures [158, 160]. It is potentially difficult to differentiate what subpopulations exist in the heterogeneous samples encountered in a mutational study. The differentiation of spectra of various mixed broth samples needs much more complex work compared to the differentiation of spectra of various pure samples. When spectra from micro colonies grown on agar plates are obtainable, colonies with different spectrum compared to the spectrum of the original strain may constitute mutated strains. However, the micro colony may be a mixture of original and mutated strain.

2.5.4. Feasibility of Nuclear Magnetic Resonance (NMR) Spectroscopy as a Monitoring Method

Nuclear Magnetic Resonance (NMR) spectroscopy is widely used to determine the structure of a molecule. NMR is a phenomenon experienced by the nuclei of some atoms when they are placed in a static or oscillating magnetic field. Different nuclear spin alignment possesses different energy levels. Absorption of radio frequency radiation by the nuclear of an atom allows the nuclear spin to realign or flip to a higher energy state. Reemission of radio frequency radiation then allows the nucleus to return to a lower energy state. The transition energy of an atom depends on the position of the atom in the molecule [163-165].

NMR is capable of producing a spectrum unique to the bacterium. The spectrum would be a unique combination of signals depending on the constituents of the cell wall and cytoplasm. Accordingly, bacteria harvested from plate culture were used as samples when NMR was used as the identification method [166-168].

The position and intensity of the spectral peaks, however, was dependent on the growth condition. Relative shift of the position of the peak was less significant compared to the change of intensity when two samples of the same species were grown in two different media [167, 168]. Therefore, there was a need to standardize

the growth medium in the NMR study. The scientists interested in the bacterial study using NMR could perhaps agree on a particular medium for a particular bacterium.

NMR spectra allow assignment of peaks to different kind of molecules. Therefore, the technique can be used to study the biochemistry nature of the bacterial sample. The NMR equipment was, however, still rather expensive and not commonly available in small scale laboratories [166]. Pattern recognition work was also required to classify closely related species due to the low dissimilarity of the magnetic resonance spectra.

Ohara *et al.* observed that Nuclear Magnetic Resonance (NMR) spectra could be used to tell the differences in cell composition in distinct isolates of the same species. They also suggested that there might be correlation between DNA and NMR spectra [169]. Although NMR possessed some advantages for identification purpose, there had not been any NMR studies on sample containing more than one type of bacteria.

Bourne *et. al.* encountered difficulties in identifying closely related species. They did not manage to separate three bacterial species based on their NMR spectra since the spectra were similar. The metabolites produced by the three species were physiologically homogeneous and therefore all those species produced the same spectra [167]. However, using a different pattern recognition technique, NMR spectra from two Methicillin resistant *Staphylococcus aureus* (MRSA) strains with identical DNA patterns were considered similar in another study. There were even similarities between NMR spectra of living and disintegrated bacteria [169]. Hence, it was not possible to use NMR spectra to differentiate live and dead mutant strains in a mixed sample. Considering that mutation-in-progress could easily involve many mutants, a detection of mutants in a sample might not indicate the existence of a thriving mutant.

2.5.5. Feasibility of UV-Visible Spectroscopy as a Monitoring Method

UV and visible range of radiation were of interest in the project since the interactions between matter and electromagnetic radiation in the visible region manifests visibly as colour [170]. However, changes in the electronic level of an atom can be accompanied by changes in the vibrational quantum level. Therefore, the whole energy changes manifested as visible colour, which are contributed by factors more than just the electronic level [171].

Visible spectrum is just one small range inside the wide range of electromagnetic radiation. Though the end point for visible spectrum is normally set at 400 and 800 nm in Figure 7, the range considered in the project was 400-700 nm since 700 nm is considered as a starting point for near Infrared region [150].

Figure 7. Range of visible spectrum [148]

UV-Visible spectroscopy is built upon Beer-Lambert law which can be described by Equation (2) [172]:

$$A = \epsilon lc \quad (2)$$

Where,

- A = Absorbance
- l = Pathlength of absorbing solution (cm)
- ϵ = Molar absorption coefficient ($\text{mol}^{-1} \text{dm}^3 \text{cm}^{-1}$)
- c = Concentration of absorbing species (mol dm^{-3})

Beer-Lambert law applies strictly to monochromatic light which impinges homogenous and isotropic solutions. If polychromatic light and/or concentrated analyte solution is used, the law is not obeyed. The law is obeyed for polychromatic light only if the test is performed on an instrument where pathlength could be changed with tests on different concentration of the analyte solution [173].

UV-visible spectroscopy was used in a study to monitor streptococci's activity [62]. In that study, pH changes correlated well to the spectral intensity change over the incubation time. Using the fiber optic set up, the bacterial activity was monitored for two hours. In another study, growth kinetics of *Chlorobium thiosulfatophilum* was monitored via changes in turbidity [63]. The effect of light attenuation due to the formation of sulfur particle upon photosynthetic capability of the bacterium was monitored for more than three days.

Visible spectroscopy had also been used to study the interaction of *Desulfovibrio desulfuricans* during its interaction with Molybdenum (Mo) thin films. The colour difference of the different complexes produced during exposure of the bacterium to different Mo environment was well recorded [174]. Production of indigo had also been followed for >2 hours during bacterial expression of recombinant mammalian P450 enzyme by monitoring the absorbance value at 665 nm [175].

Considering the ability of UV-visible spectroscopy, particularly visible spectroscopy, in performing detection and short-time monitoring studies, visible spectroscopy could feasibly be used to monitor mutation in progress. The ability of a spectrometer to record minuscule colour and intensity changes may allow detection of small physiological changes happening in a bacterium if the changes were reflected in the spectroscopic sample.

2.6. Mathematical Model of the Emergence of Antibiotic Resistance

There are a number of published mathematical models describing microbial growth in relation to various growth conditions. However, few models were developed

solely to describe monitoring of mutation. This section of the report describes some basic bacterial growth models and other mathematical models related to the emergence of antibiotic resistance.

Various parameters were introduced by different groups of researchers according to specific test conditions and apparatus. Unfortunately, different groups used different symbols for the same parameters. This section reviews those models and uses the same symbol for the same parameter. A symbol used for more than once will only be described when it first appears.

2.6.1. General Growth Model

Bacteria normally grow by following four phases of life (Figure 8) [1]:

1. Lag phase is experienced when the bacteria adjust their metabolism machinery to allow themselves to live in the new environment. This phase is defined by a slow or no division
2. Exponential phase is experienced when the bacteria start to grow exponentially after the adjustment performed in the lag phase. In this phase, growth rate far exceeds the killing rate
3. Stationary phase is experienced when nutrients are exhausted and bacterial growth is inhibited by the metabolites or other types of inhibition. Therefore, stationary phase is indicated by an equal growth rate and killing rate.
4. Death phase is experienced when the growth rate is exceeded by the killing rate due to the growth inhibition caused by the unfavourable environment

Figure 8. Typical bacterial growth curve in a batch culture

Bacteria grows by binary fission and therefore the increase in bacterial number can be expressed as [1]:

$$N(t) = N_0 \cdot 2^n \quad (3)$$

Where,

$N(t)$ = number of bacteria at a point of time (CFU/ml)

N_0 = number of starting bacteria (CFU/ml)

n = number of generations

Since it was easier to get the generation time instead of number of generations, the following equation was introduced

$$n = \frac{t}{g} \quad (4)$$

Where,

t = time (h)

g = generation time (h)

Therefore,

$$N(t) = N_0 \cdot 2^{t/g} \quad (5)$$

In order to ease the calculation, a growth rate, μ , was introduced as follows

$$\mu = \frac{\ln 2}{g} \quad (6)$$

Where,

$$\mu = \text{growth rate (h}^{-1}\text{)}$$

Introduction of Equation (6) to Equation (5) changed the growth equation to a familiar exponential growth equation,

$$\begin{aligned} N(t) &= N_0 \cdot 2^{t/g} \\ \Leftrightarrow \ln N(t) &= \ln N_0 + \frac{t}{g} \cdot \ln 2 \\ \Leftrightarrow \ln N(t) &= \ln N_0 + \mu \cdot t \\ N(t) &= N_0 \cdot e^{\mu \cdot t} \end{aligned} \quad (7)$$

Therefore, the change of bacterial number over time can be described as:

$$\frac{dN(t)}{dt} = \mu N(t) \quad (8)$$

Exponential growth described in Equation (8) was used to determine the number of bacteria. It only worked well when the bacteria in the population divided exponentially. The equation could not, however, describe the growth at other phases of bacteria culture (Figure 8). In fact, there was a limit or a maximum amount of bacteria when the population was grown in a batch culture. The limit was imposed by nutrient limitation, metabolite inhibitions, quorum sensing, and other growth limiting factors [1].

2.6.2. Mathematical Model of Growth in the Absence of Antibiotic

The various limiting conditions posed by a growth environment dictated that the growth rate was by no means constant. Therefore, the terms μ was set to vary with time although there was a maximum value of $\mu_{\max}$. Baranyi and Roberts introduced a general function to describe the inhibition function affecting the lag and stationary phase [176]. The function was generally an extension of Equation (8) and can be seen in the following equation:

$$\frac{dN(t)}{dt} = \mu_{\max} \alpha(t) u(N(t)) N(t) \quad (9)$$

Where,

$$\begin{aligned} \mu_{\max} &= \text{maximum growth rate (h}^{-1}\text{)} \\ \alpha(t) &= \text{adjustment function for lag phase} \\ u(N(t)) &= \text{inhibition function for stationary phase} \end{aligned}$$

Equation (9) describes a function where the bacterial number $N(t)$ grows with time to approximate $N_{\max}$ asymptotically as time, t , increases. Richard's family of growth curves are one of the common growth models which follow Equation (9) although the curves family was developed some time before the introduction of Equation (9). Richard's family of growth curves can be described as follows [176]:

$$\frac{dN(t)}{dt} = \mu_{\max} \left(1 - \left[\frac{N(t)}{N_{\max}} \right]^R \right) N(t) \quad (10)$$

Where,

$$\begin{aligned} N_{\max} &= \text{maximum bacterial number} \\ R &= \text{curvature parameter} \end{aligned}$$

Equation (9) and many other functions includes an inhibition function described as $u(N(t))$. The inhibition function used in Richard's family of growth curves is

$$\left(1 - \left[\frac{N(t)}{N_{\max}}\right]^R\right) \quad (\text{Equation (10)}). \text{ When } R = 1, \text{ Equation (10) was called logistic or}$$

Pearl-Verlhurst growth model.

Since it was known that previous growth condition would affect the current growth especially in terms of the lag time, Baranyi and Robert introduced adjustment function, $\alpha(t)$, to the general model (Equation (9)). The function was to indicate the gradual adaptation of the bacterial cell to the new environment since the bacteria might come from different environment in the previous passage. A monotone increasing function, the adjustment function is described as follows [176, 177]

$$\alpha(t) = \frac{q(t)}{1 + q(t)} \quad (11)$$

Where,

$$q(t) = \text{physiological state}$$

Developing Baranyi and Roberts model further by incorporating more terms, Van Impe *et. al.* introduced what they called as more deterministic terms into the equation [178]. Product and substrate inhibition effect on the growth of a culture was included by formulating a monotonous decreasing effect for each factor. The Van Impe model can be expressed as

$$\frac{dN(t)}{dt} = \mu_{\max} \mu_Q(t) \mu_P(t) \mu_S(t) N(t) \quad (12)$$

Where,

$$\mu_Q(t) = \text{a term for adjustment function in lag phase}$$

$$\mu_P(t) = \text{a term for inhibition by toxic product}$$

$$\mu_S(t) = \text{a term for inhibition by substrate limitation}$$

It was clear that both Baranyi and Roberts model; and Van Impe model tried to explain the change of bacterial number as deterministic as possible. Inclusion of terms involving the components of the growth medium seemed to be the common idea. Simplification of Van Impe model for special cases was discussed [178]. Nevertheless, complete curve fitting required the researcher to define the inhibiting metabolites and the effect of diminishing substrate on bacterial growth. Such measurements, although was possible, would need quite a number of experiments which require a complete understanding of the medium and the metabolism of the bacteria. At the time of writing (January 2007), the metabolism of *Staphylococcus aureus* was still poorly understood. It was an irony since *Staphylococcus aureus* had been an important opportunistic pathogen for quite long [101].

A model with a total departure from the conventional growth rate type of growth model was introduced by Doona *et. al.* [179]. They modeled bacterial growth using the analogy of chemical reaction where the progress from one state to another (Figure 8) proceeded with a particular rate. The chemical reaction analogy was provided in Table 3.

Table 3. Quasi chemical growth model [179]

Stage	Reaction	Rate equation
Activation	$M \rightarrow M^*$	$v_1 = k_1.M$
Multiplication	$M^* \rightarrow 2M^* + A$	$v_2 = k_2.M^*$
Sensitized death	$M^* + A \rightarrow M^{**} + D$	$v_3 = (10^{-9}).k_3.M^*.A$
Natural death	$M^* \rightarrow D$	$v_4 = k_4.M^*$

“Quasi chemical” model described the activation of bacteria M to become M^* . After which activation, the bacteria was in the exponential phase and multiplying. Product A was produced as a metabolite and was hypothesized to sensitize the bacteria to death D via the intermediate M^{**} . Natural death could also be triggered directly regardless of the effect of the metabolite A . The model seemed to perform well in describing the stationary and death phase of a culture. The growth model with its wealth of variables can be described with Equations (13) to (16) [179].

$$\frac{dM(t)}{dt} = -v_1 \quad (13)$$

$$\frac{dM^*(t)}{dt} = v_1 + v_2 - v_3 - v_4 \quad (14)$$

$$\frac{dA(t)}{dt} = v_2 - v_3 \quad (15)$$

$$\frac{dD(t)}{dt} = v_3 + v_4 \quad (16)$$

Bacterial growth model described so far involves terms that describes the growth/multiplication and growth inhibition/killing of bacteria. It was commonly perceived that some metabolites contributed to the growth rate reduction and even bacterial killing. Hence, antibiotics were inherently of the same nature as the inhibiting metabolites even though antibiotics itself came from an external source, not produced by the bacteria in study. Most models presented to this point do not include a limiting value for the maximum bacterial density except the Richard's family of growth curves.

2.6.3. Mathematical Model of Growth in the Presence of Antibiotic

Growth models developed for bacterial growth in the presence of antibiotic were not very different from the models reviewed in section 2.6.2. The most obvious differences among the models were the inclusion of killing rate and antibiotic effect. Due to the lack of standards, different parameters were used in different equations [180, 181].

Any models had the tendency to be limited to a particular experiment due to the use of a particular method and equipment. Therefore the models seemed to lose a certain level of versatility. Nevertheless, the common idea for models to account for the antibiotics action was the inclusion of Hill function to describe the rate of change of viable bacterial number. Hill function was an increasing sigmoidal function which response was limited with an upper bound value. The function was almost predominantly used to describe the antibiotic concentration effect on

bacterial growth. More commonly used in enzyme studies [182], the function is described in Equation (17).

$$y = V_{\max} \frac{x^H}{a^H + x^H} \quad (17)$$

Where,

$V_{\max}$ = maximum value of the response

a = constant

H = Hill coefficient

The use of Hill function in a growth model created the so called $E_{\max}$ model [183]. The model was a saturating function. In Hill function, Hill coefficient was used to describe the steepness of the linear phase of the sigmoid curve. The constant defined the lag portion of the curve.

The most simplistic model which utilized Hill function and a modification of Equation (8) was presented by Mouton *et. al.* [181]. In the model, Equation (8) was basically supplemented with the effect of antibiotics by the means of a killing rate. The terms, EC_{50} , which described the antibiotic concentration at which 50% of maximum killing rate was obtained, was used in the model as well. The model was

$$\frac{dN(t)}{dt} = \left(\mu - k_{\max} \frac{C^H}{C^H + EC_{50}^H} \right) N(t) \quad (18)$$

Where,

$k_{\max}$ = maximum killing rate (h^{-1})

C = concentration of the antibiotic (mg/L)

EC_{50} = concentration at which 50% of maximum killing rate was obtained (mg/L)

Hill function was also used by de la Pena *et. al.* in their study with cefaclor, a cephalosporin antibiotic [184]. Unfortunately, the fitting done on growth curves of

four different strains during their exposure to cefaclor was limited to 8 hours of incubation. Simulations on 24 hours exposure were performed based on the parameters obtained. More comprehensive result could have been presented if more data points were used in the curve fitting.

Growth rate was dependent on the initial inoculum, the maximum density that a test vessel could contain, and the nutrient limitation. De la Pena *et. al.* included a modifying term into Equation (18) to account for maximum bacterial number that the vessel can support. The modified equation was

$$\frac{dN(t)}{dt} = \left(\mu \left[1 - \frac{N(t)}{N_{\max}} \right] - k_{\max} \frac{C^H}{C^H + EC_{50}^H} \right) N(t) \quad (19)$$

Where,

$$N_{\max} = \text{maximum number of bacterial density (CFU/ml)}$$

The value of $N_{\max}$ depends upon the particular bacteria in that particular setting. It is generally agreed that a particular set of environment can only support a bacterium to a certain maximum density. Therefore, if the model is to be used in a different experimental vessel, it is warranted that a characterization study is performed with the check on the maximum density in particular.

Yoshitaka *et. al.* [185] proposed a model similar to Mouton model. The model can be represented as follow:

$$\frac{dN_1(t)}{dt} = \left(\mu N_{\max} \left[1 - \frac{N_1(t)}{N_{\max}} \right] - k_{\max} \frac{C}{C + EC_{50}} \right) N_1(t) - \frac{dN_2(t)}{dt} \quad (20)$$

$$\frac{dN_2(t)}{dt} = K_1 N_1(t) - K_2 N_2(t) \quad (21)$$

Where,

$$K_1 = \text{transfer rate constant from compartment 1 to compartment 2 (h}^{-1}\text{)}$$

$$K_2 = \text{transfer rate constant from compartment 2 to compartment 1 (h}^{-1}\text{)}$$

Instead of having only $N_1(t)$ as the modifier for the first component in the model, as in Mouton model (Equation (18)), Yoshitaka *et. al.* [185] used the maximum density. Yoshitaka model also showed a preference for a simple saturating exponential effect to illustrate the effect of antibiotic concentration on the killing rate. The concentration effect on the model was therefore more pronounced than the Mouton model, which was sigmoidal in nature. Yoshitaka model incorporated two compartments with antibiotic exposure at one compartment only. Unfortunately, no explanation was given about how both compartments functioned or related to each other. The curve fitting was performed using five points per curve and might be the reason of why the R^2 value is < 0.9 .

Departing from the common usage of growth rate, μ , Meagher *et. al.* [186] in their study of extended release ciprofloxacin formulation on *Escherichia coli* introduced few unique terms. It was once again noticed in Meagher model that the usage of Hill function was indeed very common. The model is shown below

$$\frac{dN(t)}{dt} = \left(VG_{\max} \frac{1}{N_m + N(t)} - K_d \cdot \left[1 + K_{d_{\max}}^f \frac{\left[\frac{C}{MIC} \right]^H}{SIT_m^H + \left[\frac{C}{MIC} \right]^H} \right] \right) N(t) \quad (22)$$

Where,

- $VG_{\max}$ = maximal velocity of bacterial growth (CFU/ml.h)
- N_m = bacterial number at which replication rate is half maximal (CFU/ml)
- $K_{d_{\max}}^f$ = maximal fractional increase in non-antibiotic bacterial death rate
- SIT_m = serum inhibitory titer, the value of $\frac{C}{MIC}$ at which
the replication rate is half maximal
- K_d = non-antibiotic bacterial death rate (h^{-1})

Two subpopulations, sensitive and intermediately sensitive, model was found to fit the experimental data better than single population model. Therefore, the model produced two values of SIT_m . Nevertheless, it was observed that introduction of

half maximal effect complicated the curve fitting since the estimation of SIT_m and $K_{d_{\max}}^f$ were difficult [185].

In general, all models presented could be said to follow the general equation presented in Equation (9). The models uniformly relied on a growth term and some limiting functions. There was no metabolites related function included in any of the models presented for studies with antibiotics. Perhaps it was generally felt that the killing posed by the antibiotics was far more dominant compared to the inhibition by the metabolites. The models also revealed that the only measure of bacterial growth to the point of writing (January 2007) was viable bacterial count.

2.6.4. Mathematical Model of the Emergence of Antibiotic Resistance

The emergence of antibiotic resistance during or after antibiotic exposure complicated the decision on treatment regimes. Therefore, many studies were conducted to assess the amount of antibiotic resistant mutants produced after the exposure to certain antibiotics. The study on the emergence of antibiotic resistance during antibiotic exposure normally included some mathematical models. Generally, incorporation of an adaptation rate to the general models presented in the previous section was done.

The most simplistic growth model testing on the possibility of emerging resistant mutants was presented by Wiuff *et. al.* [187]. The study was conducted to understand the declining antibiotic sensitivity of a culture over time. One of the hypotheses put up was the emergence of resistant mutants. The model for the hypothesis split the populations into two subpopulations. Nonetheless, the simple population model did not account for how resistant mutants could occur. Wiuff model is as follows:

$$\frac{dS(t)}{dt} = (\mu_s - k_s) S(t) \quad (23)$$

$$\frac{dR(t)}{dt} = \mu_R R(t) \quad (24)$$

Where,

μ_S = growth rate of the sensitive subpopulation (h^{-1})

k_S = death rate of the sensitive subpopulation (h^{-1})

μ_R = growth rate for resistant subpopulation (h^{-1})

Growth model by Lipsitch and Levin [188] also introduced two subpopulations, i.e. sensitive and resistant subpopulations. Since the dosage regimen was an intermittent one, the antibiotic concentration was increased by C^0 at each dosing interval, τ , i.e. $C(t = n.\tau) = C(t = n.\tau - dt) + C^0$. The value of C^0 was a product of D/V , drug dosage over the distribution volume. Lipsitch and Levin model is as follows:

$$\frac{dS(t)}{dt} = \left(\mu_S - k_S \frac{C(t)}{C(t) + EC_{50}} \right) S(t) \quad (25)$$

$$\frac{dR(t)}{dt} = \left(\mu_R - k_R \frac{C(t)}{C(t) + EC_{50R}} \right) R(t) \quad (26)$$

$$\mu_S = b - K_d \quad (27)$$

Where,

$S(t)$ = amount of sensitive subpopulation (CFU/ml)

$R(t)$ = amount of resistant subpopulation (CFU/ml)

k_R = killing rate for resistant subpopulation (h^{-1})

EC_{50R} = concentration at which 50% of maximum killing rate was obtained for resistant subpopulation (mg/L)

b = rate of cell division (h^{-1})

In the model, the emergence of resistant subpopulations was modeled by a stochastic component. The conversion from susceptible to resistant occurred as a Poisson process with rate μ when the sensitive bacteria replicated. The amount of

R was set to increase by 1, $R+1$, in time interval dt , with a probability equal to $\mu bS(t)dt$ [188].

Lipsitch and Levin model was nearly identical with Mouton model (Equation (18)). The main exception was the split of populations into two subpopulations and the accommodation of the model for the stochastic emergence of resistant mutant from the sensitive subpopulations. No experimental data was fitted in the publication although some simulations were performed. The model also assumed the emergence of a single resistant mutant every time interval dt , which implied that there would be a lot of resistant mutant strains at the end of the study. The model also did not to include the popular $N_{\max}$ term.

Instead of introducing stochastic component, Mouton used adaptation rate to indicate the gradual increase in resistance over time. Equation (28) shows how the adaptation rate increases the contribution of the first term to increase the amount of population. The increase of the population number was again used to indicate the emergence of resistant mutants.

$$\frac{dN(t)}{dt} = \left(\mu \left[1 - \frac{N(t)}{N_{\max}} \right] \left[1 - e^{-\alpha t} \right] N(t) - k_{\max} \frac{C(t)^H}{C(t)^H + EC_{50}^H} \right) N(t) \quad (28)$$

Where,

$$\alpha = \text{adaptation rate}$$

The same usage of adaptation rate was found in the work by Liu *et. al.* [189] though in Liu model, the term $(1 - e^{-\alpha t})$ was also used as a modifier of the antibiotic concentration effect. The inclusion of an exponentially saturating adaptation rate to the killing rate was arguably more plausible than what was done in Mouton model. The adaptation rate could be said to contribute to the declining antibiotic effect on the population since mutants emerged. The emergence of resistance mutants reduced the killing rate effect so as to increase the bacterial number. Liu model was presented as follows:

$$\frac{dN(t)}{dt} = \left(\mu - k_{\max} \frac{C_f^H(t)}{EC_{50}^H + C_f^H(t)} [1 - e^{-\alpha t}] \right) N(t) \quad (29)$$

Where,

C_f = unbound antibiotic concentration at the site of action (mg/L)

Different from other works, Liu and coworkers explicitly acknowledged that only unbound antibiotic concentration would be interacting with the bacteria. Moreover, the unbound antibiotic concentration was known to differ according to the site of infection [7]. Liu model did not consider the maximum capacity of the test vessel as well.

Another unique inclusion of adaptation rate was done by Tam *et. al.* [190]. In the model, a special adaptation function was used to modify the contribution of EC_{50} in the Hill function. The Tam model was a simple modification of Mouton model (Equation (18)). Tam model is as follows:

$$\frac{dN(t)}{dt} = \left(\mu \left[1 - \frac{N(t)}{N_{\max}} \right] - \left[k_{\max} \frac{C(t)^H}{C(t)^H + (\alpha EC_{50})^H} \right] \right) N(t) \quad (30)$$

$$\alpha = 1 + \beta [1 - e^{-C(t)\tau}] \quad (31)$$

Where,

β = maximal adaptation

τ = rate of adaptation factor (L/mg.h)

Another obvious departure from the model used in Mouton model [181] and Yoshitaka model [185] was the exclusion of $N(t)$ in the first component of the differential equation. Tam model hypothesized that higher antibiotic concentration would make the adaptation process faster. The adaptation function envisioned an almost instant adaptation at very high antibiotic concentrations. This idea was in contrast with the proposal of Mutant Prevention Concentration (MPC) [93, 96, 191], where it was argued that high enough antibiotic concentration would provide

no chance for the bacteria to adapt or even mutate. The bacteria would be simply killed too fast, which was the reason why contamination of high concentration antibiotic stock solution was considered extremely rare [192].

A more complex model was proposed by Campion *et. al.* [53]. A total of three models with three different levels of complexities were proposed. It was found that model belonging to the intermediate complexity level could perform better. The model was as follows:

$$\frac{dS(t)}{dt} = \left(\mu_s \left[1 - \frac{S(t) + R(t)}{N_{\max}} \right] - k_s \frac{C(t)}{C(t) + EC_{50}} \right) S(t) \quad (32)$$

$$\frac{dR(t)}{dt} = \left(\mu_R \left[1 - \frac{S(t) + R(t)}{N_{\max}} \right] - k_R \frac{C(t)}{C(t) + EC_{50}} \right) R(t) \quad (33)$$

The model was clearly a combination of Lipsitch model (Equations (25) and (26)) and Mouton model (Equation(18)). Mainly derived from Lipsitch model, the model from Campion and co-workers included the maximum carrying capacity of the vessel. A more complex model, which accounted for the emergence of mutants by the rate of m was found to have less optimal curve fit.

$$\frac{dS(t)}{dt} = \left(\mu_s \left[1 - \frac{S(t) + R(t)}{N_{\max}} \right] - m \left[1 - \frac{S(t) + R(t)}{N_{\max}} \right] - k_s \frac{C(t)}{C(t) + EC_{50}} \right) S(t) \quad (34)$$

$$\frac{dR(t)}{dt} = \left(\mu_R \left[1 - \frac{S(t) + R(t)}{N_{\max}} \right] - k_R \frac{C(t)}{C(t) + EC_{50}} \right) R(t) + m \left[1 - \frac{S(t) + R(t)}{N_{\max}} \right] S(t) \quad (35)$$

Where,

m = the appearance rate of resistant mutant R from susceptible parent S

The probable failure of the more complex model to fit better than the intermediate complex model could have stemmed from the use of m . The determination of mutation rate, m , was performed by incubating the bacteria under 2-times the MIC value [193]. On the other hand, *in vitro* test performed by Campion and co-workers were using various antibiotic concentrations [51-53]. The mutation rate under

different antibiotic concentration could be different. Furthermore, fluoroquinolone used in their study, as being said earlier in section was known to produce higher mutation rate during prolonged exposure to the antibiotics [88, 89]. The more complex model could have performed differently under more suitable test conditions.

It could be seen that most of the growth model used to describe the emergence of mutants indeed stems from the notion of an exponential growth with the effect of growth rate reduced by the killing rate. The most general form of the equation can be described as follows:

$$\frac{dN(t)}{dt} = \left(\mu \cdot [U_{\mu}(N(t), N_{\max})] - k \cdot [U_k(C(t), EC_{50})] \right) \cdot N(t) \quad (36)$$

Where,

$$U_{\mu}(N(t), N_{\max}) = \text{modifier of growth rate}$$

$$U_k(C(t), EC_{50}) = \text{modifier of killing rate}$$

Another modifier terms for emergence of resistance was then included in many ways. Nevertheless, most of the model incorporated empirical terms without providing much explanation on what the terms would mean. Inclusion of more realistic terms, which could be derived from better understanding of the interaction of antibiotics and bacteria, might provide a better model.

Chapter 3

MATERIALS PREPARATION

It was mentioned earlier in Chapter 1 that the focus of the project would be on the use of an *in vitro* system to monitor mutation-in-progress. Taking the emergence of antibiotic resistance as a pilot model of mutation-in-progress, the phenotypic method was employed. The advantages of phenotypic method included the possible monitoring of yet-unknown new phenotypic traits, the viability study of the mutant, and the reduction of resources needed. Furthermore, at the time of writing (January 2007), the conventional culturing methods were still the gold standard in medical diagnostics laboratories. It was expected that microbiological culture methods would probably never be completely abandoned [45].

The project aimed to devise/introduce a novel system capable of performing the monitoring of mutation-in-progress. Therefore, the project involved a new system, the corresponding materials, and the test protocol. In the study, culturing methods used for the determination of the antibiotic resistance were based on standards championed by CLSI (Clinical and Laboratory Standards Institutes) [192, 194-196]. The standards advocated the use of Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) as the growth media.

In the study of the emergence of antibiotic resistance, the broth medium used was Mannitol Salt Broth (MSB) instead of MHB. The salt content in the mannitol salt medium is selective for Staphylococci [93]. Furthermore, the metabolism of mannitol is the specific characteristic of *Staphylococcus aureus* which is well known in its identification [197]. The colour production due to the production of acetate was instrumental towards the use of spectroscopy in the monitoring technique. On Mannitol Salt Agar (MSA), *Staphylococcus aureus* normally produces circular, smooth, and opaque yellow colony with yellow zone indicating mannitol fermentation [2].

3.1. Preparation of Antibiotic Stock Solution

Antibiotics were used in the study to induce mutation. Oxacillin and Ciprofloxacin were the antibiotics of choice in this work. Oxacillin stock solution was prepared by dissolving oxacillin powder (Sigma-Aldrich) in sterile deionized water. A few drops of 0.1 N sodium hydroxide (NaOH) was added to help the dissolution of ciprofloxacin powder (Fluka) in sterile deionized water. The amount of powder required for a particular amount of diluent was calculated according to the following equation [198]:

$$V = \frac{m.P}{c} \quad (37)$$

Where,

- V = Volume of diluent, in this case sterile deionized water (ml)
- m = Mass of antibiotic powder (mg)
- P = Potency of antibiotic powder ($\mu\text{g}/\text{mg}$)
- c = Concentration of stock solution needed ($\mu\text{g}/\text{ml}$)

The manufacturers reported that the potency of the oxacillin powder was 939 $\mu\text{g}/\text{mg}$ while the potency of the ciprofloxacin powder was 986 $\mu\text{g}/\text{mg}$. Antibiotic stock solution was made with the concentration of 1,000 $\mu\text{g}/\text{ml}$. The solution was stored in Eppendorf tubes at -80°C .

3.2. Preparation of Growth Media

3.2.1. Preparation of Agar Media

The agar growth media used in this project were Mannitol Salt Agar (MSA) and Mueller Hinton Agar (MHA). Both agar media were purchased in dehydrated form from Merck and Remel respectively. For dehydrated MSA, 108 g of granules were dissolved per 1 liter of deionized water while for dehydrated MHA, 38 g of powder was used. The diluted slurry was stirred manually to achieve complete dissolution

in a Pyrex bottle. The agar solution was sterilized at 121 °C for 20 minutes. After that, the solution was cooled to around 40 °C and poured into 10 mm diameter Petri dishes until the agar solution reached a depth of around 2-3 mm. The whole process was carried out in a biological safety cabinet to prevent contamination.

The lid of the Petri dish was then kept ajar to help the agar solidify. The lid was put back to its covering position once condensation was not visible on the lid. The prepared agar plates were incubated in air at 36-37 °C for 24 hours and checked for contamination. Plates were considered as contaminated when one or more colonies were found on it. Contaminated plates were discarded while sterile plates were then stored at 4 °C for not more than two weeks before used.

3.2.2. Preparation of Agar Slants

Agar slant was used to keep a working culture of bacteria. The method to prepare agar slants of both MSA and MHA was similar to the method described in section 3.2.1. However, for slants, sterile test tubes were used to contain the medium instead of Petri dishes. Each tube contained around 15 ml of agar solution. An empty culture tube was used as a resting place to help the agar form the slant as described in Figure 9.

Figure 9. An agar slant is positioned for cooling

The slant was air cooled by loosening the screw cap of the test tube. Pouring and cooling processes were done inside biological safety cabinet. The slants were incubated in air at 36-37 °C for 24 hours and checked for contamination as indicated by the existence of any colonies. Contaminated slants were discarded

while sterile slants were then stored at 4 °C for not more than two weeks before used.

3.2.3. Preparation of Broth Media

Besides solid media, liquid media were also used, namely Mueller Hinton Broth (MHB), Mannitol Salt Broth (MSB), and Luria Bertani Broth (LB broth). MHB was prepared from dehydrated media purchased from Remel while MSB and LB broth were prepared by mixing several ingredients in the proportion described in Appendix A. In preparing MHB, 21 g of the MHB powder was dissolved in 1 liter of deionized water at room temperature while ingredients of MSB and LB broth were dissolved and boiled by using a microwave. All broth solutions were then cooled to room temperature. The pH of MHB and MSB was adjusted to 7.2 ± 0.2 while the pH value of LB broth was adjusted to 6.8 ± 0.2 . Since pH measurement was dependent of the temperature, all adjustments were made at room temperature.

The broth was then dispensed into clean test tubes according to the desired amounts. The tubes containing the broth were sterilized at 121°C for 20 minutes. During sterilization process, the screw cap of the tubes was not tightened. After sterilization, the tubes were screwed tightly and the sterile medium was stored at 4 °C for not more than two weeks before used.

3.2.4. Preparation of Glycerol-Containing Broth Media

In order to preserve some culture, glycerol was added into LB broth which was then stored at -80 °C [199]. The LB broth was mixed with 98% glycerol (Duchefa Biochemie) to reach 80% v/v concentration based on Equation (38). The final glycerol concentration used in this study is 40 % v/v after equal volume of culture was mixed with the prepared LB broth.

$$V_1 \cdot M_1 = V_2 \cdot M_2 \quad (38)$$

Where,

V_1 = Volume of antibiotic stock solution (ml)

M_1 = Concentration of antibiotic stock solution ($\mu\text{g/ml}$)

V_2 = Volume of broth (ml)

M_2 = Concentration of antibiotic in the broth ($\mu\text{g/ml}$)

The broth containing the glycerol was dispensed into Eppendorf tubes and then sterilized at 121 °C for 20 minutes. The lids of the Eppendorf tubes were kept ajar to prevent pressure build up. After sterilization, the tube were capped and kept in 4 °C for not more than two weeks before use.

3.2.5. Preparation of Antibiotic-Containing Agar and Broth Media

Antibiotic stock solution, which was stored at -80 °C (section 3.1), was thawed in room temperature. The solution was then added into cooled sterile broth (< 40 °C) according to Equation (38). Generally, the antibiotic concentration of the broth was prepared in two times the final concentration required for the experiments. An equal volume of sample, which is prepared in antibiotics-free broth, was then added to the antibiotic-containing broth to achieve the final concentration.

Antibiotic-containing agar was prepared using the same method as the broth. However, in contrast to the preparation of antibiotic-containing broth, the concentration was prepared at its final amount. The antibiotic was added into lukewarm agar solution before the agar solution was poured into Petri dishes and allowed to solidify. The prepared agar was kept in 4 °C for not more than two weeks before use. The antibiotic dilution was calculated according to Equation (38). All antibiotic inoculation processes were performed inside a biological safety cabinet.

3.3. Preparation of Culture Stock Solution

Reference bacterial strains were used in the study to eliminate the need to characterize the strains. *Staphylococcus aureus* ATCC strains were purchased from ATCC via Remel or Biogen in the form of culti-loops® or freeze dried powder. Commonly used methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300, a heterogeneous oxacillin resistance strain, and methicillin sensitive *Staphylococcus aureus* (MSSA) ATCC 29213 were purchased. Both strains were recommended by CLSI for use in quality control.

If the strain came in the form of a culti-loops®, the loop was lightly pressed to the surface of a MHA plate for around 10 seconds and then streaked across the plate surface for several times. If the strain is provided in freeze dried form, the isolates are hydrated with 2 ml of LB broth. The suspension was then vortexed before a loopful suspension was streaked across MHA plate surface. The streaked MHA plates were then incubated in air for 24 hours. Some of the resulting colonies were subjected to one more passage on a sterile MHA plate for another 24 hours before being inoculated to LB broth. All incubations were performed in air at 30 °C.

After the growth in the LB broth was confluent, 0.5 ml of the broth was mixed with 0.5 ml sterile glycerol-containing LB broth prepared in section 3.2.4. Consequently, the final glycerol concentration of the 1 ml solution was 40%. The tubes were then kept in -80 °C.

3.4. Preparation of Working Culture

A working culture was defined as the culture kept alive by re-culturing them in a fresh medium in a regular interval so as to provide bacteria for experiments. The stock culture was first thawed inside biological safety cabinet and 10 µl inoculating loop was then used to streak the culture to an MHA plate. The plate was incubated at 30 °C for 24 hours. Some of the resulting colonies were then cultured to agar slants.

One slant for each strain was stored in a refrigerator at 4 °C and re-cultured into fresh slant every week. The working culture is then re-cultured in air at 30 °C for 24 hours to provide colonies for the experiments. A maximum of four passages of the working culture were performed for each stock culture. Therefore, working culture had to be prepared from stock culture each month.

Chapter 4

SYSTEM FOR MONITORING MUTATION-IN-PROGRESS

It is earlier mentioned in the objectives (Section 1.1.) that the project intended to devise a novel system and technique to monitor mutation-in-progress. The description of the system is provided in this section along with the preliminary setup leading to the final design of the system.

4.1. Initial Optical Set-up

Based on the literature review (Chapter 2), UV-Visible spectroscopy was selected as the method of choice in building the system for monitoring mutation-in-progress. The spectroscopy method was chosen since the method was used in several bacterial characterization studies. To further investigate the possibility of using the method in the system, some initial characterization works were performed. An optical set-up was prepared according to Figure 10 for use in the experiments.

Figure 10. Optical set-up used in characterization works

The set-up shown in Figure 10 above consisted of a light source, a sampling unit, a spectrometer (S2000), a data acquisition unit, patch cord optical fibers (Ocean Optics), and a focusing lens. Earlier in the study, tungsten halogen lamp (LS-1, Ocean Optics) was used as the light source. The media was shown to experience changes mainly in the green colour region. Therefore, a green LED whose emission peaked at 575 nm was used to replace the tungsten halogen lamp as the light source. To ensure a stable LED emission, an electrical circuit was designed to control the LED. The circuit is shown in Figure 11.

Figure 11. Schematic of the circuit for the LED used in the initial optical set-up

The characterization works confirmed the possibility of using visible spectroscopy to monitor physiological changes of the viable bacteria levels in liquid culture. These tests were correlated with lab-based viable count technique. Therefore, a novel system was devised to harness the ability of the spectroscopy technique in monitoring mutation-in-progress.

4.2. Schematic of the System for Monitoring the Mutation-in-Progress (SMMIP)

The optical set-up shown in previous section was successfully used to monitor physiological changes of the bacteria. Therefore, the set-up was incorporated into the design of the SMMIP whose schematic of the system is shown in Figure 12. It can be seen that the components set-up contained in the blue dotted box is similar to that of Figure 10.

Figure 12. Schematic of the System for Monitoring Mutation-in-Progress (SMMIP)

The system consisted of the following components:

1. Detection System (DS)

The set-up of the Detection System (DS), components in blue dotted box, were based the arrangement shown in the initial optical set-up (Figure 10). In the characterization work, sample incubation was interrupted by spectral reading activity since the detection unit (Figure 10) was not placed inside the incubator. Since SMMIP was designed to enable a continuous monitoring, the detection unit was housed inside the Controlled Environmental Chamber (CEC). Nevertheless, the electronic components, i.e. light source and spectrometer, were not housed inside the CEC to prevent the electronic components from experiencing heat related variation. Two detection units was made and incorporated into the CEC.

Black colour Delrin® or polyoxymethylene was used to manufacture the detection unit. An LED with emission peak at 575 nm was used for the light source. Solid

state timer with an 8-bit programmable micro controller (AT89C51 from Atmel) was used to control the active time of the LED. For the detector, the SD 2000 spectrometer (Ocean Optics) was utilized. The specification for the spectrometer can be found in Appendix B.

2. Controlled Environmental Chamber (CEC)

The Controlled Environmental Chamber (CEC) was made to provide a stable culture environment. In the author's laboratory setting, the chamber could be set to provide incubation temperature ranging from room temperature to 40 °C. The heating power of the chamber was provided by four pieces of 10 W (12 V) lamps. The lamps were installed in the center of the four sides of the chamber's stainless steel body. The temperature of the chamber was measured by a solid state precision temperature sensor, LM35CZ (National Semiconductor), which was mounted to the mid section of one of inner body's four corners. The sensors provided temperature measurement with ± 0.2 °C accuracy (at 25 °C). A programmable micro controller was used to control the heating provided by the lamps. Therefore, the temperature of the chamber was within ± 0.4 °C of the intended incubation temperature.

A shaker unit was also installed based on the assembly shown in Appendix D to provide intermittent orbital mixing to the sample. The mixing would help to homogenize the sample. The orbital movement was produced by off-centering the motor shaft. The moment produced by the off-centering was balanced by the counterweight. A ball bearing was also installed to balance the platform while mixing process was in place and the orbital movement was present. The control for the shaker unit was integrated with the temperature control circuit.

3. Replenishing System (RS)

The nutrient contents of a growth medium gradually depletes as the bacteria grows. At the end of an incubation period of 24 hours, the content of the bacterial growth medium becomes significantly depleted. Therefore, in order to compare the bacterial growth between days, the starting nutrient content should be similar. A nutrient replenishing process was therefore required.

A programmable NE-100 Syringe Pump (New Era Pump Systems, Inc.) was used with the Replenishing System (RS). As seen in Figure 12, a 0.22 μm syringe filter was fitted to the syringe pump. The filter was then connected to a tube which was then connected to the sample. A sterile 20 ml syringe (Terumo) was used to contain 20 ml sterile fresh broth for replenishing purpose. The syringe filter further ensured the sterility of the medium that was going into the sample. The tube was replaced each day with a new sterile tube to prevent contamination. New syringe containing sterile broth was also supplied each day. At the start of each day, the replenishing system provided 4 ml of sterile broth to the sample.

4. pH Control System (pHCS)

In this study, growth between days were compared and studied. In order to do so, the starting pH of the sample must be similar to provide a similar starting environment. However, at the end of an incubation period, which was normally 24 hours, the pH of the sample might be different from the starting pH. Therefore, pH adjustment might be necessary to return the pH of the sample to the pH at the start of the study. The pH was adjusted only after the nutrient replenishing process by Replenishing System was done.

A NE-100 Syringe Pump (New Era Pump Systems, Inc.) was used to adjust the pH of the sample every time replenishing process was done. A sterile 10 ml syringe (Terumo) was used to contain sterile 0.01 M NaOH. Considering the small volume needed for pH adjustment and the rare contamination of highly alkali NaOH solution, no syringe filter was needed. However, sterile Nalgene tubing was used to pass the alkali solution to the sample. Once the peak of the spectral reading of the sample coincided with the initial spectral reading, the syringe pump was set to extract the remaining NaOH solution from the tubing.

5. Extraction System (ES)

The replenishing process by the Replenishing System (RS) provided 4 ml of extra volume to the sample. Therefore, an Extraction System (ES) was needed to return the volume of the sample to 1 ml. Extraction System (ES) had an NE-100 Syringe Pump (New Era Pump Systems, Inc.). No syringe filter was used in this case. A

sterile 10 ml syringe (Terumo) was used to withdraw the excess volume of the sample after nutrient replenishing in order to return the volume of the sample to 1 ml. The extracted sample was then used for Population Analysis Profile (PAP) test and other tests.

6. Control Unit (CU)

A Pentium IV with processor speed of 3.20 GHz and RAM of 512 MB with OOIBase32 software (Ocean Optics, Inc.) installed was used to acquire the data obtained by the spectrometer. The spectral reading from the spectrometer was continuously acquired every 30 minutes. Each reading was stored under a format readable by Microsoft Excel® and then analyzed accordingly.

4.3. Electrical Circuitry of the System for Monitoring Mutation-in-Progress (SMMIP)

Three control circuits were utilized for the SMMIP, namely LED Control Circuit, Shaker Control Circuit, and Temperature Control Circuit. The schematic of the control circuits are shown in Appendix E-1, E-2, and E-3 respectively.

4.3.1. LED Control Circuit

The LED Control Circuit was used to regulate the active time of the light source such that it would be active for 4 minutes every 30 minutes. The on and off timing was controlled by an 8-bit micro controller (AT89C51) from Atmel. The step function graph programmed into the micro controller is described in Figure 13. The timing was made such that the LED was provided with 2 minutes warming up time for spectral stabilization before data acquisition was performed. Overall, a total of 4 minutes of the LED service life was used for every spectral reading. The schematic of the control circuit for the LED Control Circuit is shown in Appendix E-1.

Figure 13. State of the light source used in SMMIP

4.3.2. Shaker Control Circuit

In order to mix the sample, an orbital platform was integrated into the Controlled Environment Chamber (CEC). The orbital movement was provided by off-centering the shaft connecting the motor shaft and the counterweight. The moment produced by the platform on the motor was balanced by a counterweight. The motor shaft was secured to the shaft attached to the counterweight by an M3 set screw. The exploded view of the assembly of the shaker unit is provided in Appendix D.

The mixing action lasted for 3 minutes and ended 1 minute before every spectral reading was taken. First reading was taken without orbital mixing since the sample was already mixed before being placed into the detection unit. Therefore, the first value of the step function graph on the micro controller program was set to low as shown in Figure 14.

Figure 14. State of the shaker used in SMMIP

The schematic of the circuit for the shaker control is shown in Appendix E-2. The micro controller was programmed to produce high and low signal accordingly such as to control the relay for the motor. A geared 12 V DC motor (Crouzet) capable of producing up to 27 rpm was used in the shaker assembly. A variable resistor allowed the user to tune the speed of the orbital shaking movement by varying the voltage supplied to the motor.

4.3.3. Temperature Control Circuit

Precision centigrade temperature sensor, LM35CZ (National Semiconductor), produced output voltage which was linearly correlated with the temperature it senses. The sensor featured a scale factor of 10.0 mV/°C and a capability to sense - 40 °C to 110 °C. The voltage produced by the sensor was digitized by a 16-bit Analog to Digital Converter (ADC), LTC1605, from Linear Technologies. The digitized voltage value was then sent to the 8-bit micro controller that converted the value into centigrade temperature. The temperature sensed was displayed on an alphanumeric 8x2 LCD display (Powertip Technology) up to one decimal value. The schematic is shown in Appendix E-3.

Due to the high current needed for the operation of the lamp used for the heater, two step-down transformers (Block) with 5 A current rating were utilized. Each transformer produced 1 output of 12 V to power 2 lamps set in series (Appendix E-3). The CEC walls consisted of two layers of stainless steel 13 mm thickness. Each sheet was separated by a 15 mm empty space. Each lamp was placed at the centre of the empty space. The contacts of the voltage supply to the lamp were facilitated by two relays (Siemens) with switching current up to 8 A.

The lamp control was programmed into the micro controller. For incubation temperature of 30 °C, electrical connections to the lamps were simultaneously stopped when the temperature of the chamber reached 29.9 °C. The electrical connections were restored when the temperature of the chamber was lowered to 29.5 °C or below. Any change of incubation temperature could be performed by changing the program burnt into the micro controller.

Chapter 5

EXPERIMENTAL PROCEDURES

5.1. Procedures of Standard Plate Count Technique

In this study, the amount of viable bacteria was determined by standard plate count technique. The sample was diluted serially and then plated on agar plates. The dilution was performed by mixing 1 ml of bacterial suspension to 9 ml of sterile broth or sterile deionized water. This way, 10 times dilution was achieved. Before dilution, the suspension was vortexed in order to homogenize the sample. Each agar plate was then inoculated with 100 μ L of suspension taken from the appropriate dilution step. Duplicate of plates were used for each dilution step to provide better counting accuracy. The plates were then incubated in air at 30 or 36 °C and counted after 24 hours or more. Normally, four to six dilution steps were necessary to get a reasonable amount of colonies on the agar.

Each colony found on an agar plate was considered to have originated from a single bacterium. Since the colony might have also resulted from a cluster of bacteria, the measurement unit used in the plate count technique is colony forming unit (CFU). Colony number was only noted if the amount was between 3 and 300. If the colony number was below three, the counting result was commonly considered as an error since the colonies could have been the result of a contamination. On the other hand, if there were more than 300 colonies present on an agar plate, the amount of bacteria was noted as “too many to count” (TMTC). It was acknowledged that such huge amount of bacteria was difficult to count with reasonable accuracy.

The colony counting was performed by the help of a colony counter (Stuart). A 10^{-5} dilution plate with 30 colonies indicated that the original 10^0 suspension might have 30×10^6 CFU/ml since only 100 μ l was used for inoculation. Therefore, the amount of viable bacteria per ml in original suspension was calculated by taking the average of the amount of colonies on the incubated plates.

5.2. Procedures of Latex Agglutination Test

To confirm that the bacteria used in the experiments were *Staphylococcus aureus*, latex agglutination tests were sometimes performed. Some circular opaque yellow colonies were tested for clumping factor and surface antigen specific to *Staphylococcus aureus* using latex agglutination test kit. The latex agglutination test kits were purchased from Dry Spot Staphytest Plus (Oxoid).

According to the directions from the manufacturer, 50 µl of 0.85% saline solution was introduced to each blue reagent dot. Colonies were picked from an agar plate and dispersed in the saline homogeneously. The slide with the dot was then rocked for 10-30 seconds. The formation of blue latex beads during the rocking process indicated the positive presence of tested factors. No negative test results were found throughout the course of study.

5.3. Procedures of Scanning Electron Microscopy (SEM) Imaging of *Staphylococcus aureus*

Scanning Electron Microscopy (SEM) was performed for some samples of the *Staphylococcus aureus* strains to understand the morphological change caused by the exposure to antibiotics. A broth culture was used for the SEM imaging. Firstly, the bacterial suspension was centrifuged at 8,000 rpm for 10 minutes in a 4 °C using a refrigerated centrifuge (Kubota). The supernatant was then discarded and the pellet was washed by 1 x Phosphate Buffered Saline (PBS) prepared in-house from 10 x PBS buffer of cell culture grade (US Biological). The centrifuging and washing process was repeated for two times.

After three times of washing using sterile PBS, the bacterial pellet was fixed in 2.5% Glutaraldehyde (Sigma) solution. The suspension was incubated in 4 °C for 3 hours. Thereafter, the bacterial suspension was again washed and centrifuged for three more times. Glutaraldehyde fixed the bacterial cells by introducing cross-links

between different tissue components, particularly proteins, nucleic acids and lipids [200].

The Glutaraldehyde was washed away by using sterile PBS solution. The washing process was repeated for 3 times. The final bacterial pellet was then ready for dehydration. A gradual dehydration process was performed by washing the bacterial pellet with 30%, 50%, 70%, 80%, 90%, and 100% Ethanol (Fluka) [200]. The bacterial suspension in each ethanol concentration was incubated for five minutes in room temperature. Following the incubation, the suspension was centrifuged at 8,000 rpm in 4 °C and the supernatant was extracted. The next dehydration step would then follow.

The final bacterial suspension in 100% Ethanol was then processed onto a cover slip after the incubation period was done. For each SEM sample, 30 µl of the suspension was dropped onto a cover slip. Both sides of the cover slip were pre coated with gold. The gold coater was set to 20 mA and 80 seconds duration in order to produce a gold thin film of 10 nm thickness. The cover slip containing the suspension was then dried in air by putting it inside Biological Safety Cabinet (BSC) for 30 minutes. Then, the cover slip was kept in dry cabinet before gold coated with the same parameter as the previous coating process. After that, the sample was ready for microscopy.

5.4. Procedures of the Detection of Antibiotic Resistance

5.4.1. Procedures of Agar Diffusion Susceptibility Test

Agar diffusion susceptibility test was another common method used to determine antibiotic susceptibility of bacteria [194]. In this study, agar diffusion susceptibility test was used to determine the oxacillin resistance of *Staphylococcus aureus*. In the test method, bacterial suspension containing $\sim 5 \times 10^5$ CFU/ml was inoculated and spread on MSA. One piece of 1 µg oxacillin disk (Oxoid) was placed at the centre of the plate. Upon 24 hours incubation, the size of the inhibition ring was measured.

The end of hazy appearance was taken as the end of the inhibition zone. An inhibition zone diameter of <16 mm was used to indicate oxacillin resistance [77].

5.4.2. Procedures of Minimal Inhibitory Concentration (MIC) Dilution Test

Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration of an antimicrobial agent that prevented visible growth of a microorganism in an agar or broth dilution susceptibility test [192]. In this study, both agar and broth dilution tests were performed to determine the ciprofloxacin resistance of the strains but not the oxacillin resistance. Only disk diffusion susceptibility was performed to determine oxacillin resistance since the study involving oxacillin did not require information on how resistant the bacteria was. On the other hand, during the monitoring of the emergence of ciprofloxacin resistance, the degree of ciprofloxacin resistance was needed. MIC dilution test was conducted to provide such information.

The MIC dilution test method used in this study was the macro broth dilution method. Colonies were again suspended in MHB to the number $\sim 10^8$ CFU/ml. Serial dilution was then performed by using sterile MHB to produce a bacterial suspension of 10^6 CFU/ml. 1 ml of the suspension was then mixed with another 1 ml of MHB containing two times the intended ciprofloxacin concentration. The broths were then incubated in air at 30 °C for 24 hours or 48 hours. The broth producing no visible growth was considered to contain ciprofloxacin MIC of the strain. The final ciprofloxacin concentration used in the broth dilution tests are 0.0625, 0.125, 0.25, 0.5, 1, 2 µg/ml.

5.4.3. Procedures of Population Analysis Profile (PAP) Test

It was not possible to obtain the MIC of the bacteria if the final bacterial suspension contained $< \sim 5 \times 10^5$ CFU/ml. In addition, MIC value only described the overall apparent antibiotic resistance. In order to determine the heterogeneity of the population in terms of antibiotic resistance, Population Analysis Profile (PAP)

testing was the only available method [201]. PAP tests entailed the incubation of several dilution steps (ideally all) to agar containing various antibiotic concentrations.

To perform a PAP study, serial dilution was performed using sterile deionized water. 100 µl suspension from various dilution steps were plated on the agar plates containing ciprofloxacin from 0.0625 µg/ml to 2 µg/ml. The plates were incubated for 48 hours before counting was performed due to the slow growth resulting from growth inhibition by the antibiotic. The counting procedure followed the standard plate count technique described in section 5.1. A plot of bacterial number (CFU/ml) against antibiotic concentration was produced at the end of PAP test. Since the least amount of valid bacterial count on a plate was 3, the detection limit of PAP test was 300 CFU/ml. The plating was performed in duplicates.

5.5. Procedures of the Media and System Characterization Studies

5.5.1. Procedures of the Experiments to Study the Change of Medium pH during Bacterial Growth

Some tests were conducted in order to understand how the medium's pH changed during bacterial growth process. Various amounts of bacteria were inoculated into 30 ml of MSB. A suspension of 1 ml was extracted to provide the initial inoculum. Another 10 ml of the suspension was then poured into a Pyrex beaker. The rest of the suspension was incubated and used to determine the development of viable bacterial count during the test.

A pH probe of HI98230 (Hanna Instruments) pH Meter was then placed into the beaker containing 10 ml of the suspension. The pH probe was fastened by a parafilm to ensure constant immersion in the bacterial suspension. The whole progress of pH change of the sample was logged at a 30 minutes interval. The samples were incubated in air at 36 °C. The pH data was retrieved and processed by Microsoft Excel. Both MSSA ATCC 29213 and MRSA ATCC 43300 were tested.

5.5.2. Procedures of the Experiments to Determine the Light Source

In the early part of the characterization studies, a LS-1 tungsten halogen lamp (Ocean Optics) was used as a light source in order to narrow down the crucial wavelength regions. After some calibration tests, it was learned that the tungsten halogen lamp needed a warming up time of 30 minutes to produce relatively stable spectral reading. Therefore, the first spectral plot was taken after the lamp had been turned on for 30 minutes. The tungsten halogen lamp provided emission at the wavelengths between 360 and 2000 nm.

Using the tungsten halogen lamp, spectral plots of sterile broth and broth spiked with suspected *Staphylococcus aureus* colony obtained from nasal swab culture were obtained. The sterile broth was taken out from refrigerator and incubated in air at 36 °C for around 4 hours prior to the taking of its spectral plot. After that, 1 ml of the broth was pipetted into a cuvette and incubated in air at 36 °C for the duration of the test. The sterility of the broth was confirmed by incubating it for 10 days. The broth was confirmed sterile if it experienced no colour change and showed no visible growth at the end of the incubation period.

The result from the trials using tungsten halogen lamp indicated that changes occurred in the green colour region. Therefore, a light emitting diode (LED) with emission in the green colour region could be used as a light source. Thus, the characterization studies were then continued using an LED with peak intensity at 575 nm as the light source. An LED circuit was wired as shown in Figure 11 to ensure the emission stability of the LED. Calibration tests were performed on the LED to study how long it took for the LED to reach a relatively stable emission. The readings were taken without any sample. Then, a set of blank broths were spectral analyzed and compared with the spectral plots obtained when the light source was a tungsten halogen lamp. In each reading, only 1 ml of broth was used. The comparison results showed that the green LED was a suitable light source for this study.

5.5.3. Procedures of the Experiments to Study the Effect of pH Change on Recorded Spectra

The pH change of the growth media was crucial for this study since the change was used to determine bacterial growth. Thus, some tests were performed to study how different pH affected the spectral plot of the MSB. In the tests, 20 ml of MSB with an initial pH of 7.2 was placed in a beaker. The MSB was heated and the pH at the corresponding temperature was recorded. Such a study gave an understanding on the temperature dependency of the pH measurement of MSB. The effect of such dependency was also observed via the change in the spectral plots of the MSB solution.

Besides temperature dependency, effect of the broth acidification on the spectral plots of the broth was also studied. The acidity level of the 20 ml of MSB with initial pH of 7.2 was varied by adding a few drops of 0.1 M HCl solution at a time. This experiment studied the colour development of the broth as the pH of the broth went from relatively high pH to lower pH. Every time some HCl solution was added, the broth solution was stirred and the pH was measured. After the pH was stable, the spectral plot of the MSB solution was then taken.

Another experimental set was performed with the MSB initially set at pH of 4.5 and the pH adjustment was made with 0.1 M NaOH solution. The procedure for this test was the same as the procedures stated in the previous paragraph. This experiment, using NaOH for the pH adjustment, studied the spectral changes as the pH of the MSB solution was changed from relatively low pH (6.17) to higher pH (7.54). All pH measurements were performed by using a HI98230 pH meter (Hanna Instruments).

5.5.4. Procedures of the Experiments to Study the Stability of Broth

Spectral plots of sterile broths and broths spiked with non viable bacteria were taken to assess the stability of the broths upon incubation. Spectral plots were

obtained for the antibiotics-free MSB, MSB containing oxacillin (MSBOxa) and MSB containing ciprofloxacin (MSBCip). Broth with dead bacteria was also used in the experiments to understand the effect of static amounts of dead bacteria on the spectral plots over time. Spectral plots of MSBOxa were obtained using the setup as described in Figure 10 while spectral plots of MSBCip were obtained using SMMIP (Chapter 4). 1 ml of broth was used for each reading. The incubation was in air at 36 °C or 30 °C for 10 days.

To describe the antibiotic-containing broth, the broths would be abbreviated with the following syntax: [broth][antibiotic]-[concentration]. Therefore, MSBOxa-4 was used to denote the MSB solution which contained 4 µg of oxacillin per ml of broth. The broths spiked with non viable bacteria were obtained by sterilizing the broths which was spiked with viable bacteria. The sterilized broths containing dead bacteria were sometimes stored in 4 °C before utilized. The broth was suitable for use in this study only when the spectral plot of the broth was not affected by the incubation and non-viable bacteria.

5.5.5. Procedures of the Experiments to Study the Temperature Stability of the Controlled Environmental Chamber

The temperature stability of the incubation chamber affected the performance of the culture. Therefore, it was necessary to monitor the temperature fluctuation of the chamber over time. The temperature inside the CEC was recorded based on the temperature displayed in the LCD of the temperature control circuit. The temperature monitoring was performed for a few hours at a pre set temperature of 30 °C.

5.6. Procedures of Growth Monitoring

5.6.1. Procedures of the Experiments to Develop a Growth Curve of *Staphylococcus aureus* in Mannitol Salt Broth (MSB)

The incubation chamber was tested and was shown to be capable of providing a relatively stable incubation temperature. Tests were then conducted to understand the effect of the presence of viable *Staphylococcus aureus* on the spectral plot of the MSB solution over an incubation period of 24 hours. The growth curves of MSSA ATCC 29213 and MRSA ATCC 43300 were thus obtained in terms of their spectral plots.

To obtain the spectral plots, a small inoculum from a working culture was first inoculated on MHA and incubated in air at 36 °C. Some colonies were then inoculated into MSB. The bacterial suspension was also diluted using sterile deionized water for plate counting. 1 ml of the suspension was pipetted into a cuvette and was subjected to incubation in air at 36 °C. Every half an hour, the sample was removed from the incubator and the spectral plot was captured over 24 hours. Plate counting was also performed for some intervals.

5.6.2. Procedures of the Experiments to Study the Possibility of Direct Clinical Identification

First of all, *Staphylococcus aureus* were isolated from the anterior nares of volunteers. For each volunteer, a sterile cotton swab (Copan) was rolled on the side of one nostril for 5 to 10 times. The swab was then streaked across a Mannitol Salt Agar (MSA) plate for specific isolation of *Staphylococcus aureus*. After 24 hours of incubation in air at 36 °C, several colonies were subjected to latex agglutination test (section 5.2). The nasal swab culture on MSA was found to consistently produce yellow colour colonies characteristic of *Staphylococcus aureus*. All colonies tested for agglutination factor unfailingly yielded positive agglutination tests indicating that the colonies were indeed *Staphylococcus aureus*.

Several other colonies were used to inoculate 10 ml of Mannitol Salt Broth (MSB). The broth was vortexed for around 5-10 seconds to properly disperse the bacteria. A suspension of 1 ml was then pipetted into a cuvette. The cuvette was incubated in air at 36 °C and taken out regularly to obtain the spectral signature. Standard plate count was conducted by performing serial dilution using sterile MHB. The antibiotic resistances of the strains involved in these experiments were not established.

The possibility to perform direct detection of *Staphylococcus aureus* based on the nasal swab was then investigated. Firstly, nasal swabs were taken from some volunteers by using sterile cotton swabs (COPAN Innovation). The swab was rolled in the two nasal cavities and care was taken such that no mucus or hair was retained by the swab. The cotton swab was then dipped and swished into 10 ml of MSB to disperse the bacteria retained by the swab. The swab was then pressed to the inside wall of the test tube to remove excess liquid before it was discarded. The broth was vortexed for around 5-10 seconds to properly disperse the bacteria. 1 ml of the suspension was then pipetted into a cuvette and incubated in air at 36 °C. At a regular interval, the cuvette was taken out to obtain a spectral plot. Standard plate count was conducted on the initial suspension to obtain the initial viable bacterial count.

5.6.3. Procedures of the Experiments to Study the Possibility of Oxacillin Resistant Strain Identification by Monitoring the Growth

Since the spectroscopic reading was not affected by non viable bacteria and the broth media were stable, it was then decided to investigate further on the identification of resistant strains. To monitor mutation in progress, there was a need to understand how to differentiate the mutants and the parent strain. Therefore, the possibility of using MSB to differentiate *Staphylococcus aureus* mutants from the original strain was explored.

Methicillin Susceptible *Staphylococcus aureus* (MSSA) and its resistant strain, Methicillin Resistant *Staphylococcus aureus* (MRSA) were used in the trial. Two ATCC strains, MSSA ATCC 29213 and MRSA ATCC 43300, were used. For any tests, a set of four tubes containing 10 ml of MSB, six tubes containing 9 ml of MSB, and six tubes containing 9 ml of MSB containing oxacillin were taken out from the storage and left to reach room temperature for around 30 minutes. Two tubes of 10 ml and twelve tubes of 9 ml of MSB without oxacillin were used to establish the initial amount of bacteria used in each experiment.

Colonies were picked by a 10 µl disposable inoculating loop from plates obtained from a working culture (section 3.4). The colonies were then dispersed in 10 ml of antibiotic-free MSB to form a suspension. The broth was vortexed for at least 15 seconds to disperse the organisms. 0.5 ml of MSB containing oxacillin was pipetted to a 70 µl micro cuvette (Brand). The oxacillin-containing broth was then mixed with 0.5 ml of the bacterial suspension. Consequently, the final oxacillin concentration was half of the original concentration. The amount of bacteria in the broth was also reduced to half the amount obtained from the standard plate count of the bacterial suspension.

In all experiments, the cuvette was then capped and sealed with parafilm. The broth in the cuvette was then vortexed to further ensure the dispersion of bacteria. Incubation of the broth was done in air at 36 °C. The spectral plot of the broth was obtained by using a spectrometer. The spectrum displayed by the OOIBase32TM software was an average of 20 spectra and the smoothing parameter for the boxcar algorithm was set to 10. The plots were obtained at 0, 30, 60, 90, 120, 150, 180, 240, 300, 360, 420, and 480 minutes of incubation. Before the reading was taken, the broth was removed from the incubator and vortexed to provide aeration and dispersion. After the bubble resulted from the vortexing process was let to settle for around 5-10 seconds, the spectra were recorded.

The recorded readings from the spectrometer were in the form of intensity against wavelength. In order to observe the changes on the sample during the incubation, Equation (39) was introduced to calculate the intensity ratio (IR).

$$IR(t)_{\lambda} = \frac{I(t_i)_{\lambda}}{I(t_0)_{\lambda}} \quad (39)$$

Where,

$I(t_i)_{\lambda}$ = Intensity at time i and wavelength λ (count)

$I(t_0)_{\lambda}$ = Intensity at reference time and wavelength λ (count)

Thus, from Equation (39), it was observed that the IR value is a relative value taken by comparing the transmitted intensity at a particular time with the reference transmitted intensity. The reference time at first was taken as 0th minute or the starting point. To account for the temperature adjustment period, the reference time at the later experiments was set to 30th minute. The ratio was calculated for each wavelength recorded.

5.6.4. Procedures of the Experiments to Determine Effect of Temperature to Growth

Both MSSA ATCC 29213 and MRSA ATCC 43300 were incubated under two temperatures, 30 °C and 36 °C. Some colonies were obtained from a working culture and streaked on an MHA plate. The plate was incubated in air for 24 hours at 36 °C. Some of the resulting colonies were then used to inoculate a tube of MSB. 1 ml of the bacterial suspension was then placed into a cuvette and incubated in air at either 30 °C or 36 °C. Spectral signature of the sample was then obtained by taking the sample out from the incubator at a 30 minutes interval and placed into the sample holder (Figure 10). Standard plate count technique was performed on the bacterial suspension by performing dilution steps before the samples were pipetted into the sample holders. The growth of the bacteria at the two different temperatures was then compared based on the development of the spectral signature.

5.6.5. Procedures of the Experiments to Study the Contribution of the Acidity and Turbidity of the Media to the Spectral Reading

Increase of the viable bacterial number was expected to increase the overall turbidity of the growth medium. The increase in the light absorption by the sample due to the higher turbidity was expected to span over a wavelength range. Nevertheless, bacterial growth did not only increase the bacterial number, the bacterial metabolism also produced acid which increased the acidity of the medium as well. The pH change caused the pH indicator in MSB to experience a colour change. The wavelength region of the spectral plot, which was affected by the colour change, might overlap with the wavelength range affected by the change of the turbidity of the medium.

Phenol red was the chemical that caused colour change in MSB. Experiments were then conducted to understand the contribution of phenol red to the overall transmittance change in MSB. In the experiments, colonies from a working culture were used to inoculate an MHA plate. The incubation of the plate was in air at 30 °C for 24 hours.

Colonies were then obtained from the MHA plate and was inoculated into a tube which contained MSB deficient of phenol red (MSBw/oPR). 0.5 ml of the inoculated MSBw/oPR was then mixed with another 0.5 ml of sterile MSBw/oPR to create 1 ml of bacterial suspension in MSBw/oPR. Another 1 ml of bacterial suspension was made by adding 0.5 of the inoculated MSBw/oPR to 0.5 ml of sterile MSB containing two times the normal phenol red concentration (MSBw2PR). The resulting broth was the conventional MSB. All 1 ml bacterial suspensions were then incubated in air at 30 °C. SMMIP was used in this test and therefore, a continuous monitoring could be performed.

Standard plate count technique was performed on the original bacterial suspension to obtain initial bacterial count. Six cuvettes containing 1 ml of bacterial suspensions were also incubated. One of the several suspensions was taken out

from the incubator at the chosen interval for testing. Standard plate count technique was then performed on the suspension to obtain the bacterial count at that particular time.

5.7. Procedures of the Studies on the Emergence of Antibiotic Resistance

5.7.1. Procedures of Monitoring the Emergence of Antibiotic Resistance

Ciprofloxacin MIC of MSSA ATCC 29213 was determined to be 0.5 µg/ml. Based on the Mutant Selection Window (MSW) hypothesis [56], MSBCip-1 and MSBCip-0.5 were used for the monitoring study. For each monitoring test, the initial culture was obtained by passaging a stock culture in air at 30 °C for two incubation period of 24 hours each. Some of the colonies obtained from the final plate were then inoculated into antibiotic-free MSB. The resulting bacterial suspension was subjected to 1:1 serial dilution with 0.5 ml of sterile MSBCip containing two times the final concentration. Hence, MSBCip-2 would be used to produce MSBCip-1 and MSBCip-1 would be used to produce MSBCip-0.5.

A special cuvette set-up was devised in order to allow the cuvette to contain up to 7 ml of liquid sample. The cuvette was integrated with some tubes to allow infusion of the nutrient broth, pH adjustment, and sample extraction. The cuvette designed is described in Figure 15 below.

Figure 15. Cuvette designed for use with SMMIP

The cuvette containing the sample was placed into the sample holder inside the CEC (Figure 12). A spectral plot was recorded with an interval of 30 minutes. After 24 hours had passed, RS (Figure 12) was activated in order to inject 4 ml of sterile MSBCip into the cuvette. The syringe pump was set to inject with the speed of 1 ml/min. A 20-ml sterile syringe was used to contain the sterile MSBCip. MSBCip-1.25 was used in a test to simulate an increasing antibiotic pressure while MSBCip-0.5 was used in a test to simulate a constant antibiotic pressure.

After the sterile medium had been injected, the pH of the solution was checked by comparing the spectral plot of the sample with the spectral plot at the 0th hour. If there was a wavelength shift between the two plots, pHCS was activated. Slow stream of 0.1 M NaOH was injected by pHCS (Figure 12) in order to adjust the medium pH to 7.2, which is the pH value of the original broth. During pH adjustment, the spectral plot of the sample was continuously compared with the plot at the 0th hour. Once the peak location was similar, the pHCS was deactivated.

After the pH of the sample was adjusted, ES (Figure 12) was activated to extract 4 ml of the bacterial suspensions. The extracted bacterial suspension was used to obtain the bacterial count at the point of extraction. Since the extraction followed the activation of RS and pHCS, the process was thus conducted every 24 hours. The bacterial count obtained was indicative of the inoculum size of the sample for the new 24 hours cycle. On the other hand, the sample count at the end of the previous 24 hours incubation, i.e. before activation of RS, was five times the bacterial count obtained. The chain of process from RS activation to ES activation was repeated every 24 hours. The flowchart of the protocol utilized was described in Figure 16.

Every 24 hours, the plate count of up to the previous three days was compared to understand the development of viable bacterial count. If it was observed that the viable bacterial counts of two consecutive days were below the detection limit, the test would be stopped. The sample would have failed to produce resistant mutants. In addition to the plate count result, the decision to stop the test was also made by observing the development of the spectral plots. The absence of wavelength shift

signified insufficient viable bacteria to produce observable acidification effect. Therefore, the test was also stopped if the spectral plot of the sample showed no wavelength shift over two consecutive 24-hours incubation cycles. Based on the results obtained in the monitoring test, the final protocol for SMMIP was then developed and shown in Figure 16.

Figure 16. Experimental protocol used to monitor the emergence of antibiotic resistance using SMMIP

5.7.2. Procedures of Experiments to Determine the Stability of the Ciprofloxacin Resistant Mutants

It was acknowledged that mutants might experience further mutations when the antibiotic pressure was stopped [202]. Therefore, it was necessary to assess the stability of the mutants produced during the study of mutation in progress. The mutants recovered from the plates containing highest ciprofloxacin concentration were assessed for the stability of their resistance. The colonies were passaged for seven incubation periods of 24 hours each on antibiotic-free MHA before the MICs of the mutants were determined [203-205]. During each passage, five colonies from the current passage were picked randomly by an inoculating loop and streaked on an MHA plate for the next passage. Colonies with different phenotype might be found on the same plate. Hence, multiple colonies were taken with the loops to introduce randomness.

After the seven passages, macro dilution tests [192] were performed to obtain the MICs of the mutants. PAP was also performed to assess the possible changes in the composition of the populations. MHB was used for the MIC test while PAP was conducted using MHA. Inoculum size of $\sim 5 \times 10^5$ CFU/ml was used in the macro dilution test. A bigger inoculum size of $\sim 10^8$ CFU/ml was used in PAP since it was hypothesized that such inoculum size gave a better analysis of the population.

5.7.3. Procedures of the Experiment to Characterize the Mutants at Molecular Level

Some mutants in the monitoring study using SMMIP were subjected to genetic tests to understand the types of mutation they experienced. The parent strain, ciprofloxacin susceptible MSSA ATCC 29213, was also included in the molecular characterization as a reference. Tube and slide coagulase test [206] was performed to ensure that all isolates were *Staphylococcus aureus*. Molecular typing of the mutants and MSSA ATCC 29213 indicated that they are indeed the same isolates.

After DNA chromosomal extraction, Polymerase Chain Reaction (PCR) was performed on the QRDRs and the *norA* promoter region (*flqB*) using some pairs of primers listed in Table 4. DNA extraction was performed by using QIAamp DNA mini-kit (QIAGEN, Research Biolabs). The PCR process involved an initial melting at 95° C for 5 minutes and then followed by 25 cycles of melting (95° C for 30 seconds), annealing (55° C for 30 seconds), and elongation (72° C for 1 minutes). Final elongation was set at 72° C for 7 minutes. Agarose gel electrophoresis (SeaKem LE agarose for a 1.5% gel) was used to determine that the PCR products were present.

Table 4. Primers used to perform PCR on QRDR and *norA* promoter region [207]

Gene amplification (nucleotide positions)	Primer	Sequence (5' to 3')
<i>grlA</i> (2347 to 2870) ^a	GrlAF2 GrlAR2	AATGATCAATTTGATGAGGAGG CACTAGTAAGTTAGGAAATCT
<i>grlB</i> (1562 to 1877) ^a	GrlBF2 GrlBR2	GTGAAGATGCTCGTTTCAGG ACGATTATAATTACTATCTT
<i>gyrA</i> (2152 to 2658) ^b	GyrAF1 GyrAR1	ATGGCTGAATTACCTCAATCAAG GGCTAATAAGTTAGGGAATCGA
<i>gyrB</i> (1379 to 1686) ^b	GyrBF1 GyrBR1	GTGAAGTAACACGTCGTA TTTGTGATATCTTGCTTTCGC
<i>flqB</i> (129 to 546) ^c	NorAL2 NorAL3	AACGTCATCACATGCACCA TATTACTAAACCGATAACC

^a Nucleotide positions are indicated according to GenBank sequence number D67075.

^b Nucleotide positions are indicated according to GenBank sequence number D10489.

^c Nucleotide positions are indicated according to GenBank sequence number D90119.

The PCR product was then purified using Qiagen PCR purification kit (Qiagen, Research Biolabs). The purified PCR product was loaded on a 1% agarose gel to check for the quality and quantity of the product. Comparison with 100 bp ladder marker was also performed. The PCR product was sequenced by using ABI Genetic Analyzer 3100 (Applied Biosystems). The sequencing work was outsourced to a commercial company (Research Biolabs).

The electropherogram of the sequences was analyzed with FinchTV software (Geospiza) and ambiguous nucleotide was assumed. The nucleotide sequences was then analyzed by Sequence Manipulation Suite 2 [208]. ClustalX v1.81 [209] was used to perform both nucleotide and protein alignment. GenBank accession numbers D67075, D10489, and D90119 [210, 211] were used to obtain the coding sequence (CD) and amino acid sequence for DNA topoisomerase IV, DNA gyrase, and *norA* gene respectively. The sequences from the GenBank database were used in the sequence alignment as well.

Chapter 6

MEDIA AND SYSTEM CHARACTERIZATION STUDIES

6.1. Acidification of MSB during Bacterial Growth

Mannitol Salt Broth (MSB) provides mannitol, a sugar alcohol, as the main carbohydrate source for the bacteria. *Staphylococcus aureus* can be identified by measuring the pH of the MSB since it carries out mannitol fermentation and the resulting acetate production will acidify the medium [197]. This is shown in Figure 17.

Figure 17. Temperature and pH development of MSB inoculated with 7.1×10^7 CFU/ml of MSSA ATCC 29213 and incubated in air at 36 °C

Due to the large amount of liquid needed to immerse the pH probe, 10 ml of MSB was used. As a result, more than 2 hours were needed for the liquid to reach the incubation temperature. The results in Figure 17 show that pH of the medium decreased while the temperature of the medium increased before it reached equilibrium. As the broth temperature approached 36 °C, the reduction of pH value slowed down as well. After 120 minutes of incubation, the pH value steadily decreased while the temperature of the broth stayed within the incubation

temperature. The pH development was an inverted sigmoid curve. The inverted sigmoid curve is a mirror image of bacterial growth phase. This is expected since acid production during growth lowers the medium pH. Therefore, in the absence of pH buffer, the pH development should be affected by the population size and hence, the inverted sigmoid curve. The formulation of a complete inverted sigmoid curve (inverted S-shape) is dependent on whether the exponential and stationary phase (Figure 8) are reached within the 800 min of incubation period shown in the Figure 17 to 19. Lag phase may not be seen if the period is shorter than the pH reading period.

Figure 19 shows that relatively low inoculum size produces a prolonged lag phase (~200 min) and at the end of 800 min, the stationary phase is not yet reached. While Figure 18A shows the absence of lag phase and Figure 18B shows a short lag phase of < 100 min. The lag phase is not clearly shown in pH development curve partly because of the warming up time. As will shown later in the thesis, medium pH is more acidic at higher temperature. Hence, it can be seen from Figure 17 to 19 that the pH development at $t < 100$ min is a mirror of the temperature development. The warming up time, therefore, masks the formation of lag phase. To obtain a more complete inverted sigmoid curve, warming up time can be minimized by heating the medium to incubation temperature although the temperature equilibrium between broth and incubator will still take some time. This activity is not done in this study since the intention is to show that the medium pH is indeed acidified as the incubation time increases.

Figure 18. Temperature and pH development of MSB incubated in air at 36 °C and inoculated with (A) 4.0×10^6 CFU/ml of MSSA ATCC 29213, (B) 3.6×10^6 CFU/ml of MRSA ATCC 43300

Figure 18 shows two other plots of MSB inoculated with 4.0×10^6 CFU/ml of MSSA ATCC 29213 and 3.6×10^6 CFU/ml of MRSA ATCC 43300. As with Figure 17, the temperature of the medium required 2 hours to reach the incubation temperature and a similar sharp decline of pH value can be observed. A total of 6 sets of experiments were performed, of which 3 were of MSSA ATCC 29213 and 3 were of MRSA ATCC 43300, to understand the pH development. The gradient of

the pH development was obtained from the trend line plotted based on 20-25 data points (250-500 minutes) of the linear portion of the pH data. A summary of the data obtained from the experiments is shown in Table 5.

Table 5. A summary of the data obtained from pH development tests at 36 °C

Strain	pH gradient (min ⁻¹)	R ²	Viable count at 0 th hour (CFU/ml)	Viable count at 8 th hours (CFU/ml)
MSSA ATCC 29213	-0.0008	0.98	1.3 x 10 ⁶	5.5 x 10 ⁶
	-0.0016	0.98	4.0 x 10 ⁶	3.2 x 10 ⁷
	-0.0023	1.00	7.1 x 10 ⁷	1.6 x 10 ⁸
MRSA ATCC 29213	-0.0011	0.99	9.7 x 10 ⁵	9.3 x 10 ⁵
	-0.0011	0.98	3.6 x 10 ⁶	1.3 x 10 ⁷
	-0.0015	0.99	1.5 x 10 ⁸	9.5 x 10 ⁷

As seen in Table 5, the increase in pH gradient generally correlated to the increase in starting inoculum. The data also showed that the growth of MRSA ATCC 43300 generally resulted in only a slightly slower pH drop compared to the growth of MSSA ATCC 29213 when they are subjected to incubation in air at 36 °C. It appeared that starting inoculum in the mid range of 10⁶ CFU/ml can produce relatively more viable bacteria after 8 hours of incubation. No increase of viable bacterial count was observed for sample with inoculum size at the order of 10⁵ and 10⁸.

Figure 19. Temperature and pH development of MSB inoculated with 9.7×10^5 CFU/ml of MRSA ATCC 43300 and incubated in air at 36 °C

Figure 19 shows that an inoculum of 9.7×10^5 CFU/ml did not produce enough acid to result in a measurable pH change in the MSB up to 300 minutes of incubation. There was, however, a reduction of 0.11 pH value per 100 minutes of incubation after the short period of constant pH (100th-280th minutes). The viable count obtained for MRSA ATCC 43300 in the pH development test suggested that mannitol metabolism occurred though there was no increase in the viable count. Therefore, instead of viable count, pH measurement could be used as an indication of bacterial viability during growth in MSB for MRSA ATCC 43300. Since the pH development was observed in MSSA ATCC 29213 as well, pH change could also be used as an indication of bacterial viability.

6.2. Determination of the Appropriate Light Source

6.2.1. Tungsten Halogen Lamp

Initially, a wide spectrum light source was used in the experiments to determine the wavelength region of interest. Tungsten halogen lamp LS-1 (Ocean Optics) was chosen for the light source as it emitted light from 360 nm to 2000 nm. As

recommended by the manufacturer, the lamp was subjected to a warming up time of 30 minutes so that the tungsten halogen could emit stably.

Although tungsten halogen emits at a wide spectral range, only the visible region was of interest in this study. Hence, the spectra discussed in this report would correspond to the visible region of 400 – 700 nm (Figure 7). A further reduction of wavelength region of interest might be possible if there were some wavelength regions which contained mainly noise.

A spectral plot produced by incubating 1 ml blank Mannitol Salt Broth (MSB) in air at 36 °C is shown in Figure 20. MSB typically produced two peaks, one at ~ 518 nm and another at ~ 628 nm. The figure shows that the peak at the red colour region (~ 628 nm) was ~ 6 times stronger than the peak at the green colour region (~ 518 nm). The transmitted spectrum showed that bulk of transmitted light was in the red colour region which corresponded with the red colour of the broth.

Figure 20. Spectral reading of blank MSB incubated for 720 minutes in air at 36 °C under illumination by tungsten halogen lamp

All five trials conducted with blank MSB gave similar graphs as shown above. It was observed in Figure 20 showed that there were small transmitted intensity changes in the range of 525-575 nm. In order to understand how the intensity

changes over time, intensity ratio (IR) was then calculated according to Equation (39).

Figure 21. Intensity ratio (IR) of blank MSB under illumination by tungsten halogen lamp

A peak and a flat region could be observed in Figure 21, which describes a typical IR plot. A wavelength band with relatively higher noise as compared to other bands was also observed at the 400-480 nm region. The instability of the lamp output in this region could have caused the noise and was most likely due to the low output efficiency ($\sim 10\%$) in this region (Appendix F).

It was observed during the incubation that the colour of the broth changed according to the temperature of the broth. The ability of the spectrometer to detect slight colour variation due to temperature drift was well testified by the changes observed in the IR plot. The broth experienced colour changes as it underwent heating from room temperature ($\sim 23\text{ }^{\circ}\text{C}$), which was the temperature during broth manipulation, to the incubation temperature ($36\text{ }^{\circ}\text{C}$). It was known that the measured pH of a solution changed as its temperature changed [212]. A higher temperature was observed to increase the acidity of MSB while a lower temperature increased the alkalinity.

The change of broth pH changed the colour of the phenol red indicator. The temperature adjustment resulted in a slight colour change to the more yellowish region as captured by the spectrometer. Therefore, the spectral reading of the 1st hour would be expected to experience some shift compared to the starting spectral reading. The changes in the IR plot focused within the wavelength region of 525-575 nm as observed earlier in Figure 20.

The peak indicates an increase of intensity in 480 to 580 nm region which corresponds to green and yellow band (Figure 7). The highest value of intensity ratio was found to be at 557.47 nm, indicating the wavelength at the green colour region. This corresponded to the fact that a yellow colour solution would transmit both green and red light while originally, the broth was of red colour, which transmitted only red light. The proportion of the transmitted green and red light determine what shade of yellow was produced [173].

The temperature adjustment of the broth to the higher temperature resulted in a slightly more acidic broth and therefore a minute colour change from red to more yellow. Therefore, transmission of the green component of the light source increased. This peak was thus expected to occur in all subsequent tests since mannitol acidification would affect the phenol red indicator in the medium to change from red to yellow.

The temperature adjustment effect on the IR plot can be observed in Figure 21 by the appearance of a broad peak at ~ 557 nm obtained after 60 minutes of incubation. If the spectral reading at the 60th minute was taken as the denominator of Equation (39), the peak would flatten and variation of IR value would be reduced by > 90%. In fact, the drift at wavelength > 610 nm disappeared.

Figure 22. Intensity ratio (IR) plot of a blank MSB by setting 60th minute as the reference time

The IR plot using 60 minutes as the reference time is shown in Figure 22. As can be seen, there are still small peak in the 557 nm region. Taking the intensity at 557.47 nm for calculation purposes and making use of the intensity ratio at 1st hour as reference, the drift over 8 hours of incubation resulted in maximum IR value of 1.05 ($\sigma = 0.02$). The drift could be contributed partly from the variation in broth temperature since the reading was performed at room temperature. The slight change of colour due to temperature was said earlier to affect the spectral plot around the peak region. The drift could not have been resulted from emission drift since emission drift was expected to occur across all wavelengths while no variation was found for wavelengths > 610 nm. Nevertheless, the drift was insignificant and changes attributed to the presence of bacteria resulted in maximum IR value far exceeding 1.05.

6.2.2. Light Emitting Diodes (LED)

Using a tungsten halogen lamp as the light source in the optical setup as shown in Figure 10, the trials as described in the previous section, showed that changes could be expected in the green colour region. However, tungsten halogen lamp has a low

green light component (Figure 20). Hence, a small intensity fluctuation in the region would contribute to large percentage of change relative to the real intensity change. On the other hand, a light emitting diode (LED) with emission in green light region would provide a narrower emission focusing on the region of interest in this study. Thus, LED with an emission peak at 575 nm was chosen for subsequent trials and experiments. The emission spectra of the LED used is shown in Figure 23.

Figure 23. Spectral reading of LED with emission peaked at 575 nm

Calibration was then conducted on the LED to decide on the length of time needed for the LED to produce stable emission. The readings were taken over time by switching on the LED for the whole test duration. The IR plot of the LED over time is shown in Figure 24. From the plot, the x-axis ranged from 540 to 600 nm since the region beyond that band produced a relatively high amount of noise due to emission instability.

Figure 24. Intensity ratio of LED with emission peak at 575 nm for the first 10 minutes of activity

It was found from Figure 24 that the intensity ratio from 2nd minute to 10th minute of activity was reduced by 3.63% (at 552.16 nm) while the intensity ratio at the 0th minute to the 2nd minute experienced a reduction of 10.29%. It was then decided to take 2 minutes as a warming up time for the LED. Thus, it was decided that the time lapse between the switching on of the LED and the recording of spectra should be 2 minutes. It would therefore be logical to expect the intensity of the LED not to reduce by more than 3.63% if the reading was completed within 8 minutes.

6.3. Effect of Temperature Change on Measured pH

It was shown earlier that the growth of *Staphylococcus aureus* acidified the pH of MSB. It was therefore of interest to show how the spectral readings of MSB changed with respect to different medium pH. Since it is known that pH value is dependent on temperature, experiments were conducted to understand how pH varied with temperature. A green LED was used in the experiments.

The pH of a solution depends on its ionization constant while the ionization constant varies with different temperature. Therefore, the measured pH of a solution varies with different temperature [212]. Through heating up MSB in several

temperature intervals, it was observed that the measured pH was indeed sensitive to temperature since the pH varied with different temperature. The plot of the temperature effect on measured pH is provided in Figure 25. It can be seen from the plot that the higher the temperature of the broth, the lower its pH is. The data points obtained during triplicates tests showed nearly overlapping points.

Figure 25. Effect of the temperature of the broth on the measured pH

The data points obtained in Figure 25 can be easily fitted with linear trendlines resulting in trendlines with excellent fit ($R^2 = 0.9990 \pm 0.0004$). The gradients of the linear trendlines have a close range as well ($m = -0.0178 \pm 0.0001$). The results show that an increase of broth temperature from room temperature of 23 °C to incubation temperature of 36 °C would be expected to change the measured pH value of the broth by 0.23.

Figure 18 shown previously, confirmed that broth pH was lowered by 0.20 when the broth temperature was increased from 24.3 to 36 °C. The observation that temperature indeed affected the spectral reading was translated into a wavelength shift. Generally, a lower pH produces a spectral peak at a lower wavelength as shown in Figure 26.

Figure 26. Effect of the pH of the MSB solution on the peak wavelength of the spectral reading

6.4. Effect of pH Change on Recorded Spectra

Tests were also conducted to understand how spectral reading changed with respect to pH variation at a fixed temperature. Obvious changes in the spectral reading could be observed when the pH of the MSB was modified by adding HCl or NaOH. The test was conducted in room temperature and therefore no temperature effect contributed to the spectral change. Increasing the acidity of MSB resulted in a negative wavelength shift, i.e. the peak was shifted to the left. Figure 27 clearly shows that the peak of the transmitted light intensity shifted to a lower wavelength over the green colour region.

Figure 27. Spectral readings of MSB with different pH value in room temperature

The IR was again plotted to allow a closer look at the intensity changes over different pH. The ratio was taken by dividing the spectral reading of MSB at various pH with the spectral reading of MSB at the initial pH value as the reference. The IR plot can be seen in Figure 28.

Figure 28. Intensity ratio plot of the effect of pH change on the spectral reading with reference to MSB at pH 7.23 ($T \sim 23^\circ\text{C}$)

The IR plot in Figure 28 reveals a figure similar to Figure 22. A peak is clearly seen at ~ 560 nm. The more acidic the pH was, the higher the intensity of the spectral reading for all wavelengths. The spectral intensity at 557.47 nm of MSB with pH 6.17 was higher by 15.5 times compared to the spectral intensity of MSB with pH 7.23.

Figure 29. Normalized wavelength shift of MSB with different pH measured under room temperature

The negative shift of the spectra due to an increase in acidity level is shown in Figure 29. Taking pH 7.23 as the reference spectra, the shift of the spectral peak's location was normalized. The more alkali MSB at pH 7.3 – 7.4 produced a spectral reading with peak shifted to a higher wavelength. The three trials produced a closely overlapping normalized wavelength shift with a downward exponential shift as the MSB was getting more acidic. The change in the normalized wavelength shift between pH 6.9 and 7.2 followed a linear trend. The temperature effect seen in the previous section (Figure 26) concurred with the observation in Figure 29 that MSB with a higher measured pH produced spectral reading which is shifted to a lower wavelength.

Figure 30. Development of intensity ratio (IR) at 557.47 nm of blank MSB under pH adjustment using 0.1 M HCl

Based on Figure 28, the IR value at 557.47 nm increases as pH gets lower (Figure 30). The increase seemed to be steeper at around pH 6 and 7. A lower IR change was observed when the pH value changed from 7 to 7.2. The increase in IR value at 557.47 seemed to be quite stable among the triplicates.

Both trials under this and the previous section was started with $\text{pH } 7.23 \pm 0.01$ and 7.26 ± 0.02 respectively. The starting broth temperature was 22.3 ± 0.1 °C in the study of pH effect and 22.7 ± 0.4 °C in the study of temperature effect. Thus, the starting temperature and pH were similar. In addition, the MSB used in the study of the pH effect had an initial spectral peak at 586.94 ± 0.20 nm while the MSB used in the study of the temperature effect had an initial spectral peak at 586.36 ± 0.35 nm.

Despite the similarities among the starting pH, temperature, and initial spectral reading, the spectral readings obtained at similar measured pH in the two studies (section 6.3 and 6.4) did not overlap. The reading at the measured $\text{pH} \sim 7.01$ produced by heating the broth to ~ 36 °C gave a spectrum with a peak at ~ 584.25

nm while the acidification of MSB to pH 7.01 by HCl produced a spectrum with a peak at ~ 581.1 nm.

The difference could have been a result of complication due to the inherent temperature adjustment programmed into the pH meter. Nevertheless, the differences observed were not expected to affect subsequent trials since the incubation was performed at a static temperature. Nonetheless, both studies showed a general trend of spectral shift to the left when pH was lowered. Temperature adjustment was also accounted for by taking the reference spectral reading at 30 minutes instead of 0 minute.

6.5. Stability of Broth

6.5.1. Effect of Incubation on Spectral Reading of Blank Broth Containing No Antibiotics

Using blank broth as the sample, stability of broth subjected to incubation was determined. All IR plots produced were found to be similar in shape with those obtained when the tungsten halogen lamp was used. One of the five IR plots obtained from the trials is plotted in Figure 31.

Figure 31. Intensity ratio of blank MSB with LED as light source

Similar to the IR plot obtained when the tungsten halogen lamp was used as the light source, the highest value of the intensity ratio was at ~ 557.47 nm, which was the highest point of the peak formed in the plots. On the other hand, the lowest value obtained in LED calibration was at 552.16 nm, which was in the negative peak region (Figure 24). Since the positive and negative peaks obtained in the blank broth trials and LED calibration were located at different wavelengths, the variation in the IR values of blank broth at the trials were not due to the variation in LED emission.

If the variation was considered by comparing the data at the 30th minute and the data at the 360th minute, the intensity ratio at 557.47 nm, which had the highest variation, had an IR value of 1.03 ($\sigma = 0.015$). The variation could not be compared directly with the experiments with the tungsten halogen lamp since the green LED was only turned on 2 minutes before every spectral reading was taken. Meanwhile, the tungsten halogen lamp was active for the whole incubation period. Nevertheless, using the LED, a small variation was expected though it was insignificant compared to the changes induced by colour change which normally reached an IR value > 1.5 .

Figure 32. Normalized wavelength shift of blank broth containing no antibiotics under incubation in air at 36 °C

Over the incubated period, a slight shift in the peak location of the transmitted intensity graph was experienced (Figure 32). Five experiments were conducted in order to study the wavelength shifts of blank MSBs. Normalization was performed for the wavelength shift data by subtracting the location of the peak of the transmitted intensity after 30 minutes of incubation from the location of the peak of the transmitted intensity at any point of interest. As stated earlier, 30 minutes was chosen as the temperature adjustment period. Consequently, normalizing the wavelength shift would allow examination of the broth's pH value based on the wavelength obtained at the incubation temperature. It is seen in Figure 32 that the shifts are generally within ± 0.35 nm although there is an outlier value at 0.7 nm. Therefore, only a negative shift above -1 nm was considered meaningful.

6.5.2. Effect of Incubation on Spectral Reading of Blank Broth Containing Antibiotics

At the time of writing (January 2007), no reference was found on the growth of *Staphylococcus aureus* in Mannitol Salt Broth (MSB) with oxacillin and ciprofloxacin. This section reports on the effort to assess the stability of sterile antibiotic-containing broths before such broths were studied with bacterial inocula.

Incubating each blank broth for 8 hours, the wavelength shifts of the blank broth with antibiotics were monitored. Four different oxacillin concentrations and one ciprofloxacin concentration were investigated. Similar with the blank antibiotic-free broth, normalized wavelength shift experienced by the blank broth containing antibiotics was also within 1 nm (Appendix C-1). It was stated earlier in section 6.4 that a spectral shift to the left was the expected growth indication. For that reason, the normalized wavelength shift for positive growth indication was of negative value. Therefore, only when there was a consecutive negative normalized wavelength shift of more than 1 nm, a positive metabolism was indicated.

Figure 33. Normalized wavelength shift experienced by blank MSBOxa-12

Figure 34. Normalized wavelength shift experienced by blank broth of MSBCip-1.25

Although there was one point for blank MSBOxa-12 (Figure 33) that exceeded 1 nm, the threshold value of -1 nm was not altered. The point was positive in value while the expected wavelength shift was negative. Therefore, the outlier did not affect the setting of the threshold.

6.5.3. Effect of Non Viable Bacteria on Spectral Reading of Blank Broth

Experiments were conducted with non viable MSSA and MRSA to ensure that fixed amount of non viable microorganisms did not induce turbidity change during any incubation. The broths containing viable organisms used for some experiments were steam sterilized and then stored in 4 °C before being used for tests at the next day. Only broths containing the upper and lower limit of the oxacillin concentration tested were used in the trial. The concentrations were namely 4 and 12 µg/ml.

Figure 35. Normalized wavelength shift experienced by MSBOxa-4 spiked with non viable MSSA ATCC 29213 and MRSA ATCC 43300

Figure 36. Normalized wavelength shift experienced by MSBOxa-12 spiked with non viable MSSA ATCC 29213 and MRSA ATCC 43300

Blank broth inoculated with non viable microorganisms varying over two orders of magnitude did not produce significant wavelength shifts and turbidity changes to the broth (Figure 35-39). The normalized wavelength shift varied within ± 1 nm. Therefore, the threshold for indication of positive growth remained at -1 nm for two consecutive readings (1 hour).

6.6. Temperature Stability of the Controlled Environmental Chamber (CEC)

To establish the temperature development of the Controlled Environmental Chamber (CEC) upon heating up from room temperature, a temperature recording with 10 minutes interval was manually taken. The temperature seemed to develop in a hyperbolic manner. The temperature rose relatively fast initially before slowing down slightly and finally saturating and fluctuating around the pre-set temperature (Figure 37). By controlling the power to the heater to resume at 35.4 °C and stop at 35.9 °C, it was possible to control the temperature variation to within 35.4 °C and 36.1 °C.

Figure 37. Temperature profile of the Controlled Environmental Chamber (CEC) set at 36 °C

Figure 37 also shows that one hour was needed to warm up the chamber from room temperature to 36 °C. Therefore, the heat supply was normally turned on overnight before any sample was put into the chamber. The temperature deviation was deemed acceptable and so, no further adjustment was made on the cut off temperature.

6.7. Summary of the Characterization Studies

Staphylococcus aureus indeed acidifies MSB and higher starting inoculum produces faster pH reduction. The medium acidification produces an observable colour change from red to yellow. The colour change indicates that there is a change in the proportion of green and red light transmission. Such change is easily detectable by tungsten halogen lamp which produces a wide visible wavelength emission. Nevertheless, the warming up time of the lamp is prohibitively long (~ 60 minutes). The wavelength band affected by the colour change indicates that LED at 575 nm is suitable as the light sources. The relatively short warming up time of 2 minutes resulted in the decision to use the LED as the light source for the SMMIP system development.

It was also learned that the pH of MSB is dependent on temperature. Conditioning of MSB to reach incubation temperature of 36 °C from 23 °C will reduce the pH value by 0.23. Hence, a slight colour change is expected during the temperature equilibrating process. A normalization using the wavelength at a preset warming time is needed to obtain the wavelength shift produced by the sample during a constant incubation temperature. Further investigation using a sample of 1 ml of blank broth indicated that spectral reading at 30 minutes can be used as a normalization reference.

To ensure that the broth is stable in terms of turbidity change during a prolonged incubation period, the spectral readings of blank broths with and without antibiotics were obtained for the duration of 8 hours. The wavelength shift of the blank broth as well as when non viable bacteria were spiked into the broth produces a fluctuation of less than ± 1 nm. As seen later in the thesis, this fluctuation is not significant compared to the expected wavelength shift during incubation with actively metabolizing cultures. The threshold for indication of positive growth was set at -1 nm for two consecutive readings (1 hour).

A study on the temperature characteristics of the incubation chamber of SMMIP was then performed. The chamber needs 1 hour warming up time if the incubation temperature is set at 36 °C while the temperature varies between 35.4 °C and 36.1 °C.

Chapter 7

GROWTH OF *STAPHYLOCOCCUS AUREUS* IN MANNITOL SALT BROTH (MSB)

7.1. Establishing the Growth Curve of *Staphylococcus aureus* in MSB

It had been shown in Chapter 6 that the incubation temperature was reachable by the Controlled Environment Chamber (CEC). Broth stability had also been investigated and threshold value of normalized wavelength shift had already been set. In addition, phenol red indicator had been tested on its ability to indicate pH change. Therefore, it was imperative to understand how growth of *Staphylococcus aureus* in Mannitol Salt Broth (MSB) could be monitored.

Figure 38. Changes of the spectral readings of MSB inoculated with MSSA ATCC 29213

It is seen in Figure 38 that the spectral reading shifts to lower wavelengths over the incubation period. This phenomenon is the same as the observation in Figure 27. Acidification of mannitol shifted the peak of the spectral reading to the left, which indicated a higher transmittance of the green component of light and a reduction in the transmission of the red component (higher wavelength). Nevertheless, in

contrast with Figure 27, the presence of bacteria in MSB lowers the intensity of the spectral reading. While mere acidification of the medium actually increases the peak intensity of the spectral reading, the presence of bacteria increases the turbidity as well. The presence of an intensity drop in Figure 38 denotes the dominance of turbidity over the spectral reading compared to the acidification effect since the overall effect on the spectral reading is the lowering of the intensity of the transmitted light.

Figure 39. Growth curve of *Staphylococcus aureus* MSSA ATCC 29213 and MRSA 43300 under incubation in air at 36 °C based on the normalized wavelength shift of MSB

Incubating in air at 36 °C, it is seen clearly how the system could perform a growth monitoring by recording the wavelength shift of the broth's spectral reading (Figure 39). Taking a one hour interval reading, a growth monitoring of 24 hour duration could be performed. It is also observed in Figure 39 that MSSA ATCC 29213 and MRSA ATCC 43300 generally had nearly identical wavelength shift profiles. The medium acidification rate of both strains was therefore comparable.

Figure 40. Viable count development during the growth in MSB of *Staphylococcus aureus* MSSA ATCC 29213 and MRSA 43300 under incubation in air at 36 °C

Although there is active metabolism indicated by pH change monitored by the negative normalized wavelength shift (Figure 39), the development of viable bacterial count reveals a static number of viable bacteria in the population. This finding supports the data obtained during the study on pH development (Table 5). In the pH study, bacterial inoculum of $\sim 10^8$ CFU/ml was found to produce a pH change but no increase in viable count was determined by standard plate count. Consequently, an inoculum of $\sim 10^8$ CFU/ml was the carrying capacity of the sample holder in this study. Carrying capacity denotes the number of maximum living organism that the environment can carry. This concept was advocated widely and is included in many mathematical models of population growth [178, 213]. The viable count obtained in Table 5 and Figure 40 seemed to suggest that the number of viable bacteria declined at the end of incubation period. The initial inoculation at the carrying capacity could have caused a fast nutrient depletion and therefore prompted the bacteria to enter a death phase (Figure 8) before 24 hours was reached.

7.2. Growth Monitoring and Detection of *Staphylococcus aureus* from Clinical Samples

Besides using established strains, experiments were also conducted using samples from overnight cultures obtained from nasal swabs. The study was aimed at understanding the lower limit of bacterial inoculum size which would enable clinical growth monitoring of *Staphylococcus aureus*. Therefore, at the start of the study, colonies of *Staphylococcus aureus* were obtained from MSA plates streaked with nasal swabs.

Anterior nares were chosen for the isolation site because of it is a preferential niche for *Staphylococcus aureus* [110]. Normal nasal flora include *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Proteus sp.*, *Haemophilus influenzae*, and *Corynebacteria* [214]. Nevertheless, the presence of high concentration of sodium chloride in mannitol salt broth or agar inhibited most of the organisms but not Staphylococci. *Staphylococcus epidermidis* was also selected by MSB. However, it did not ferment mannitol aerobically and thus did not change the colour of the broth. Hence, mannitol salt medium was relatively selective for *Staphylococcus aureus* among the common nasal flora.

Mannitol salt agar (MSA) had been proven to be useful for *Staphylococcus aureus* isolations from nasal cultures in neonates [32]. Furthermore, some suspected colonies, which were obtained from the plates that provided colonies used in tests, were also used in clumping factor and surface antigen tests. All colonies tested with Dry Spot Staphytect Plus™ latex agglutination test kit produced positive reactions. Therefore, anterior nares are satisfactory sources for *Staphylococcus aureus*. Nasal swabs from three volunteers were taken and cultured.

Figure 41. Spectral reading of MSB spiked with 3.5×10^6 CFU/ml of *Staphylococcus aureus*

Figure 42. Intensity ratio of MSB spiked with 3.5×10^6 CFU/ml of *Staphylococcus aureus*

It was clearly shown earlier (Figure 39) that a growth monitoring of 24 hours duration could be performed. Therefore, the monitoring work for samples of clinical

origin was performed for only 8 hours since it was deemed sufficient to indicate growth for most inoculum sizes. Figures 41-42 shows that the peak in the spectral reading again shifted to the lower wavelength as incubation time increased. Other spectral readings from various inoculum sizes can be found in Appendix C-2. The shifting was reflective of the trial on the acidification of MSB by HCl reported in section 6.4.

As the culture went through incubation, growth was expected and thus turbidity of the broth medium was expected to increase. The change in turbidity was manifested by an overall reduction in light transmissibility of the broth. Intensity of light transmitted was thus decreased as the bacteria proliferated. The IR plot showed the increase in turbidity by a decrease in value of the whole enquired wavelength region. As the number of initial inoculum increased, the spectral peak shifted to the left faster and the turbidity increased faster as well. The summary of normalized wavelength shift experienced is plotted in Figure 43.

Figure 43. Normalized wavelength shift experienced by MSB spiked with *Staphylococcus aureus* obtained from nasal swab

Generally, a higher amount of bacteria produces more acid and therefore leads to a faster acidification of the medium. It is shown in Figure 43 that after 7 hours of incubation, an inoculum of *Staphylococcus aureus* in the order of magnitude of 10^6 CFU/ml produces -12 nm normalized wavelength shift compared to -0.7 nm normalized wavelength shift produced by the inoculum in the order of magnitude of 10^5 CFU/ml. The plot also implies that it would be difficult to obtain normalized wavelength shift indicative of positive growth from inocula smaller than 5.0×10^4 CFU/ml within 8 hours of incubation.

Figure 44. Intensity ratio at 557.47 nm of MSB spiked with *Staphylococcus aureus* obtained from nasal swab

The IR plot obtained also shows the characteristic peak around 550 nm and valley in the wavelength region larger than 590 nm. Based on the intensity ratio (IR) at 557.47 nm, it is understood that the IR value at a particular wavelength does not give any overall trend (Figure 44). A higher inoculum seems to produce higher gradient in the time frame though the points belonging to samples with inoculum of 5.2×10^4 CFU/ml and 4.0×10^5 CFU/ml seem to overlap. An obvious difference of ~ 0.3 IR value is only experienced after 8 hours (480 minutes) of incubation.

Figure 30 (section 6.4) shows that acidification of MSB increases the IR value at 557.47 nm. However, it is observed in Figure 44 that even though inocula in the order of 10^6 CFU/ml tend to have higher gradients, the increase in IR value at 557.47 nm is a lot more subdued compared to the data obtained for acidification of the blank MSB. The increase of turbidity due to the presence of bacteria seems to have affected the rising of IR value at 557.47 nm. Eventually, a decline in IR value at the wavelength is experienced at the 4th and 5th hour for inocula of 8.9×10^6 CFU/ml and 3.5×10^6 CFU/ml respectively.

Figure 45. Intensity ratio at 600 nm of MSB spiked with *Staphylococcus aureus* obtained from nasal swab

The trend of IR value at the valley region of the IR plot was obtained. The plot of intensity ratio at 600 nm against time gives an obvious trend, showing a decline in the value with time. Higher inoculum size produces faster reduction of IR at 600 nm. Therefore, the reduction of IR value at 600 nm can be used along with the normalized wavelength shift for growth monitoring.

An attempt was then conducted to investigate the possibility of growth monitoring using direct nasal swabs. Direct inoculation of nasal swabs was performed into a 10

ml MSB and 1 ml was then taken for spectral reading. It is seen in Figure 46 that a healthy volunteer in the study seems to have around 10^4 - 10^5 CFU/ml per swab. The presence of such amount of *Staphylococcus aureus* enables positive growth indication to be determined after 7 hours of incubation. The confirmation of the culture being *Staphylococcus aureus* was provided by the positive agglutination result from the agglutination test kit when colonies obtained from standard plate count technique were used.

Figure 46. Normalized wavelength shift of sample from nasal swab of healthy volunteers

Therefore, determination of *Staphylococcus aureus* colonization via broth cultures of nasal swabs was possible. The detection could be easily performed within 7 hours if the amount of *Staphylococcus aureus* was $\sim 10^5$ CFU/ml. The direct inoculation of the nasal swab to a 1 ml broth might allow faster detection since the amount of bacteria present in the broth was increased by 10 fold compared to the result obtained in Figure 46.

7.3. Use of Growth Monitoring to Detect Oxacillin Resistance in *Staphylococcus aureus*

Since the antibiotics needed to treat infection by methicillin resistant *Staphylococcus aureus* (MRSA) are different from the ones used for its methicillin susceptible counterpart, it is important to identify the infectious agent before treatment is given. Though the resistance of MRSA is originally established against methicillin, the detection of MRSA nowadays is conducted by using oxacillin instead of methicillin. Both oxacillin and methicillin belong to the beta-lactam class of antibiotics and have similar bactericidal activity. However, methicillin is no longer available in USA and oxacillin maintains its activity during storage better than methicillin. Hence, oxacillin is used in the detection of methicillin resistance [215]. Consequently, the terms of methicillin resistant and oxacillin resistant are used interchangeably.

It was seen earlier that growth monitoring could be performed by monitoring the spectral reading of MSB. Important parameter that was monitored was the wavelength shift. Therefore, an investigation was made on the use of MSB in the detection of oxacillin resistant *Staphylococcus aureus* strain.

Tests were conducted with broth containing oxacillin with concentrations of 4, 6, 8, and 12 $\mu\text{g/ml}$ respectively. The set up used in these tests was shown in Figure 10. SMMIP was not yet used in these works. The oxacillin MIC for MSSA ATCC 29213 and MRSA ATCC 43300 was determined using the macro dilution method earlier described to be 0.5 $\mu\text{g/ml}$ and $> 128 \mu\text{g/ml}$. Example of the plots of the normalized wavelength shift and normalized intensity ratio (IR) at 600 nm against time are shown in Figures 47-50. Other plots can be found in Appendix C-3.

Figure 47. Normalized wavelength shift experienced by MSBOxa-4 spiked with MSSA ATCC 29213

Figure 48. Normalized intensity ratio at 600 nm experienced by MSBOxa-4 spiked with MSSA ATCC 29213

Figure 49. Normalized wavelength shift experienced by MSBOxa-4 spiked with MRSA ATCC 43300

Figure 50. Normalized intensity ratio at 600 nm experienced by MSBOxa-4 spiked with MRSA ATCC 43300

It is shown in Appendix C-3 that the initial inoculum of 4×10^5 CFU/ml for MSSA and 5×10^5 CFU/ml for MRSA failed to result in a positive detection within 8 hours. Normalized wavelength shift experienced a downwards shift of less than 1 nm. The result was contrary to the tests of direct inoculation using nasal swab. In the trials, an initial inoculum of 10 times lower in size (5×10^4 CFU/ml) resulted in a positive detection within 7 hours (Figure 46).

A higher initial inoculum was required for oxacillin-containing broth was expected for MSSA since the strain was susceptible to oxacillin. Growth inhibition of MRSA strains was again observed and the inhibition effect concurred with one study [216]. Results of all tests using MSSA ATCC 29213 indicated an existence of growth and thus not all bacteria were killed by oxacillin. The presence of colour change was detectable even though the concentration of the oxacillin was as high as 12 µg/ml (Appendix C-3).

Generally, there is a decline in the intensity ratio (IR) at 600 nm as well. The reduction in the light intensity transmitted by the medium was more prominent for lower oxacillin concentration. The reduction slowed down and saturated after some time for MSSA ATCC 29213 samples. On the other hand, the IR at 600 nm of MSBOxa-4 inoculated with MRSA ATCC 43300 (Figure 50) seemed to continue declining. This trend was less well produced in MSBOxa-6 and MSBOxa-8 (Appendix C-3). The high oxacillin concentration in MSBOxa-12 was seen clearly to inhibit even MRSA ATCC 43300. The IR value at 600 nm saturated after a decline in the first 3 hours of incubation for the inoculum size of 2.6×10^7 CFU/ml.

The observation of wavelength shift and reduction in IR value confirmed the existence of “persisters”. Not all bacteria from a particular strain would die when exposed to antibiotics. Some amount of sensitive strain (up to 0.1% or more) could survive even after a 24 hour incubation with oxacillin [217, 218]. Their acidification of mannitol would still change the pH of the medium and this was detected as a negative wavelength shift.

Across different oxacillin concentrations, it was generally noted that higher amounts of initial inoculum would generally produce faster colour change and faster reduction in IR value for the same oxacillin concentration. This phenomenon concurred with expectations that higher amount of bacteria would produce more acid as they fermented mannitol and thus produced faster colour changes. A higher inoculum also meant that more bacteria would be produced during replication.

The experiment using MSBOxa-12 showed that the bacterial growth was very much inhibited. MSSA with initial inoculum of 1.1×10^7 CFU/ml resulted in -5.62 nm normalized wavelength shift instead of -6.67 nm for MSBOxa-4 at the 480th minute of incubation time. The shift at MSBOxa-12 thus dropped to 84.3% of the shift at MSBOxa-4. The inhibition at MSBOxa-12 occurred for MRSA as well. For the initial inoculum of 2.6×10^7 CFU/ml, it was observed that the normalized wavelength shift was -8.08 nm for MSBOxa-4 and down to 82.5% or -6.67 nm at the 480th minute of incubation time. To compare the growth of MSSA ATCC 29213 and MRSA ATCC 43300 among the MSB with different oxacillin concentrations, plots of normalized wavelength shift and normalized intensity ratio from the tests using MSBOxa with similar inoculum were consolidated in Figures 51-54 for comparison.

Figure 51. Normalized wavelength shift of MSSA ATCC 29213 with similar inoculum during growth in MSB with different oxacillin concentrations

Figure 52. Normalized intensity ratio at 600 nm of MSSA ATCC 29213 with similar inoculum during growth in MSB with different oxacillin concentrations

Figure 53. Normalized wavelength shift of MRSA ATCC 43300 with similar inoculum during growth in MSB with different oxacillin concentrations

Figure 54. Normalized intensity ratio of MRSA ATCC 43300 with similar inoculum during growth in MSB with different oxacillin concentrations

Figures 51-54 shows that the points obtained from experiments using 6 μg , 8 μg , and 12 μg of oxacillin per ml of MSB are closely located and difficult to differentiate from one another. The results plotted also clearly shows a slightly faster drop of normalized wavelength shift for growth in MSBOxa-12. The data shows that the inhibition effect produced by oxacillin concentration above 4 $\mu\text{g}/\text{ml}$ seems to be similar. This observation agrees with the understanding that the bacterial killing rate of oxacillin, whose antibiotic class is β -lactam, is concentration independent [219]. Therefore, above a certain antibiotic concentration, it is expected that increasing the antibiotic concentration will not increase the amount of bacteria killed and the spectral reading produced for those concentrations should be similar.

It is seen that although the turbidity, shown by the IR value at 600 nm, does not increase, the normalized wavelength shift seems to indicate an ongoing production of acid by the bacteria. The increase in the turbidity is very low compared to the change of turbidity during growth in MSB without antibiotics (Appendix C-3). Although the monitoring work was performed for only 8 hours, a clear inverse exponential trend of the normalized IR value can be observed. Therefore, a longer monitoring period is expected to improve the trend even though 8 hours seem to be enough for detection purposes.

So far, it has been shown that the visible spectroscopy setup arrangement in this project was capable of discriminating growth from different initial inoculum sizes by monitoring the wavelength shift and turbidity development. The data plotted showed that all oxacillin concentration was unable to kill all susceptible bacteria within 8 hours of incubation time although inhibition effect was clearly observed. Nevertheless, it was noted in Appendix C-3 that MSBOxa-4 had the least inhibition effect for both MSSA ATCC 29213 and MRSA ATCC 43300. Thus, using MSBOxa-4, discrimination of MRSA from MSSA could be done.

Since the reduction in the intensity ratio (IR) value at 600 nm was not as steep as the reduction experienced during growth under MSB containing no antibiotics, the

IR value at 557.47 nm was revisited. A consistent reduction in normalized wavelength shift was shown earlier during growth in MSBOxa-4 (Figure 47 and Figure 49), while the IR value at 557.47 nm was expected to increase as shown in Figure 44. However, in contrast to the growth in a non-antibiotic medium (Figure 42), the trend of IR value during growth in MSBOxa-4 was not very much affected by the turbidity as shown by the slow reduction in IR value at 600 nm. Therefore, an equation was introduced to utilize the IR value at both points.

$$II = \frac{IR_{557.47}}{IR_{600.00}} \quad (40)$$

Where,

$$\begin{aligned} II &= \text{Intensity Index} \\ IR_{557.47} &= \text{Normalized intensity ratio at 557.47 nm} \\ IR_{600.00} &= \text{Normalized intensity ratio at 600.00 nm} \end{aligned}$$

By plotting the normalized value of Intensity Index (II) against the normalized wavelength shift obtained for similar inoculum of MSSA ATCC 29213 and MRSA ATCC 43300, it is seen that differentiation of both strains could be performed (Figure 55). The figure clearly shows that MRSA ATCC 43300 has a faster change of normalized II at any particular normalized wavelength shift and thus the points belonging to MRSA ATCC 43300 are above the points belonging to MSSA ATCC 29213. It shows that MSBOxa-4 inhibited MSSA ATCC 29213 more than MRSA ATCC 43300 since the production of the same level of acidity corresponds to slower increase in the II (mainly lower IR value at 600 nm).

Figure 55. Normalized intensity index changes compared to normalized wavelength shift for MSBOxa-4

At normalized wavelength shift of -1.05 nm, MRSA ATCC 43300 with an initial inoculum of 5×10^6 CFU/ml has a normalized intensity index of 0.62 while MSSA ATCC 29213 with a similar amount of initial inoculum has a value of 0.43. At normalized wavelength shift of -3.16 nm, the difference is larger since the normalized intensity index of MRSA ATCC 43300 is 1.53 while the normalized intensity index of MSSA ATCC 29213 is 1.20. Thus, the experimental setup could discriminate between both strains under standardized initial inoculum.

7.4. Effect of Different Incubation Temperature to Bacterial Growth

Several reports in the past documented how different incubation temperature affected the detection of antibiotics resistance. In general, it was suggested that relatively higher antibiotic resistance was detected at lower incubation temperature. Canawati *et. al.* [220] suggested that at 30 °C, cell wall synthesis was at a relatively higher rate compared to other cell structures and therefore contributed to higher resistance of MRSA to an antibiotic class called cephalosporin. Cephalosporins are similar to penicillins chemically, in mechanism of action, and toxicity [221].

Incubation in 30 °C had also been shown to increase the production of PBP2a, the binding protein held accountable for methicillin resistance in MRSA [222].

Considering that antibiotic resistance could be detected better at lower incubation temperature, it was then decided to perform the monitoring work of “mutation-in-progress” at 30 °C instead of the conventional 36 °C. Lower incubation temperatures might allow the detection of low resistance level due to the relatively higher production rate of cell components contributing to resistance. A study was performed to understand the effect of incubating MSSA ATCC 29213 and MRSA ATCC 43300 at 30 °C and 36 °C. Since lower incubation temperature or non optimal growth temperature translated to lower growth rate, a lower growth inhibition effect on MRSA in 30 °C compared to MSSA would support the idea of performing the monitoring work at lower temperatures. The lower incubation temperature would then help in the inhibition of the susceptible strain and allow the generally slower growing mutant strain to compete with susceptible parent strain [87, 223].

Figure 56. Normalized wavelength shift of MSB inoculated with various initial inoculum sizes of MSSA ATCC 29213 and incubated under two different temperatures

Figure 57. Normalized intensity ratio at 600 nm of MSB inoculated with various initial inoculum sizes of MSSA ATCC 29213 and incubated under two different temperatures

Figure 58. Normalized wavelength shift of MSB inoculated with various initial inoculum sizes of MRSA ATCC 43300 and incubated under two different temperatures

Figure 59. Normalized intensity ratio at 600 nm of MSB inoculated with various initial inoculum sizes of MRSA ATCC 43300 and incubated under two different temperatures

In general, Figures 56-59 shows that growth at 30 °C was much slower for MSSA ATCC 29213 as compared to MRSA ATCC 43300. The normalized wavelength shift and normalized intensity ratio of MRSA ATCC 43300 under incubation at 30 °C (Figures 58-59) were less separated compared to the data at 36 °C when similar inoculum sizes were compared (1.0×10^8 CFU/ml and 1.3×10^8 CFU/ml at 36 °C and 30 °C respectively). On the other hand, farther separation was noted for MSSA ATCC 29213 when inoculum sizes of 1.4×10^8 CFU/ml and 1.0×10^8 CFU/ml was compared for incubation at 30 °C and 36 °C respectively (Figures 56-57). Therefore, the findings suggest that incubation temperature at 30 °C may allow better competition between mutant and susceptible strains. When different temperatures are desired, the CEC of the system designed can be set to provide incubation from room temperature to 40 °C.

7.5. Separating Contribution of Acidity and Turbidity to the Spectral Plots

The spectral changes during acidification of MSB indicated that IR increases when pH of the medium was lowered. The increase occurred in the whole wavelength range (Figure 27). In contrast, inoculation of viable *Staphylococcus aureus* into the medium resulted in an increase of the intensity ratio at ~ 550 nm although the intensity ratio at wavelength > 580 nm declined. The decline happened due to the increase of the turbidity of the medium (Figure 38). Therefore, there was an interaction between colour and turbidity. An attempt was made to understand the interaction by performing tests on conventional MSB and MSB devoid of phenol red (MSBw/oPR) using the same source of inoculum. The experiments were to provide an understanding on the contribution of phenol red in the spectral changes.

Figure 60. Plot of the change of intensity ratio (IR) experienced by MSBw/oPR spiked with 2.7×10^8 CFU/ml of MSSA ATCC 29213

The spectral plot of MSBw/oPR shows no wavelength region where the IR increases with time (Figure 60). The overall reduction of IR was caused by the increase in the turbidity of the broth due to bacterial growth. A plot of the development of IR over some wavelengths is provided in Figure 61. The IR

generally decreased and eventually reached saturation. It is seen that maximum IR decline rate was experienced at ~ 531.42 nm and ~ 561.01 nm. Conversely, the IR around 561.01 nm has been shown to indicate the colour changes of MSB (Figure 28). Therefore, the change of IR around 561.01 nm of MSB containing viable *Staphylococcus aureus* would be greatly affected by the change of the turbidity of the medium.

Figure 61. Development of intensity ratio (IR) of MSB without phenol red spiked with 2.7×10^8 CFU/ml of MSSA ATCC 29213 at various wavelengths

The study using blank MSB under different pH suggested a slight increase of IR at 600 nm as the pH of the medium was lowered. The IR plots of MSB and MSBw/oPR revealed that the colour change experienced by the phenol red reduced the IR changes at 600 nm. The increase in IR > 580 nm seen during the acidification of blank MSB (Figure 28) compensated the reduction in IR value > 580 nm when bacteria increased the turbidity of the medium (Figure 42).

Figure 62. Development of IR_{600}^- of samples with similar initial inoculum of MSSA and MRSA

To analyze the data further, the intensity ratios at 600 nm of MSB and MSBw/oPR were plotted. IR_{600}^- was then calculated by subtracting IR at 600 nm of MSBw/oPR from IR at 600 nm of MSB. IR_{600}^- plot of samples inoculated with similar inoculum of MSSA ATCC 29213 and MRSA ATCC 43300 was shown in Figure 62. For the tested inocula, the increase of IR_{600}^- started to slow down after 300 minutes of incubation and after around 600 minutes, IR_{600}^- decreased with a decreasing rate.

IR_{600}^- was plotted along with the wavelength shift of the inoculated MSB. For samples with initial inoculum of 2.7×10^8 CFU/ml of MSSA ATCC 29213, it was seen that the rate of change of the normalized wavelength shift seemed to dictate the rate of change of IR_{600}^- (Figure 63A). Once IR_{600}^- reached the maximum, normalized wavelength shift seemed to decline with a slower rate. The trend of the IR_{600}^- plot was similar with the trend of the normalized IR at 561.01 nm plot (Figure 63(B)). Since the IR at 561.01 nm was related to colour change, it could be

deduced that IR_{600}^- was contributed by phenol red as the agent that produces the colour change in MSB.

Figure 63. (A) Comparison of IR_{600}^- and normalized wavelength shift experienced by MSB; (B) Comparison between normalized IR at 561.01 nm experienced by MSB and IR_{600}^-

However, the bell shape trend shown in Figure 62 is not replicated faithfully in other inoculum size. Figure 64 shows that only inoculum size of 4.6×10^7 CFU/ml displays a rather bell shape trend of IR_{600}^- over the incubation period. In contrast, the inoculum size of 4.5×10^5 CFU/ml displayed the trend in the form of inverted bell shape. Much more irregularities were present in culture with starting inoculum of $< 10^8$ CFU/ml. There were even small negative values observed in Figure 64 which was resulted from the uncertainties at the lag phase of the culture. Therefore, there was no correlation between IR_{600}^- trend over time and inoculum sizes. Nonetheless, when the samples were of similar inoculum sizes, the IR_{600}^- plots were similar irrespective of the inoculated strains.

Figure 64. IR_{600}^- plot when the broths were inoculated with MSSA ATCC 29213

To understand clearly the role of turbidity changes caused by bacterial growth in the development of IR, for each inoculum, normalized wavelength shift of MSB was plotted against normalized IR at 561 nm (Figure 65). The plot showed that in MSB, colour change dominated the IR change. Increases in turbidity should have decreased the IR at 561.01 nm as seen in Figure 60. However, Figure 65 showed that the IR at 561.01 nm of the MSB continued to increase before the wavelength shift reached the maximum value of -19.35 ± 1.41 nm. Therefore, the increase in the turbidity of the medium was unable to reduce the IR at 561.01 nm before no more colour change could be experienced by the medium. When the wavelength shift was at the maximum, the IR at 561.01 nm continued to drop consistently as more and more bacteria was produced and the medium was more turbid.

Figure 65. Development of IR at 561.01 nm with respect to colour change in MSB

7.6. Summary of the Growth of *Staphylococcus aureus* in MSB

This chapter shows that it is possible to monitor the growth of *Staphylococcus aureus* in MSB over a long period of time by monitoring the wavelength shift and intensity ratio changes of MSB. After normalization of wavelength shift with data obtained at the 30 minutes of incubation, the wavelength shift is observed to be a negative trend due to a left shifting of the spectral peak as shown earlier in many figures, e.g. Figure 38. The shifting to the left indicates a higher transmittance of the green component of light and a reduction in the transmission of the red component. It is observed that after a period of increase in spectral peak intensity, the intensity drops as the spectral peak shift is no longer observed. It was also learned that $\sim 10^8$ CFU/ml is the carrying capacity of the sample holder in this study as sample higher than that amount did not produce increase in viable count over 24 hours incubation despite inducing a colour change.

Since it was established that the monitoring of growth of reference strains, attempts were performed to investigate the possible of growth monitoring on clinical

samples. First type of experiments involved inoculating of MSB with colonies obtained from nasal swab. The second type of experiments involved the direct inoculation of MSB with nasal swab. The results of both experiments show that the determination of *Staphylococcus aureus* colonization via nasal swabs is possible. Directly inoculating the nasal swab into the broth gives us a 10 times increase of sample concentration since the sample broth is 10 times less than the broth needed if the colonies are obtained from agar. The growth detection was possible within 7 hours if the amount of *Staphylococcus aureus* in the swab was $\sim 10^5$ CFU/ml.

Extending the growth monitoring concept, an attempt was performed to understand whether, using the same concept, detection of oxacillin resistance in *Staphylococcus aureus* can be performed. MSSA ATCC 29213 and MRSA ATCC 43300 were incubated in MSB containing oxacillin with the concentration of 4, 6, 8, and 12 $\mu\text{g/ml}$. The observation of wavelength shift and IR reduction confirmed the existence of “persisters” in the sample. Persisters are antibiotic sensitive populations that survive even after 24 hours of antibiotic exposure. The physical confirmation of persisters is seen at the ultrastructural observation.

Growth of both MRSA ATCC 43300 and MSSA ATCC 29213 were inhibited in all oxacillin MSB broth tested. Higher starting inoculum was observed to produce higher wavelength shift and IR change over times since more acid are produced by higher amount of bacteria. In the experiments, it was also observed that wavelength shift can be used to indicate the growth of bacteria before IR change is observed. The inhibition effect produced by oxacillin with concentration of $> 4 \mu\text{g/ml}$ was largely similar. This observation is enforced by the understanding that the bacterial killing rate of oxacillin is concentration independent [219]. When the starting inoculum is standardized, the study using MSB with oxacillin shows that MSSA ATCC 29213 and MRSA ATCC 43300 can be discriminated if they are grown in MSBOxa-4. Further experiment to understand the effect of incubation temperature on oxacillin inhibition of both strains revealed that incubation at 30 °C may allow better competition between both strains. Hence, the CEC of the system is set to provide incubation temperature at 30 °C.

To provide better understanding on the dynamics of wavelength shift and IR change, spectral plot obtained from MSB containing no phenol red (MSBw/oPR) and MSB were compared. From this study, it is understood that the wavelength shift reduced the amount of IR reduction that should have been produced by increase in turbidity. The turbidity dominated the IR change only when the wavelength shift is near the maximum. Armed with all these understanding, a prolonged monitoring of the emergence of antibiotic resistance was conducted.

Chapter 8

MONITORING OF THE EMERGENCE OF ANTIBIOTIC RESISTANCE

8.1. Ultrastructural Changes of *Staphylococcus aureus* at Exposure to Ciprofloxacin

The change in the ultrastructure of *Staphylococcus aureus* was studied by using Scanning Electron Microscopy (SEM). The study focused on bacteria incubated in media containing ciprofloxacin since the antibiotic was used in the study of the emergence of resistance (Figure 12). The ultrastructural images were taken at 10,000 X and 20,000 X magnifications.

It was suggested that ciprofloxacin causes bacterial apoptosis since DNA ends are released at the failure of DNA supercoiling during DNA replication [61]. The production of some proteins during the apoptosis event then caused the subsequent bacterial lysis [224]. SEM pictures (Figure 66) taken on MSBCip-0.5 culture after 24 hours of incubation show clearly the lysed forms of *Staphylococcus aureus*.

Figure 66. Scanning Electron Microscopy (SEM) pictures of *Staphylococcus aureus* after 24 hours incubation in MSBCip-0.5

The SEM images above revealed extended lysis where the bacterium seemed to have deformed into random shapes. The arrows in the figure point to a circle which could be the previous round shape of each bacterium before it swelled and deformed. Under normal incubation in MSB, *Staphylococcus aureus* had a round

shape with a diameter of $0.60 \pm 0.03 \mu\text{m}$ ($n = 15$) (Figure 1). On the other hand, the circle seen in Figure 66 was of a bigger diameter ($1.2 \pm 0.28 \mu\text{m}$; $n = 15$). The standard deviation was bigger as well though in overall, the swollen shape was two times bigger than the normal shape. Despite the possible anisotropic shrinkage of up to 60% that might be caused by the dehydration process during sample preparation [200], the swelling noted by the size differences of the circle was $\sim 100\%$. Therefore the swelling was clearly due to exposure to ciprofloxacin and was not the effect of dehydration process.

Figure 67 shows the existence of bacteria with normal round shape in the populations, which was isolated after 24 hours incubation in MSBCip-0.5. The population of bacteria with normal morphology might be persisters. Persisters were defined as viable antibiotic susceptible bacteria which can be recovered after hours of exposure to the antibiotic [225]. Fluoroquinolone was known to kill non-growing cells but not persisters [226]. Some reports had also studied the existence of persisters in *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* during the culture in antibiotics (oxacillin, ciprofloxacin, and ampicillin) containing medium [217, 218, 226-229].

Figure 67. Scanning Electron Microscopy (SEM) pictures of *Staphylococcus aureus* with normal and filamentous morphology after 24 hours incubation in MSBCip-0.5

Besides intact persister cells, the image also showed filamentous clusters of *Staphylococcus aureus*, which failed to complete the cell division. In *E. coli* grown in nalidixic acid, the cellular filamentation was thought to occur due to DNA synthesis failure [224]. Since ciprofloxacin also inhibited DNA synthesis in *Staphylococcus aureus*, the filamentation shown in Figure 67 might have occurred due to the same reason. Further study, which was outside the scope of this study, could be performed to investigate the event.

Figure 68 also confirmed the existence of persisters in the population of *Staphylococcus aureus* grown in MSBCip-2. The existence of intact *Staphylococcus aureus* cells with their round morphology was seen in the figure. The persisters contributed to the changes in normalized wavelength shift and increased in IR reported earlier during study of growth in antibiotics-containing medium. Persisters were known to have gradually reduced their metabolism rate during antibiotic exposure [226]. Their metabolism, though was slower than normal population, should have contributed to the acidification of medium.

The indication of positive metabolism despite exposure to oxacillin has been reported in section 7.3. Similar observations could be seen in the later part of this report on the growth in medium containing ciprofloxacin. Comparison of the image taken on growth in MSB without antibiotics (Figure 1) and the finding of bacteria with normal morphology in MSBCip-2 (Figure 66) confirmed that the bacterial lysis was caused by ciprofloxacin.

Figure 68. Scanning Electron Microscopy (SEM) pictures of *Staphylococcus aureus* after 24 hours incubation in MSBCip-2

8.2. SMMIP in Monitoring the Emergence of Antibiotic Resistance

It has been argued so far that using the set-up shown in Figure 10, visible spectroscopic method allowed growth monitoring of *Staphylococcus aureus* in Mannitol Salt Broth (MSB). The determination of growth was performed by monitoring the normalized wavelength shift and normalized intensity ratio at 600 nm. The threshold of normalized wavelength shift was determined as -1 nm while the threshold for normalized intensity ratio at 600 nm to indicate positive growth could be set at -0.05 (Figure 50).

The temperature of the Controlled Environmental Chamber (CEC) in the SMMIP had been shown to stay within 35.4 °C and 36.1 °C when 36 °C was set. When the control was set to 30 °C, the temperature was found to stay within 29.6 °C and 30.1 °C. The range of temperature fluctuation was smaller since the lower temperature limit for cutting off the power supplied to the heater was set at 29.6 °C. The result shown in section 7.4 had also argued that incubation at temperature 30 °C would help the mutant strains, which was normally of lower fitness, to compete with the susceptible parent strain [87, 223].

Study was then performed using SMMIP to investigate its ability to monitor of the emergence of antibiotic resistance. In the study, MSSA ATCC 29213 was incubated in MSB containing ciprofloxacin. The mutagenic ability of ciprofloxacin on *Staphylococcus aureus* had been shown in the work by Allen *et. al.* [55]. The ability of ciprofloxacin to induce adaptive mutation was also mentioned by Riesenfeld *et. al.* [88].

The Minimum Inhibitory Concentration (MIC) of Ciprofloxacin for MSSA ATCC 29213 was determined by using macro dilution method [192]. Using inoculum size of 5×10^5 CFU/ml, the MIC was determined as 0.25 µg/ml and 0.5 µg/ml after incubation in air at 30 °C for 24 and 48 hours respectively. Considering that incubation 30 °C caused a slower growth rate, the considered MIC value was the one obtained at 48 hours, which was 0.5 µg/ml. Few studies showed that

ciprofloxacin induced resistance when *Staphylococcus aureus* was subjected to the antibiotics at the concentration close to the strain's MIC value [51, 56]. Therefore, in the study on the emergence of antibiotic resistance, the progress of mutation was investigated using SMMIP for MSSA ATCC 29213 incubated in air at 30 °C using MSB containing 0.5 µg/ml and 1 µg/ml.

At the first part of the study using SMMIP, MSSA ATCC 29213 was inoculated into MSBCip-1 and the ciprofloxacin concentration was subsequently increased up to 1.25 µg/ml by adding fresh MSBCip-1.25 every 24 hours. Therefore, there was an increase in the ciprofloxacin concentration from 1 µg/ml to 1.2 µg/ml, 1.24 µg/ml, and 1.25 µg/ml at day 2, 3, and 4 respectively. The medium above day 4 would then contain ciprofloxacin with the concentration of 1.25 µg/ml.

Negative gradient of normalized wavelength shift and normalized intensity ratio (IR) have so far been used for positive growth indication. Since ciprofloxacin was known to be bactericidal, fewer and fewer bacteria were expected to survive if the culture was maintained for few days [61]. Therefore, a reduction in the negative gradient, i.e. less steep, in the next incubation period indicate less amount of bacterial activity. However, an increase in the gradient, i.e. higher rate of change, of the normalized wavelength shift and/or normalized IR at 600 nm would be indicative of higher amount of surviving bacteria. This change of trend of the normalized wavelength shift and IR value would be used to indicate the presence of more resistant mutants in the otherwise single susceptible population.

Figure 69. Development of normalized wavelength shift of MSB containing increasing concentration of ciprofloxacin which was spiked with MSSA ATCC 29213

The normalized wavelength shift at the first day (Figure 69) was of similar trend compared to the growth in MSB containing no antibiotics (Figure 43). However, the amount of shift that the spectral reading experienced was generally reduced as the culture was incubated further. The plot of normalized wavelength shift was discontinuous every 24 hours interval since the pH of the medium was always adjusted back to the initial pH value. The adjustment was achieved by performing titration using 0.1 M NaOH. The end point of the titration was checked by comparing the resulting spectral reading of the sample with the spectral reading at the 30th minutes, which would be used as a reference.

Culture with starting inoculum of 6.4×10^8 CFU/ml ceased to have any indication of active metabolism at the 5th day of incubation while the culture containing 6.4×10^7 CFU/ml had normalized wavelength shift consistently ~ -1 nm at the 4th day of incubation. Though rather large amount of *Staphylococcus aureus* ($\sim 10^9$ CFU/ml) was inoculated, no mutants with ability to grow in ≥ 0.5 $\mu\text{g/ml}$ was recovered. The large size of the initial inoculum was chosen so as to exceed the mutation frequency

of *Staphylococcus aureus*, which was $\sim 10^6$ CFU/ml in environment containing two times its MIC value [230].

The presence of normal bacteria in the culture (Figure 67-Figure 68) even after 24 hours of incubation in ciprofloxacin explained the normalized wavelength shift seen in Figure 69. However, there was a small increase of normalized wavelength shift denoting shifting of the spectral peak to the right at the end of day 1 for inoculum size of 6.4×10^7 CFU/ml. The upward shift of normalized wavelength was again seen in day 2 and day 3 of the monitoring period. The upward shift meant that the medium had more alkali pH.

The alkalization of the medium was brought about by the bacterial lysis. During the lysis of bacterial cell, cytoplasm contents of the cells were released into the medium [231]. The cytoplasm of bacteria with optimum growth around neutral pH was known to be of pH 7 – 8 [1]. Extrusion of the cytoplasm contents of the lysed *Staphylococcus aureus* (Figure 66) would move the pH of the medium to a more alkali region. Therefore, the more dead bacteria the culture produced, the more alkali content was released into the medium. Such process would move the normalized wavelength shift upwards (more positive).

The growth monitoring was observed as well via the development of normalized intensity ratio (IR) at 600 nm (Figure 70). The profile of the IR plot was the same as what had been shown before in several previous sections. Nevertheless, if the IR plot was taken as a growth determinant, the test of the sample with initial inoculum of 6.4×10^7 CFU/ml would have been stopped at 2nd day of incubation. However, normalized wavelength shift trend of the culture of 6.4×10^8 CFU/ml showed no more active growth indication only after three days of incubation. Therefore, plot of normalized wavelength shift was more superior in determining the presence of viable bacteria in the culture.

Figure 70. Development of normalized intensity ratio (IR) at 600 nm for MSB containing increasing concentration of ciprofloxacin which was spiked with MSSA ATCC 29213

From Figure 70, it is observed that the IR increased after 10 hours of incubation for the smaller inoculum size. The breaking of cell wall (Figure 66 and Figure 68) released the bacterial cell content to the medium. The contents released could then be dissolved into the medium easily [232]. The dissolution decreased the turbidity of the medium and in consequence, the increase of IR at 600 nm was observed. The clearing of the growth medium was known to happen on *Escherichia coli* culture on ciprofloxacin-containing medium [231].

It was therefore seen that no mutants was produced in the study using a progressively increasing ciprofloxacin concentration regime. MSBCip-1 presents twice the MIC concentration for MSSA ATCC 29213. Some earlier studies earlier have pointed out that higher antibiotic exposure reduces the chance for resistant mutants to appear. Furthermore, the increase of ciprofloxacin concentration in the growth medium also added further pressure and reduced the chance further. The antibiotic pressure might have prevented the emergence of resistant mutants.

A study using MSBCip-0.5 was then conducted. In the study, fresh nutrient added was MSBCip-0.5 as well. Therefore, the population was subjected to MSB containing constant ciprofloxacin concentration, which was 0.5 $\mu\text{g/ml}$, for the whole duration of study.

Figure 71. Development of normalized wavelength shift for MSBCip-0.5 which was spiked with MSSA ATCC 29213

Figure 71 shows the development of wavelength shift when MSBCip-0.5 was spiked with MSSA. The blank sample experienced no meaningful wavelength shift over a period of 7 days. On the other hand, discontinuity of data points was also experienced every 24 hours for broth containing samples. The discontinuity resulted from pH adjustment as sterile fresh nutrient was added into the sample. Figure 69 had earlier shown similar feature. The progress of the normalized wavelength shift was indeed similar as the trend shown during growth in MSB containing no antibiotics (Figure 46). It had been shown in many studies that 24 hours incubation in ciprofloxacin with MIC concentration did not kill all isolates in the culture [54]. Therefore, the normalized wavelength shift shown in Figures 69 and 71 showed the activity of the surviving bacteria.

The study with MSBCip-0.5 showed the ability of a culture with initial inoculum of 1.3×10^8 CFU/ml (Sample 3) to induce colour change to the medium up to day 4. Data at day 5 again showed the alkalization of the medium as the cytoplasm content of the lysed bacteria was released into the medium. The study with MSBCip-0.5 showed that a culture with initial inoculum of 1.3×10^7 CFU/ml (Sample 2) experienced a drop in the amount of normalized wavelength shift detected in the 2nd day. The reduction of normalized wavelength shift continued in day 3 and the normalized wavelength shift fell below the threshold value of a positive growth indication. Similar trend was also observed for culture with initial inoculum of 6.0×10^6 CFU/ml (Sample 1).

The rate of change for the normalized wavelength shift increased at day 6, 4, and 7 for Sample 1, 2, and 3 respectively. The increase indicated that the metabolism activity was higher and consequently, more bacteria had survived. The increase of the surviving bacterial number indicated the presence of more resistant mutant. Susceptible bacteria continued to be eradicated by the antibiotic. Such bacteria would not be able to contribute to any increase of the indication of an active metabolism activity.

Figure 72. Development of the amount of normalized wavelength shift for some selected incubation time point over five incubation days for Sample 1

The positive indication of mutation via indication of higher active metabolism is illustrated in Figure 72. The six selected incubation time points experienced a reduction of the amount of wavelength shift over four incubation days. However, there was an increase of the amount of wavelength shift at the 16th hour to 24th hour at day 6 compared to day 5. Such increase indicated that there were more acid produced at that two time points of day 6 compared to day 5. The increase of the amount of acid suggested a higher metabolism activity, which was indicative of the presence of thriving bacteria. The thriving bacteria were the emerged resistant mutants capable of growing in the antibiotic concentration provided by the medium. Indication of the emergence of resistant mutants could then be pointed to the increase in the amount of wavelength shift at the 16th hour of day 6 or 136th hour of incubation. The confirmation of the presence of mutants was provided by the seventh day data where there was a further increase of the amount of wavelength shift over the chosen time points.

Figure 73. Development of the amount of normalized wavelength shift for some selected incubation time point over five incubation days for Sample 2

Figure 74. Development of the amount of normalized wavelength shift for some selected incubation time point over five incubation days for Sample 3

The increase of the indication of active metabolism was also experienced for the sample with initial inoculum of 1.3×10^7 CFU/ml (Sample 2) and 1.3×10^8 CFU/ml (Sample 3). Compared to Sample 1, the change occurred faster for Sample 2 but

later for Sample 3. For Sample 2, increase of the slope was seen at day 4 (92nd hour) while for Sample 3, the change of the slope was experienced at day 7 (161st hour). In the test with Sample 3, the normalized wavelength shift at 144th to 160th hour was already below positive growth indication. Nevertheless, the amount of wavelength shift increased at the 161st hour onward. For both samples, the normalized wavelength shift continued to increase at the next day indicating increased active metabolism in the medium.

Figure 74 shows a plot similar to Figures 72 and 73. In the figure, there was actually an increase of the amount of wavelength shift at the 16th hour of day 3 compared to day 2. However, such increase was followed by a decrease in the amount of wavelength shift at the next day. The increase could have resulted from experimental uncertainties. The true indication of mutation was seen at day 7 where an increase of the amount of normalized wavelength shift was observed. The increase continued to day 8 where a consistent increase of the amount of wavelength shift could be noted over all the selected time points. Therefore, the positive increase of active metabolism activity was seen starting in day 7, day 4, and day 6 for culture with initial inoculum of 1.3×10^8 CFU/ml, 1.3×10^7 CFU/ml, and 6.0×10^8 CFU/ml respectively. The existence of resistant mutants could be confirmed from the data obtained at the beginning of the next incubation period.

The presence of mutants resulted in a competition between the alkalization caused by content from lysed bacteria and the acidification caused by the mutants. Day 5 data of Sample 3 indicated the lower amount of wavelength shift at the 20th and 24th hour of incubation compared to the 16th hour (Figure 74). Therefore, the resistant mutants could only be detected by a further drop in the normalized wavelength shifts when the acidification process produced more acid to counter the alkalization process. The competition was seen at day 7 of Sample 3 where the flat portion of the wavelength shift data was followed by a sudden negative wavelength shift (Figure 71). Hence, the normalized wavelength shift indicated the emergence of resistant mutants at the end of day 4 for Sample 2 and at the end of day 7 for

Sample 3. The wavelength shift at the next day confirmed the presence of such mutants.

Normalized IR at 600 nm was also plotted to determine the emergence of mutants via changes in the IR value (Figure 75). Sample 1 experienced increase of IR indicating relatively higher bacterial activity compared to the previous day at day 7 while Sample 2 experienced an increase of the IR at day 5. On the other hand, Sample 3, whose inoculum was 10 times more than Sample 2, experienced the increase of the slope only after eight days of incubation. The increase of the IR after a continuous drop of IR within the day was also observed. Hence, there were clearing of the medium.

Figure 75. Development of normalized intensity ratio (IR) at 600 nm for MSBCip-0.5 which was spiked with MSSA ATCC 29213

The reduction of the turbidity of the medium was seen at day 4 for Sample 3 (the culture with a starting inoculum of 1.3×10^8 CFU/ml). The clearing produced positive IR, indicating that the medium was less turbid compared to the starting turbidity at that particular incubation day (Figure 75). After 2 days of negative

growth indication, the IR again dropped to negative value in day 8. The clearing of the growth medium mirrored the result shown in Figure 70. The IR plot of Sample 3 indicated higher turbidity at day 3 compared to day 2. The increase could have been interpreted as the presence of more bacteria although there was no resistant yet recovered in the sample. The increase of the amount of turbidity change was resulted from the lack of homogeneity of the sample which might have resulted in the absorption of more light by the filaments of bacteria. Future design of the sample holder would be needed to prevent formation of filaments and clumps.

Table 6. Comparison of the time required to indicate the presence of mutants

Sample	Wavelength shift	IR at 600 nm
Sample 1	Day 6	Day 7
Sample 2	Day 4	Day 5
Sample 3	Day 7	Day 8

It is clearly seen, therefore, that normalized wavelength shift could indicate the presence of mutants in the sample a day earlier compared to normalized IR at 600 nm. The increase in the amount of viable bacteria could yet to increase the turbidity of the medium to a detectable level while the amount of acid produced had changed the pH of the medium to a detectable level. Metabolism activity seemed to be more useful to determine the presence of mutants in the sample.

This study of the emergence of ciprofloxacin *Staphylococcus aureus* mutants with SMMIP revealed that increasing the size of initial inoculum did not translate to faster emergence of mutants (Table 6). In fact, an optimum inoculum size for the emergence of resistant mutants might exist. Culture containing 1.3×10^7 CFU/ml produced mutants at least 2 days faster than inoculum containing 6.0×10^6 CFU/ml and 1.3×10^8 CFU/ml. A large bacterial inoculum size of 10^8 CFU/ml might have provided sub optimum growth condition since the maximum capacity of a 1 ml of MSB for the growth of *Staphylococcus aureus* was in the order of 10^8 CFU/ml. The capacity was deduced from the inability of any inoculum size tested in this study to produce 10^9 CFU/ml of *Staphylococcus aureus* at the end of 24 hours of

incubation. On the other hand, small initial inoculum size might provide too few bacteria for resistance selection.

8.3. Comparative Conventional Methods to Monitor the Emergence of Antibiotic Resistance

As stated previously in the literature review, standard plate count and Population Analysis Profile (PAP) were used to determine the emergence of antibiotic resistant mutants. In order to confirm the findings described in Figure 71 and Figure 75, those techniques were performed at the start of each incubation period (24 hours interval) as comparative methods. The viable count and PAP results confirmed the validity of using normalized wavelength shift and normalized IR at 600 nm to monitor mutation-in-progress.

Figure 76. Development of the viable count of MSSA ATCC 29213 during incubation in MSBCip-0.5

Figure 76 shows the viable count obtained at the 0th hour of each incubation period. There were two viable count values obtained at the end of each incubation period since 5-times dilution of the sample was resulted from the nutrient replenishing

process. In order to help the visualization of the development of viable count from time to time, a straight line was drawn to connect the points.

Figure 76 also shows that the sample with starting inoculum of 1.3×10^8 CFU/ml (Sample 3) experienced a continuous decline of the viable bacteria over six incubation days. The viable count then steeply increased by 100-fold at the end of day 7. The increase of viable count was clearly seen in Figure 71 as an increase in the gradient of the normalized wavelength shift.

The viable count for culture with initial inoculum of 1.3×10^7 CFU/ml (Sample 2) fell below the detection limit of plate count technique performed (300 CFU/ml) after two days of incubation. The viable count could be obtained again at the end of day 4 (96 hours of incubation). The increase in the viable count was reflected as an increase in the gradient of normalized wavelength shift in day 4. The increase was more clearly seen in the shift experienced during incubation at day 5.

The increase of viable count was encountered at the 120th hour or the end of day 5 for culture with initial inoculum of 6.0×10^6 CFU/ml (Sample 1). Nevertheless, the increase of the viable count from 1.7×10^3 CFU/ml to 2.4×10^6 CFU/ml did not produce enough acid to result in detectable colour change. Negative wavelength shift was only produced at the next day (day 6) (Figure 71). Wavelength shift data and the viable count method indicated the presence of resistant mutants at the same incubation day for Sample 2 and 3. In the study with Sample 1, the viable count increased a day earlier than the plot of the normalized wavelength shift. However, the result of the viable count was obtained at the same day as the result of the normalized wavelength shift. Therefore, the viable count method confirmed the presence of resistant mutants when an increase of wavelength shift data after a period of decline was experienced.

There was an increase of viable count in day 2 incubation of Sample 1 and day 3 incubation of Sample 3. The increase was, however, not indicated by an increase of wavelength shift shown in Figure 71. The PAP, explained at the later part of the

report, did not establish the presence of any resistant mutants as well. Hence, the slight increase did not correlate with the emergence of resistant mutants. The variation of the viable bacterial counts at those points could have been the result of inadequate mixing of the sample. This error perhaps could highlight one of the disadvantages of viable plate count technique as the main determination of the emergence of resistant mutants. Fortunately, the increase of the viable bacterial count was around one order of magnitude while the presence of resistant mutants normally increased the count by two orders of magnitude. Furthermore, the viable count at the subsequent days clearly denoted the decrease of viable bacterial number.

It was also observed that the viable count of Sample 1 was fairly similar over day 2 to 4 of the incubation. The presence of ciprofloxacin seemed to fail to eradicate all bacteria. Instead, some of the population persisted in the sample although the number was too small to produce discernable colour change. The exact reason for such persistence in this study was unknown and outside the scope of the current work. However, it was surmised that the bacteria was of slower growth rate due to the presence of ciprofloxacin. The slow growth rate perhaps implied lower activity of DNA topoisomerase IV, which was the drug target. Hence, few bacteria were killed by the drug.

Since viable count only confirmed the increase in the number of population, a more detailed study into the composition of the population was provided by PAP. PAP study was conducted every beginning of a 24 hours incubation period. The detection limit was 300 CFU/ml. Figure 77 shows the PAP result for the culture with initial inoculum of 1.3×10^8 CFU/ml (Sample 3). The PAP profile established the absence of subpopulations capable of growing in agar containing 0.5 µg/ml of ciprofloxacin up to 120 hours or day 6 of incubation. The population profile from the culture after 144 hours of incubation or at the end of day 7 then clearly showed the emergence of subpopulations capable of growing on MHBCip-0.5. Therefore, the subpopulation was a more resistant mutant, which was capable of growing at the inhibitory ciprofloxacin concentration of MSSA ATCC 29213. Therefore, the

mutants were present although the viable count yet increased (Figure 76). Viable count might miss the appearance of mutants whose amount was yet enough to counter the number of the dying susceptible strain. It was also observed that the mutants were not producing sufficient acid to affect colour change in the medium. Wavelength shift of the day 6 of Sample 3 (120-144 hours) did not show any increase in the amount of shift. The emergence of mutants capable of growing in ciprofloxacin concentration of 0.5 µg/ml was only shown as an increase of the normalized wavelength shift in day 7 (Figure 71).

Figure 77. Population Analysis Profile (PAP) of the bacterial population found in MSBCip-0.5 which was initially inoculated with 1.3×10^8 CFU/ml of MSSA ATCC 29213 (Sample 3)

The PAP analysis of the culture with starting inoculum of 1.3×10^7 CFU/ml (Sample 2) and 6.0×10^6 CFU/ml (Sample 1) further confirmed the possibility of using normalized wavelength shift as the indication of the emergence of more resistant mutant/s (Figures 78-79). Profiles of the cultures showed the presence of mutants among the populations isolated after 96 hours and 120 hours of incubation for Sample 2 and Sample 1 respectively. The PAP results indicated that resistant mutants of Sample 1 was also yet affecting the normalized wavelength shift of

SMMIP when the mutants started to emerge in the sample. On the other hand, the mutants found in Sample 2 were recovered at the same day as the positive indication of mutants provided by SMMIP.

Figure 78. Population Analysis Profile (PAP) of the bacterial population found in MSBip-0.5 which is initially inoculated with 1.3×10^7 CFU/ml of MSSA ATCC 29213 (Sample 2)

Figure 79. Population Analysis Profile (PAP) of the bacterial population found in MSBip-0.5 which is initially inoculated with 6.0×10^6 CFU/ml of MSSA ATCC 29213 (Sample 1)

The recovery of MSSA ATCC 29213 mutants with ability to grow in ciprofloxacin with concentration of $0.5 \mu\text{g/ml}$ (MIC of MSSA ATCC 29213) was clearly shown in Figures 77-79. The percentages of the mutants among the total populations were also generally found to increase over time during the incubation in MSBCip-0.5. An additional $\sim 25\%$ of the populations were able to grow in ciprofloxacin concentration of $0.5 \mu\text{g/ml}$ at the end of day 7 for the sample with starting inoculum of 1.3×10^8 CFU/ml.

During the growth in MSBCip-0.5, the subpopulations inhibited by the ciprofloxacin concentration would die. Less resistant subpopulations would be killed faster and populations would consist of those subpopulations with relatively higher resistance. The event was seen in day 3 of the culture with initial inoculum of 1.3×10^8 CFU/ml. The percentage of the subpopulation capable of growing at MSBCip-0.5 was $\sim 16\%$ higher at the 72nd hour compared to the previous day.

8.4. Superiority of SMMIP Compared to the Conventional Methods in Monitoring the Emergence of Antibiotic Resistance

In the results described in Figure 71, the presence of *Staphylococcus aureus* mutant/s capable of growing at the MIC of the parent MSSA ATCC 29213 strain was shown by the SMMIP. The emergence of mutants was then compared to the conventional plate count and PAP analysis technique (Figures 76-79). The viable count and PAP analysis confirmed the results produced by SMMIP. Consequently, the normalized wavelength shift data from the SMMIP could be used to study the progress of mutation, or termed mutation-in-progress.

The PAP analysis required 48 hours of incubation while the viable count method required 24 hours of incubation. On the other hand, SMMIP produced real time wavelength shift data to allow a real time monitoring. Therefore, SMMIP can determine the presence of mutants in the culture earlier than the conventional methods (PAP and viable count). The study using SMMIP can also be performed with fewer resources since it was unnecessary to keep track of the viable count. No PAP analysis was also needed before an indication of mutation-in-progress was provided by SMMIP. A study that took five days to complete typically required 21 ml of broth if SMMIP was utilized while 1440 agar plates or 28.8 litres of agar solution were needed if viable count was performed every hour. On the other hand, PAP analysis required 300 agar plates or 6 litres of agar solution if the analysis was performed at the start of each incubation period using 6 antibiotic concentrations. Therefore, there was a significant reduction of resources when SMMIP was utilized for mutational study.

In this study, the monitoring result with SMMIP, which was confirmed with the viable count and PAP methods, showed that Sample 2 seemed to produce resistant mutants faster than the other samples. There was no evidence to correlate the size of the starting inoculum with the speed of mutant recovery. Thus, this finding did not agree with the concept of mutation frequency, which advocated that as long as the inoculum was larger than the mutation frequency and the antibiotics induced the

emergence of mutants, mutants would emerge in the sample. Hence, the concept indicated that more resistant mutants could be found in cultures with larger inocula and mutants would intuitively emerge faster when the inoculum size was bigger. Such notion was challenged by the result of this study. Since no mutants were produced after 48 hours of incubation, the concept of mutation frequency would have ruled that the ciprofloxacin mutation frequency of *Staphylococcus aureus* ATCC 29213 was $< 10^{-8}$ CFU/ml. Therefore, it would seem counter intuitive to even try to produce mutants using any inoculum lower than 10^8 CFU/ml. However, the result produced by SMMIP clearly shows the emergence of mutants from starting inocula of $\sim 10^6$ and $\sim 10^7$ CFU/ml (Figures 77-79). The mutants even emerged faster than the starting inoculum of $\sim 10^8$ CFU/ml.

The result of the SMMIP study supported the randomness of mutation. The concept of mutation frequency disregarded the randomness of mutational event. It did not consider that mutants might fail to emerge even though the inoculum size was larger than the mutation frequency. In fact, mutation frequency of *Staphylococcus aureus* during ciprofloxacin exposure varied between studies [230, 233]. The range of the mutation frequency spanned over three orders of magnitude, which pointed out the uncertainty in determining how many mutants could be recovered and on what day they emerged. Hence, amount of inoculum should not be the only criteria to judge the possibility of the emergence of mutation to antibiotic resistance. Nonetheless, it had been shown that SMMIP was capable of monitoring mutation-in-progress with fewer resources and the results could be produced faster compared to conventional methods.

8.5. Characterization of Ciprofloxacin Resistant Mutants

8.5.1. Stability of the Ciprofloxacin Resistant Mutants Produced by SMMIP

In general, conventional studies utilized a single colony for the passaging process [203-205]. However, it was felt that a single colony would not be sufficient to represent the overall possible changes that could happen on a plate containing up to

few hundreds colonies. The work with SMMIP showed that mutation was indeed hard to predict. Mutant was known to be of lower fitness and might undergo further mutation to compensate for the loss of fitness [234]. Therefore, different colonies on the same plate might have evolved differently during the passaging on antibiotic-free agar plates. Passaging with multiple random colonies was expected to provide a better illustration of the changes happening to the mutants.

In this section of the report, another abbreviation was introduced to ease the identification of the generated mutants. The abbreviation shall be: Alphabet1 – Number – Alphabet2. Alphabet1 denoted the sample set where A was used for mutants from sample with starting inoculum of 1.3×10^8 CFU/ml, B for mutants from sample with starting inoculum of 1.3×10^7 CFU/ml, and C for mutants from sample with starting inoculum of 6.0×10^6 CFU/ml. Number denoted the ciprofloxacin concentration of the MHA on which the mutant was recovered. 0 was used to describe bacteria recovered from MHACip-0.25 while 1 described those recovered from MHACip-0.5. Alphabet2 described the day when the sample was taken where A was used for mutants obtained after 24th hour (day 1) of incubation, B for mutants obtained after 48th hour (day 2) of incubation, G for mutants obtained after 168th hour (day 7) of incubation, and so on.

Conventional methods utilized MIC value to determine the possible phenotypic changes on the resistant mutants. Consequently, the MIC value was checked for the mutants after the passages. The MICs was compared to the expected MICs, which was double the antibiotic concentration on the plate where the mutants were recovered. The comparison is shown in Table 7.

Table 7. Comparison of the expected MICs of some mutants with the MICs of the mutants after seven passages on antibiotic-free agar

Mutant	Expected MIC	MIC after 7 passages
A1F	1 µg/ml	2 µg/ml
B1D	1 µg/ml	2 µg/ml
B1E	1 µg/ml	2 µg/ml
C1E	1 µg/ml	2 µg/ml

Since the genetic inquiry was only used to confirm the presence of mutants as indicated by SMMIP, not all mutants would be tested. The mutants tested were mainly those recovered earliest from MHACip-0.5 denoting the first ciprofloxacin resistant mutants. All tested mutants were found to have a two-fold increase in their MICs (Table 7). The doubling of MIC after up to 10 times passages had been noted in some published studies [204, 205]. Such changes of MIC were considered normal though no elucidation was provided to explain the rationale. Perhaps, doubling of the MICs was considered as a probable error which occurred either during the measurement before the passages or after the passages.

All published studies mentioning the passaging to test for the MIC stability of the produced mutant reported macro dilution test for MIC determination. No study was found to report on the composition of the population. In this project, besides MIC based on the macro dilution tests, profiles of the populations were also built. The PAP was built with log inoculum size of 8.2 ± 0.3 in order to provide better detection of possible changes involving small size subpopulations.

Figure 80. Population Analysis Profiles (PAPs) of some mutants produced

Figure 80 shows the PAPs of some mutants produced during the study of the emergence of ciprofloxacin resistance in *Staphylococcus aureus* using SMMIP. There were up to 42.4% of the populations, which were capable of growing on MHACip-2. The subpopulations (5.0×10^3 CFU/ml) was two orders of magnitude smaller than the amount of inoculum (5.0×10^5 CFU/ml) used in a macro dilution test for MIC determination [192]. The subpopulation might have been too small to increase the turbidity of MHBCip-2 used in the MIC test. Therefore, the MIC was read readily as 2 μ g/ml and not a higher concentration.

Earlier PAP analysis (Figure 77 and Figure 78) performed during the study showed that the mutants used to produce Figure 80 were originally without any subpopulations capable of growing on MHACip-2. The mutants seemed to emerge during the passaging on antibiotic-free MHA. The results of the PAP analysis (Figure 80) showed that there was a risk of performing merely MIC without establishing the composition of the population. A small subpopulation with relatively higher degree of resistance could easily be missed due to their insufficient growth. The conventional method of passaging only one colony exacerbated the

problem. The resulting isolates after one passage most likely originated from a single bacterium which formed the colony.

The mutants could still undergo a phenotypic change when it was not exposed to any antibiotics for some time. Consequently, based on the result obtained in the PAP study, a mutant with a relatively intermediate level of resistance could increase its resistance level even at the absence of the selective pressure of antibiotic. However, at this point of time, the significance of this finding was not yet known.

8.5.2. Molecular Characterization of the Ciprofloxacin Resistant Mutants

Molecular characterization was performed on few mutants produced during the monitoring process. The mutants involved in the molecular study were A1F, B1D, B1E, and C1E. The parent strain, MSSA ATCC 29213, was also included as a reference. Conserved sequences of DNA gyrase, DNA topoisomerase IV, and *norA* gene belonging to *Staphylococcus aureus* were extracted from published database to be an additional reference.

Mutation on S80F of *grlA* gene, i.e. Serine at codon 80 was substituted by Phenylalanine, was by far the most common single-step mutation found in quinolone resistant *Staphylococcus aureus*. The mutation raised the ciprofloxacin MIC to 1.56 µg/ml though mutation of E84K at the same gene had been reported to raise the MIC to the same value [235]. Single-step mutation conferring increased ciprofloxacin level had never been found to occur in GyrA and GyrB subunits of DNA gyrase. Only one report was published on single-step mutation in *grlB* region, which increased the ciprofloxacin MIC to 2 µg/ml. The mutation on that particular strain occurred only in the GrlB subunit, other QRDR sites were not altered [133].

The QRDR regions and *norA* gene sections of the mutants produced in this study were sequenced and aligned with the parent MSSA ATCC 29213 and *S. aureus*' sequence from a published database. The genetic study focused on regions commonly used to study quinolone resistance [210]. A sample recovered from

MHACip-0.25 during study with Sample 3 (the sample is called C0D) was also sequenced in order to show that there was truly no genetic mutation prior to colonies recovery on MHACip-0.5. The alignment revealed that QRDR and *norA* genes were indeed highly conserved regions. The results of the alignment were included in Appendix H. The nucleotide and protein alignment showed unaltered *grlA*, *gyrA*, *gyrB*, and *norA* gene. However, a single nucleotide change was found in the coding sequence (CDs) of the *grlB* gene for all mutants. Figure 81 shows that Adenine nucleic acid at sequence number 1415 of the CDs of the published GenBank and MSSA sequence was substituted by Thymine for mutants A1F, B1D, and B1E.

Figure 81. Multiple sequence alignment of the nucleotides of the mutants, the parent strain, and the database-derived sequence which showed mutation in the *grlB* gene of mutants A1F, B1D, and B1E

The substitution at CDs position 1415 of *grlB* gene changed codon 470 of the Gr1B protein sequence from Asparagine (N) to Isoleucine (I). Mutation at codon 470 was previously reported by Fournier and Hooper [133]. In their study, however, the substitution was by Aspartate since the nucleotide change was at sequence 1408 of the CDs where Adenine was replaced by Guanine. The result of this study confirmed the increased ciprofloxacin resistance produced by *grlB* single step mutant at codon 470.

Figure 82. Multiple sequence alignment of the GrlB protein of the mutants, the parent strain, and the database-derived sequence which showed mutation in the GrlB protein of mutants A1F, B1D, and B1E

Mutants C1E, obtained from Sample 3 whose starting inoculum was 6.0×10^6 CFU/ml, was also found with single mutation in *grlB* gene. The mutation, however, was observed to occur at CDs position of 1191 of the gene instead of position 1415 that occurred in other mutants studied. Figure 83 shows that the Guanine was replaced by Cytosine in mutant C1E.

Figure 83. Multiple sequence alignment of the nucleotides of the mutants, the parent strain, and the database-derived sequence which showed mutation in the *grlB* gene section of mutants C1E

C0D sample, which were bacteria recovered a day before C1E mutant was also included in the alignment as additional reference. C0D was recovered on MHACip-0.25. The bacteria formed part of the population with the highest tolerance to ciprofloxacin prior to mutant recovery on MHACip-0.5. The sequenced *grlB* gene section confirmed that the mutation at nucleotide position 1191 was only found in C1E. Thus, the mutation conferred a two-fold increase of ciprofloxacin resistance level on C1E relative to C0D. The substitution of the nucleotide changed the amino acid at codon 396 from Alanine to Proline. As of the time of writing (January

and sub population prior to the recovery of mutants. The result confirmed the ability of SMMIP to track mutation in progress.

8.6. Summary of the Study on the Emergence of Antibiotic Resistance

Ultrastructural observation of *Staphylococcus aureus* revealed extended lysis. Filamentous bacteria were also observed which could have concurred to the failure of DNA synthesis [224]. Bacteria with intact round morphology were also seen in the SEM pictures. These bacteria might be persisters that have its metabolism rate reduced during the antibiotic exposure. The reduced metabolism rate, though slow, should have contributed to the medium acidification.

SMMIP was used to monitor the emergence of ciprofloxacin resistance of *Staphylococcus aureus* during growth in MSB. MSSA ATCC 29213 was grown in MSBCip-1 which had its concentration increased slightly every 24 hours. Such culture failed to produce resistant mutants. The failure to produce mutant likely happened due to the high ciprofloxacin concentration, which is 2 times the MIC of MSSA ATCC 29213. Despite another study reporting appearance of mutant at such concentration [230], the study was performed at constant ciprofloxacin concentration. The progressively higher ciprofloxacin concentration in this study might have exerted additional pressure every time the concentration increases. The additional pressure might have prevented the appearance of resistant mutants.

The wavelength shift development in the monitoring included some apparent alkalization of the medium. The alkalization of the medium is likely due to bacterial lysis which releases cytoplasm which pH is 7-8. The release of such relatively higher pH material should increase the medium pH and hence, produces the alkalization effect. The wavelength shift and IR development of the culture revealed both alkalization effect and medium clearing, which corresponds to effect of bacterial lysis.

During the study as well, despite the observation of wavelength shift indicating active medium acidification, IR change was not observed (Figures 69-70). Such observation revealed that wavelength shift is a better indicator of metabolism activity. Hence, wavelength shift was used as the main indicator of the presence of resistant mutant in the study on the emergence of ciprofloxacin resistance using SMMIP.

The emergence of ciprofloxacin mutant was finally observed when three different starting inocula were incubated in MSBCip-0.5 which ciprofloxacin concentration in the MIC value of MSSA ATCC 29213. The summary of the time required for the mutant production can be seen in Table 6. The tests also showed that the inoculum in the order of 10^8 CFU/ml produced mutant slower than other samples. The high concentration of bacteria, which is around the carrying capacity of the test setup, might have provided additional undesirable pressure.

The emergence of ciprofloxacin mutants were confirmed when increase of viable count was observed by standard plate count method. Furthermore, PAP detected the presence of populations with MIC higher than the original 0.5 μ g/ml. Final confirmation on the mutants produced was obtained by molecular sequencing on some samples. The exercise revealed a transversion at coding sequence 1415 of *grlB* gene from A to T in mutants A1F, B1F, and B1E. The single nucleotide change corresponds to a change of codon 470 of GrlB protein sequence from Asparagine to Isoleucine. Ciprofloxacin resistant mutant having mutation in *grlB* gene has been previously reported despite at different coding sequence [133].

Mutation in *grlB* gene of mutant C1E is slightly different from the others. The mutated coding sequence is at position 1191, which produces a transversion from G to C, corresponding to an amino acid change from Alanine to Proline. The inclusion of C0D non mutated strain further confirmed that the observed mutation confers a two-fold increase of ciprofloxacin resistance in C1E relative to C0D. The molecular confirmation along with PAP and standard plate count confirm that SMMIP is capable of tracking mutation in progress.

Chapter 9

MODELING OF THE EMERGENCE OF ANTIBIOTIC RESISTANCE

9.1. Modeling the Wavelength Shift Data of the Emergence of Antibiotic Resistance as Monitored by the SMMIP

Since there was no published data on monitoring mutation-in-progress using a system similar to SMMIP, two mathematical models were explored to understand the development of the wavelength shift. The models were adapted and modified from models proposed by Van Impe *et. al.* [178, 236]. The models were then fitted to the data obtained. In order to assess the performance of the model, the fittings were compared with some standard sigmoid curve models, i.e. Logistic function, Gompertz function, and Weibull function [237-239].

The mathematical functions were used to understand the wavelength shifts obtained during the studies using SMMIP. In general, the sigmoid curves produced by the wavelength shift were similar to the conventional growth curve, which was of viable bacterial number against time. Therefore, the equations used to describe conventional growth curves could be used as the basis to develop the model used in this study of mutation-in-progress.

The earlier model proposed by Van Impe *et. al.* [178] was a generalization of Baranyi and Robert's model [176]. The inhibition effect of limited nutrient and production of metabolites was proposed by Baranyi and Robert although more explanation, arguably, was provided by Van Impe *et. al.* in their paper. Nevertheless the general formula was a description of how bacterial number was affected by the adjustment function, nutrient limitation, and metabolite production. The adjustment function, (Q) , proposed by Baranyi and Robert [176] was derived from statistical-based Weibull function, which is used widely to fit the lifetime reliability data in many industries [238].

In this work, three models were essentially analyzed. Two models were developed for the wavelength shift data and another model was developed for the IR plot. The first model for the wavelength shift data consisted of one component while the second model consisted of two components. The first model is called Model Wv-1 and the second model is called Model Wv-2. The model for the IR plot is called model IR-1. Different models were needed for IR and wavelength shift data since both responses were indication of different characteristics. Nevertheless, it could be seen later on that the similarity of both types of plot resulted in similar models. The ability of the proposed models and the established models in fitting and explaining the experimental data will also be discussed.

9.1.1. Model Wv-1 to Explain the Wavelength Shift Data Produced by SMMIP

This is the first study on the visible wavelength shift data on the emergence of antibiotic resistance and no mathematical model is ever built yet for such data. Hence the mathematical models presented in this study are derived based on the general equation summarized in Equation (36). Model Wv-1 accounts for the acid-based indicator in the growth medium and the existence of antibiotics. The general form of the proposed Model Wv-1 was

$$\frac{dW(t)}{dt} = g(Q)(L)(C)W(t) \quad (41)$$

Where,

$\frac{dW(t)}{dt}$ = rate of change of the wavelength shift over a unit of time

g = rate of increase of the wavelength shift over time

(Q) = adjustment function to account for lag phase

(L) = limiting function to account for the maximum possible wavelength shift

(C) = inhibition function to account for the presence of antibiotic

$W(t)$ = wavelength shift at time t

9.1.1.1. The Adjustment Function of Model Wv-1

The adjustment function, (Q) , was derived from the work by Baranyi and Robert [176]. The function was used to allow for the existence of a lag period at the earlier section of the sigmoidal curve. The function was defined as follow:

$$\frac{dQ(t)}{dt} = gQ(t) \quad (42)$$

Since $Q(0) = Q_0$,

$$Q(t) = Q_0 e^{gt} \quad (43)$$

$$(Q) = \left(\frac{Q(t)}{1 + Q(t)} \right) \quad (44)$$

$$\Leftrightarrow (Q) = \left(\frac{Q_0}{Q_0 + e^{-(gt)}} \right) \quad (45)$$

Where,

Q_0 = the physiological value of the culture at time 0

The physiological value of the culture, Q_0 , was used to determine whether the population had adjusted to the new environment or not. The function of (Q) was an increasing function from Q_0 , a positive value, up to a saturation point of 1. Hence, a higher Q_0 would start the (Q) function relatively closer to 1 and the (Q) saturated faster to the value of 1. The value of $(Q) = 1$ indicated a full contribution of g to the rate of change of the normalized wavelength shift.

9.1.1.2. The Limiting Function of Model Wv-1

In the study of ciprofloxacin resistance using the SMMIP, the MSB was illuminated with green LED. The resulting spectral peaks had minimum and maximum peak location due to the limited possible colour change of phenol red. The minimum and maximum location of the spectral peak corresponded to the minimum and maximum pH that phenol red could effectively display in terms of the colour change of the solution respectively. The MSB used in this study was not at the alkali end of the effective range of phenol red while the acidification lowered the pH of MSB. Therefore, only the minimum location of the spectral peak was of concern.

The spectral reading of MSB, whose pH was 7.2 ± 0.2 , had a peak at 587.06 ± 0.35 nm after 30 minutes of incubation at 30 °C. The minimum location of the spectral peak was at 565.25 ± 0.65 nm. Therefore, using the data point at 30th minute as a reference for normalization, the spectral peak of the MSB used in the study should be able to have a maximum shift of 21.81 nm.

The maximum limit of the normalized wavelength shift was included in the mathematical model in terms of a limiting function, (L) . The function was taken as a decreasing function from 1 to 0. Consequently, the reaching of normalized wavelength shift to the magnitude of 21.81 nm ($W_{\max}$) would stop the contribution of the term g in increasing the magnitude of the shift.

It was possible for the viable bacterial number to be reduced to a number which was not enough to induce further colour change in the medium. Thus, the wavelength shift might stop before it reached the maximum. An adjustment constant, D , was included to allow the fitting of such data. The limiting function, (L) , was defined as

$$(L) = \left(1 - \frac{D * W(t)}{W_{\max}} \right) \quad (46)$$

Where,

$W(t)$ = wavelength shift at time t

$W_{\max}$ = maximum normalized wavelength shift, 21.81 nm in this study

D = adjustment constant for non maximum saturation

9.1.1.3. The Inhibition Function of Model Wv-1

The inhibition function, (C) , was intended to have its value varied depending on the antibiotic concentration at that particular point of time. Therefore, a higher (C) value was expected for sample with higher antibiotic concentration. Nevertheless, the study was conducted using the ciprofloxacin MIC value and therefore a value of 1 will be used for the (C) function.

9.1.1.4. The General Formula of Model Wv-1

Inputting all the function, the general model was therefore,

$$\frac{dW(t)}{dt} = \left(g \left(\frac{Q_0}{Q_0 + e^{-(gt)}} \right) \left(1 - \frac{D * W(t)}{W_{\max}} \right) \right) W(t) \quad (47)$$

$$g > 0; 0 \leq Q_0 \leq 1$$

The model was fitted to the magnitude of the normalized wavelength shift. $W(t)$ was then of positive value and $W_{\max}$ was 21.81 nm. The value of C remained at 1. Since the fitting was to the magnitude of change, the rate of increase, g , should always be of a positive value.

In order to obtain the explicit function of $W(t)$, Equation (47) was integrated with the help of Mathematica® 5.1.0.0. The analytical solution provided by the software was

$$W(t) = \frac{-(1 + e^{gt} Q_0) W_{\max}}{-D(1 + Q_0 e^{gt}) + e^{AW_{\max}}} \quad (48)$$

Where,

A = integration constant

It is seen in Figure 69 and Figure 70 that the plot of normalized wavelength shift starts at (0,0). However, Equation (48) clearly did not give $W(0) = 0$. Thus, a shifting of the data by an arbitrary positive value of W_0 was performed and $W'(t) = W(t) + W_0$. $W_{\max}$ was also then shifted by W_0 such that $W_{\max}^* = W_{\max} + W_0$. Consequently, at $t = 0$,

$$\begin{aligned} W_0 &= \frac{-(1 + Q_0) W_{\max}^*}{-D(1 + Q_0) + e^{AW_{\max}^*}} \\ \Leftrightarrow e^{AW_{\max}^*} &= \frac{(1 + Q_0)(DW_0 - W_{\max}^*)}{W_0} \end{aligned} \quad (49)$$

Substituting Equation (49) into Equation (48), the general equation became

$$W'(t) = \frac{-(1 + e^{gt} Q_0) W_{\max}^*}{-D(1 + Q_0 e^{gt}) + \frac{(1 + Q_0)(DW_0 - W_{\max}^*)}{W_0}} \quad (50)$$

Equation (50) was then shifted back and the final general equation was

$$W(t) = \frac{-\left(1 + e^{gt} Q_0\right) W_{\max}^*}{-D\left(1 + Q_0 e^{gt}\right) + \frac{\left(1 + Q_0\right)\left(DW_0 - W_{\max}^*\right)}{W_0}} - W_0 \quad (51)$$

9.1.2. Model Wv-2 to Explain the Wavelength Shift Data Produced by SMMIP

A more complex model was developed based on Model Wv-1 to account for the alkalization observed in the wavelength shift data (Figure 71). Another component was added into the formula to account for the reduction of wavelength shift caused by the death of the bacteria. The general form of the proposed Model Wv-2 was

$$\frac{dW(t)}{dt} = \left(g(Q)(L_g^*) - k(J)(C)(L_k^*) \right) W(t) \quad (52)$$

Where,

- $\frac{dW(t)}{dt}$ = rate of change of the wavelength shift over a unit of time
- g = rate of increase of the wavelength shift over time
- (Q) = adjustment function to account for lag phase
- (L_g^*) = limiting function to account for the maximum possible wavelength shift contributed by g
- k = rate of decrease of the wavelength shift over time
- (J) = modification function to adjust the contribution of k
- (C) = inhibition function to account for the presence of antibiotic
- (L_k^*) = limiting function to account for the maximum possible wavelength shift contributed by k
- $W(t)$ = wavelength shift at time t [nm]

9.1.2.1. The Adjustment Function of Model Wv-2

The adjustment function, (Q) , was similar as the function used in Model Wv-1.

The function was as follow:

$$(Q) = \left(\frac{Q_0}{Q_0 + e^{-(gt)}} \right)$$

Where,

Q_0 = the physiological value of the culture at time 0

9.1.2.2. The Limiting Function of Model Wv-2

Two different limiting functions were used in Model Wv-2. (L_g^*) was to limit the contribution of g . (L_k^*) was to limit the contribution of k . The limiting functions for g and k should be different since acidification of the medium decreased the ability of the bacteria to grow while the amount of bacterial death was increased due to sub optimal growth condition. In Model Wv-2, (L_g^*) was similar as the limiting function used in Model Wv-1 while (L_k^*) was similar to (L_g^*) . The functions could be defined as

$$(L_g^*) = \left(1 - \frac{D^* W(t)}{W_{\max}} \right) \quad (53)$$

$$(L_k^*) = \left(\frac{W(t)}{W_{\max}} \right) \quad (54)$$

9.1.2.3. The Modification Function of Model Wv-2

In the early stage of growth, g contributed to the acidification of the medium and bacterial death was expected to be minimum. It was observed in this study that in

growth under antibiotics pressure, the medium was still acidified by the surviving bacteria. Therefore, k would not greatly affect the wavelength shift data at the beginning of the incubation period. Hence, the contribution of k needed adjustment, which was provided by (J) . The adjustment function was formulated from an exponential function to describe the lack of response at the early part of the incubation and exponential growth at the later part. The function was as follows:

$$(J) = \left(a^{t/24}\right) \quad (55)$$

Where,

$$a = \text{adjustment constant}$$

9.1.2.4. The Inhibition Function of Model Wv-2

The inhibition function, (C) , used in Model Wv-2 was also intended to have its value varied depending on the antibiotic concentration at that particular point of time. A value of 1 will be used for the (C) function here since the antibiotic concentration was constant.

9.1.2.5. The General Formula of Model Wv-2

Inputting all the function, the general model was therefore,

$$\frac{dW(t)}{dt} = \left(g \left(\frac{Q_0}{Q_0 + e^{-(gt)}} \right) \right) \left(1 - \frac{D * W(t)}{W_{\max}} \right) - k \left(a^{t/24} \right) \left(\frac{W(t)}{W_{\max}} \right) W(t) \quad (56)$$

$$g > 0; 0 \leq Q_0 \leq 1$$

The model was also fitted to the magnitude of the normalized wavelength shift. $W(t)$ was of positive value and $W_{\max}$ was 21.81 nm. The value of C remained at 1. Since the fitting was to the magnitude of change, the rate of increase, g , should always be of a positive value. However, the value of a could be either positive or

negative depending upon how k affected the acidification of the medium. A positive k meant that there was a decrease of wavelength shift contributed by the alkalization process following the death of some bacteria.

The explicit function of $W(t)$ was obtained by integrating Equation (56) with the help of Mathematica® 5.1.0.0. The analytical solution was

$$W(t) = \frac{W_{\max} (1 + Qe^{gt})(\ln(a))(24g + \ln(a))}{24a^{1/24}k(24g + \ln(a)) + Qe^{gt} \ln(a)(24Dg + 24a^{1/24}k + D \ln(a)) + W_{\max} A \ln(a)(24g + \ln(a))} \quad (57)$$

Where,

$$A = \text{integration constant}$$

Equation (57) was again shifted to allow $W(0) = 0$. The shifting of the data by an arbitrary positive value of W_0 was performed and $W'(t) = W(t) + W_0$. $W_{\max}$ was also shifted by W_0 such that $W_{\max}^* = W_{\max} + W_0$. Consequently, at $t = 0$,

$$\begin{aligned} W_0 &= \frac{W_{\max}^* (1 + Q)(\ln(a))(24g + \ln(a))}{24k(24g + \ln(a)) + Q \ln(a)(24Dg + 24k + D \ln(a)) + W_{\max} A \ln(a)(24g + \ln(a))} \\ \Leftrightarrow W_{\max} A \ln(a)(24g + \ln(a)) &= \\ \frac{W_{\max}^* (1 + Q)(\ln(a))(24g + \ln(a)) - W_0 (24k(24g + \ln(a)) + Q \ln(a)(24Dg + 24k + D \ln(a)))}{W_0} \end{aligned} \quad (58)$$

Substituting Equation (58) into Equation (57), the general equation then became

$$W'(t) = \frac{W_{\max}^* (1 + Qe^{gt})(\ln(a))(24g + \ln(a))}{24a^{1/24}k(24g + \ln(a)) + Qe^{gt} \ln(a)(24Dg + 24a^{1/24}k + D \ln(a)) + B} \quad (59)$$

$$\text{Where } B = \frac{W_{\max}^* (1 + Q)(\ln(a))(24g + \ln(a)) - W_0 (24k(24g + \ln(a)) + Q \ln(a)(24Dg + 24k + D \ln(a)))}{W_0}$$

Equation (59) was shifted back and the final general equation was

$$W(t) = \frac{W_{\max}^* (1 + Qe^{gt}) (\ln(a)) (24g + \ln(a))}{24a^{1/24}k (24g + \ln(a)) + Qe^{gt} \ln(a) (24Dg + 24a^{1/24}k + D \ln(a)) + B} - W_0 \quad (60)$$

9.1.3. The Fitting of the Normalized Wavelength Shift with the Models

The normalized wavelength shift shown in Figure 71 was fitted with the proposed and established models. To ease the fitting process, the fitting was made to the magnitude of the shift. Data from each 24-hours interval were fitted separately. All fittings were performed by using the curve fitting toolbox of Matlab 7.1 Release 14. Sum of squares due to error (SSE) or summed square of residuals would be used to indicate the goodness of fit [240]. SSE was defined as

$$SSE = \sum_i^n \left(y_i - \hat{y}_i \right)^2 \quad (61)$$

Where,

$$\begin{aligned} SSE &= \text{Sum of Squared Errors} \\ y_i &= \text{response during experiment} \\ \hat{y}_i &= \text{response predicted by the model} \end{aligned}$$

Akaike's Information Criterion Method (AIC) was then used to compare the different models. AIC gave an indication of which model was more likely to be correct and a quantification of how much more likely was given [241]. AIC was defined as

$$AIC = N \ln \left(\frac{SSE}{N} \right) + 2K \quad (62)$$

Where,

$$\begin{aligned} AIC &= \text{Akaike's Information Criterion} \\ N &= \text{number of data points} \\ SSE &= \text{sum of squared errors} \end{aligned}$$

K = number of parameters fit by regression plus one

In this study, the experimental data possessed 25 data points per day interval and the models could consist up to four parameters. AIC produced too small a value when N was less than a few dozen times more data points than the curve parameters. Hence, a corrected AIC was used in this case, which was defined as follows [241]

$$\begin{aligned} AIC_C &= AIC + \frac{2K(K+1)}{N-K-1} \\ \Leftrightarrow AIC_C &= N \ln \left(\frac{SSE}{N} \right) + 2K + \frac{2K(K+1)}{N-K-1} \end{aligned} \quad (63)$$

Defining A to be a simpler model and B to be a more complicated model, the difference in AIC_C was calculated between the two models based on the equation below

$$\Delta AIC_C = AIC_{C,B} - AIC_{C,A} \quad (64)$$

To determine how likely a model was more correct than the other model, evidence ratio (ER) was calculated. The ratio measured the relative likelihood of the two models being correct considering the given experimental design. Thus, the ratio told that the model with the lower AIC_C score was (ER) times more likely to be correct the other model. The ER was calculated based on the absolute difference in AIC_C and defined as follows

$$ER = \frac{1}{e^{-0.5|\Delta AIC_C|}} \quad (65)$$

For ease of presentation, sample with starting inocula of 1.3×10^7 CFU/ml and 1.3×10^8 CFU/ml would be addressed as Sample 2 and Sample 3 respectively.

9.1.3.1. The Fitting of the Normalized Wavelength Shift with Model Wv-1

Substituting $W_0 = 21.81$ and therefore, $W_{\max}^* = 21.81 + W_0 = 43.62$, Equation (51) became

$$W(t) = \frac{-43.62(1 + e^{gt}Q_0)}{-D(1 + Q_0e^{gt}) + \frac{(1 + Q_0)(21.81D - 43.62)}{21.81}} - 21.81 \quad (66)$$

The fitting results of Model Wv-1 on the magnitude of wavelength shift shown in Figure 71 are presented in Figures 85-87 for three samples tested.

Figure 85. Curve fitting of Model Wv-1 on magnitude of the wavelength shift of Sample 1

Figure 86. Curve fitting of Model Wv-1 on magnitude of the wavelength shift of Sample 2

Figure 87. Curve fitting of Model Wv-1 on magnitude of the wavelength shift of Sample 3

It could be seen from Figures 85-87 that Model Wv-1 could generally describe the sigmoidal trends of the experimental data. The model provided a closer fit for

wavelength shift obtained from Sample 1 and 2 than Sample 3. The model could also be fitted well to some experimental data which was not of sigmoidal trend (day 2 to 5 of Sample 1, day 4 of Sample 2, days 6 and 7 of Sample 3). The model parameters and SSE obtained when Equation (66) was used to fit the data are presented in Tables 8-10.

Table 8. Parameters and AIC_C obtained when Model Wv-1 was fitted the magnitude of wavelength shift of Sample 1

Day	Q_0 (95% CI)	g (95% CI)	D (95% CI)	SSE	AIC_C
1	0.029 (0.018, 0.040)	0.497 (0.450, 0.544)	1.480 (1.474, 1.486)	0.676	-80.272
2	0.001 (-0.002, 0.004)	0.494 (0.259, 0.729)	1.915 (1.905, 1.926)	0.282	-102.073
3	0.012 (-0.017, 0.041)	0.377 (0.160, 0.594)	1.937 (1.927, 1.947)	0.221	-108.234
4	1 (fixed at bound)	0.040 (-0.076, 0.157)	1.801 (1.273, 2.328)	0.622	-85.199
5	1 (fixed at bound)	0.004 (0.003, 0.005)	1 (fixed at bound)	0.539	-91.359
6	0.014 (0.011, 0.0180)	0.127 (0.117, 0.137)	0 (fixed at bound)	0.643	-84.369
7	0.504 (0.292, 0.716)	0.160 (0.132, 0.188)	1.151 (1.120, 1.182)	1.664	-57.741

Table 9. Parameters and AIC_C obtained when Model Wv-1 was fitted the magnitude of wavelength shift of Sample 2

Day	Q_0 (95% CI)	g (95% CI)	D (95% CI)	SSE	AIC_C
1	0.045 (0.030, 0.060)	0.363 (0.332, 0.394)	1.081 (1.071, 1.091)	3.201	-41.385
2	0.708 (-0.789, 2.205)	0.500 (0.198, 0.797)	1.741 (1.731, 1.751)	1.399	-62.078
3	1.000 (fixed at bound)	0.330 (0.260, 0.400)	1.883 (1.877, 1.889)	0.379	-97.584
4	1.000 (fixed at bound)	0.014 (-0.101, 0.129)	0.892 (-8.169, 9.953)	0.404	-93.130
5	0.031 (0.025, 0.037)	0.256 (0.241, 0.271)	0.997 (0.979, 1.016)	1.004	-70.372

Table 10. Parameters and AIC_C obtained when Model Wv-1 was fitted the magnitude of wavelength shift of Sample 3

Day	Q_0 (95% CI)	g (95% CI)	D (95% CI)	SSE	AIC _C
1	0.043 (0.034, 0.052)	0.625 (0.594, 0.656)	0.991 (0.988, 0.994)	1.363	-62.730
2	0.265 (0.183, 0.348)	0.794 (0.726, 0.863)	0.996 (0.993, 1.000)	1.978	-53.420
3	0.900 (-0.102, 1.906)	0.626 (0.468, 0.783)	0.988 (0.978, 0.997)	13.450	-5.497
4	0.260 (0.150, 0.370)	0.539 (0.474, 0.603)	0.978 (0.971, 0.984)	5.566	-27.555
5	0.119 (0.043, 0.194)	0.557 (0.458, 0.655)	1.071 (1.060, 1.082)	10.350	-12.047
6	1.000 (fixed at bound)	0.190 (0.162, 0.218)	1.735 (1.720, 1.750)	0.975	-73.970
7	0.048 (0.006, 0.090)	0.063 (0.033, 0.093)	0 (fixed at bound)	2.676	-83.118
8	0.012 (0.008, 0.017)	0.493 (0.455, 0.532)	1.022 (1.014, 1.030)	3.761	-37.355

Starting culture of Sample 2 was a dilution of starting culture of Sample 3. Therefore, it was expected that both samples should possess similar physiological condition at the start of the culture. It could be seen that the starting physiological value (Q_0) of the two samples were indeed nearly identical. The findings confirmed that both samples were of the same past growth history. The values of Q_0 of Sample 2 and 3 increased in day 2 compared to day 1. The increase indicated that the cultures had gotten accustomed to the new environment. The lag portion of the wavelength shift was thus shorter, which was correlated with a larger Q_0 (Equation (41)). The increase was not observed in Sample 1 because the amount of bacteria at the second day was not enough to produce any detectable colour change. The starting physiological value of the culture continued to increase over subsequent incubation days as the populations were getting more and more used to the environment.

It was observed in Tables 8-10 that generation rate, g , generally declined before it increased. The increase in the generation rate happened at day 6, 5, and 8 of Sample 1, 2, and 3 respectively. There was also an increase of generation rate from day 2 compared to day 1 for Samples 2 and 3 because of a better adaptation to the growth environment. The increase of the generation rate happened when the mutants existed in a number sufficient to influence the acidification of the medium. However, the increase in the generation rate for Sample 2 and 3 occurred a day after the indication of mutant was observed by PAP (Figures 77-79). Figure 71 shows that mutants were found in day 6 of Sample 1, day 4 of Sample 2, and day 7 of Sample 3. Only generation rate of Sample 1 occurred at the same day as the indication of mutant was observed by PAP (Figure 79).

The determination of the emergence of mutants could not then be performed purely by comparing the generation rate. It was observed that parameter D increased to a value above 1 before dropping to < 1 at the day where mutants were found in Samples 2 and 3. Earlier, it was said that D was used to allow fitting of data saturating before 21.81 nm was reached. Consequently, $D > 1$ meant that the bacteria in the sample were unable to produce enough acid to allow a maximum colour change. Considering the presence of antibiotics, the number of viable bacteria would decrease as incubation time increased. Hence, once data of a day was fitted with $D > 1$, the data at the next day would be fitted with D which should be greater than the previous day's D if no resistant mutants were present. However, if $D < 1$ was fitted, it meant that the data was no longer saturating below 21.81 nm if the sigmoidal curve was completed. A further enquiry on the data was thus needed.

Taking an example of Sample 3, parameter D was 0 for day 7 data of Sample 3. The drop of D to 0 changed Equation (66) into the following equations

$$W(t) = \frac{21.81(1 + e^{gt} Q_0)}{(1 + Q_0)} - 21.81 \quad (67)$$

Equation (67) was an exponential function. It explained the shape of the fitted line for day 7 data of Sample 3. Day 6 data was fitted with $D = 1.785$ indicating early saturation of the wavelength shift curve. It meant that the acid produced by the bacteria was not enough to affect any colour changes. A drop of D to 0 at the next day meant that the normalized wavelength shift, instead of saturating, increased exponentially. With a suitable generation rate, the wavelength shift might exceed the shift fitted by Equation (66) which actually meant that more acid were produced. Such condition could happen when

$$\frac{21.81(1 + e^{gt}Q_0)}{(1 + Q_0)}_{Day(j+1)} > \frac{-43.62(1 + e^{gt}Q_0)}{-D(1 + Q_0e^{gt}) + \frac{(1 + Q_0)(21.81D - 43.62)}{21.81}}_{Day(j)} \quad (68)$$

In Equation (68), the appropriate parameters were used according to data of the day. If Equation (68) was true at more than 3 time point, it meant that at the three time points of day $(j+1)$, the bacteria in the sample produce more acid compared to day (j) . Such occurrence indicated higher active metabolism indicative of higher number of surviving bacteria. Hence, it could be said that resistant mutants had emerged in the sample and were thriving. Using such condition and applying the appropriate equation obtained according to the fitted parameters, similar condition could be confirmed for Samples 1 and 2. Model Wv-1 could then be used to determine and confirm the presence of resistant mutants. Therefore, besides checking for generation rate, a check of parameter D and the resulting condition at several time points would be needed.

The observation on the trend of D only applied if the normalized wavelength shift is above 1 nm, which is a threshold of positive growth indication. If D was to drop to 1 or below but not 0, Q_0 will be forced to 1 in order to produce positive normalized wavelength shift. In such case, to get $W(t) > 1$ nm, $g \geq 0.00488$ ($0 < D \leq 0.5$) or $g \geq 0.00733$ ($0.5 < D \leq 1$). Provided the wavelength shift is negative, D can be used to confirm emergence of mutants only when D drops after a period of increase.

9.1.3.2. The Fitting of the Normalized Wavelength Shift with Model Wv-2

A second component was added to Model Wv-1 to generate Model Wv-2. It was hoped that the new model would be able to account for the decrease of wavelength shift experienced when bacterial lysis alkalized the medium. Equation (60) shows the general form of Model Wv-2. The known constant of $W_0 = 21.81$ and $W_{\max}^* = 21.81 + W_0 = 43.62$ were put into Equation (60) to give Equation (69)

$$W(t) = \frac{43.62(1 + Qe^{gt})(\ln(a))(24g + \ln(a))}{24ka^{1/24}(24g + \ln(a)) + Qe^{gt}\ln(a)(24Dg + 24a^{1/24}k + D\ln(a)) + B} - 21.81 \quad (69)$$

Where $B = 2(1 + Q)(\ln(a))(24g + \ln(a)) - 24k(24g + \ln(a)) - Q\ln(a)(24Dg + 24k + D\ln(a))$

The equation was then used to fit the magnitude of wavelength shift shown in Figure 71. If the regression analysis of a set of data produced $k < 0.0001$, $k \approx 0$ was used. When k was 0, the second component of Equation (56) did not contribute to the fitting and Equation (56) became Equation (47). Hence, Equation (66) from Model Wv-1 would be used to fit that particular set of data. Observation of Equation (69) also revealed that when $a = 1$, for any value of t , $W(t) = 0$, therefore, the lower limit of a was set to 2. The results of the fitting are shown in Figures 88-90 while the parameters obtained in the regression analysis are provided in Tables 11-13.

Figure 88. Curve fitting of Model Wv-2 on the magnitude of wavelength shift of Sample 1

Figure 89. Curve fitting of Model Wv-2 on the magnitude of wavelength shift of Sample 2

Figure 90. Curve fitting of Model Wv-2 on the magnitude of wavelength shift of Sample 3

Table 11. Parameters and AIC_C obtained when Model Wv-2 was fitted the magnitude of wavelength shift of Sample 1

Day	D (95% CI)	Q_0 (95% CI)	a (95% CI)	g (95% CI)	k (95% CI)	SSE	AIC_C
1	1.417 (1.315, 1.519)	0.065 (-4.0×10^{-4} , 0.131)	2 (fixed at bound)	0.436 (0.353, 0.520)	0.016 (-0.007, 0.038)	0.593	-80.378
2	1.897 (1.845, 1.948)	0.015 (-0.047, 0.078)	2 (fixed at bound)	0.325 (0.080, 0.570)	1.9×10^{-3} (-0.003, 0.007)	0.251	-101.83
3	1.512 (-8.011, 11.03)	0.593 (-7.818, 9.004)	2.026 (-9.127, 13.18)	0.124 (-0.789, 1.036)	0.025 (-0.454, 0.504)	0.210	-102.81
4	0.058 (-2.4×10^4 , 2.4×10^4)	1 (-2404, 2406)	2 (fixed at bound)	0.006 (-66.59, 66.60)	0.001 (-0.317, 0.320)	0.616	-79.423
5	0 (fixed at bound)	0.9998 (-136.9, 138.9)	2 (fixed at bound)	0.006 (-0.524, 0.537)	0.003 (-0.081, 0.088)	0.500	-88.392
6	0 (fixed at bound)	0.014 (0.011, 0.018)	-	0.127 (0.117, 0.137)	0	0.643	-84.369
7	1.151 (1.120, 1.182)	0.504 (0.292, 0.716)	-	0.160 (0.132, 0.188)	0	1.664	-57.741

Table 12. Parameters and AIC_C obtained when Model Wv-2 was fitted the magnitude of wavelength shift of Sample 2

Day	D (95% CI)	Q_0 (95% CI)	a (95% CI)	g (95% CI)	k (95% CI)	SSE	AIC_C
1	0.948 (0.638, 1.257)	0.090 (-0.024, 0.203)	2 (fixed at bound)	0.325 (0.247, 0.404)	0.024 (-0.026, 0.073)	2.671	-42.753
2	1.660 (1.375, 1.945)	1 (fixed at bound)	2.583 (-6.553, 11.720)	0.408 (0.224, 0.592)	0.018 (-0.094, 0.130)	0.872	-70.732
3	1.883 (1.877, 1.890)	1 (fixed at bound)	-	0.330 (0.260, 0.400)	0	0.379	-97.584
4	0.892 (-8.169, 9.953)	1 (fixed at bound)	-	0.014 (-0.101, 0.129)	0	0.404	-93.130
5	0.715 (0.396, 1.034)	0.095 (0.023, 0.168)	2 (fixed at bound)	0.207 (0.172, 0.242)	0.024 (-8.6×10^5 , 0.049)	0.584	-80.760

Table 13. Parameters and AIC_C obtained when Model Wv-2 was fitted to the magnitude of wavelength shift of Sample 3

Day	D (95% CI)	Q_0 (95% CI)	a (95% CI)	g (95% CI)	k (95% CI)	SSE	AIC_C
1	0.991 (0.988, 0.994)	0.043 (0.034, 0.052)	-	0.625 (0.594, 0.656)	0	1.363	-62.730
2	0.996 (0.993, 1.000)	0.265 (0.183, 0.348)	-	0.794 (0.726, 0.863)	0	1.978	-53.420
3	0.988 (0.978, 0.997)	0.900 (-0.102, 1.906)	-	0.626 (0.468, 0.783)	0	13.450	-5.497
4	0.978 (0.971, 0.984)	0.260 (0.150, 0.370)	-	0.539 (0.474, 0.603)	0	5.566	-27.555
5	1.009 (0.945, 1.073)	0.204 (0.142, 0.265)	16.09 (-27.35, 59.52)	0.450 (0.409, 0.492)	3.8×10^{-3} (-0.007, 0.015)	0.886	-66.831
6	1.735 (1.72, 1.75)	1 (fixed at bound)	-	0.190 (0.162, 0.218)	0	0.975	-73.970
7	0 (fixed at bound)	0.0481 (0.006, 0.090)	-	0.063 (0.033, 0.093)	0	2.676	-45.864
8	0.998 (0.802, 1.194)	0.016 (-0.004, 0.035)	43.71 (-1174, 1261)	0.465 (0.360, 0.571)	5.2×10^{-4} (-0.016, 0.017)	3.022	-36.157

Model Wv-2, in general, was capable to fit the different shape of the data (Figures 88-90). Table 11-13 show that more than half of the data were fitted with Model Wv-2 while some data were fitted with Model Wv-1 since their fitting with Model Wv-2 gave $k \approx 0$. Model Wv-2 fitted particularly well with the wavelength shift data of Sample 3 in day 5 where a decrease in the shift was observed due to bacterial lysis. The resulting curve resulted in more than 10 times reduction of SSE compared to the fitting result of Model Wv-1.

When Model Wv-2 could fit the data with $k > 0.0001$, the fittings were generally better than Model Wv-1. However, the fitting of day 1 data of Sample 1 and 2 produced different Q_0 from the fitting by Model Wv-1. The additional second component in Model Wv-2 increased the value of Q_0 . Model Wv-2 was also yet capable of fitting the wavelength shift data of day 7 of Sample 3. The additional parameters, k and a , were seen to be of a poorer estimation properties compared the other parameters used in both Models Wv-1 and Wv-2. The curve fitting produced parameters with slightly large confidence intervals. However, the main objective of using two components model in Model Wv-2 was to explain the

reduction of the amount of wavelength shift caused by the alkalization of the medium. The use of the second component helped to improve the fitting.

Model Wv-2 also produced lower generation rate, g , of day 5 data of Sample 3 compared to Model Wv-1. The resulting generation rate was less than the generation rate of the next day, which helped to create a more consistent reduction of generate rate in Model Wv-2. Based on the generation rate, the indication of the emergence of mutants for both samples were at the day 5 of Sample 2 and day 8 of Sample 3, which was the same as the days determined by Model Wv-1.

Since the data at day 3 of Sample 2 and day 6 of Sample 3 was again fitted with Model Wv-1, similar arguments about the use of parameter D could be used. Using the combination of g and D , the emergence of mutants could therefore be determined by the help of Model Wv-2 and Model Wv-1. Model Wv-2 was seen to improve the data fitting of several weaker fitting produced by Model Wv-1.

9.1.3.3. The Fitting of Weibull Function to the Normalized Wavelength Shift Data

Weibull function was one of the comparative sigmoidal functions fitted to the magnitude of wavelength shift data. The Weibull function used in the study was derived from Weibull probability distribution function [238]. Considering the maximum value of the normalized wavelength shift achievable by phenol red indicator, the following Weibull function was obtained:

$$W(t) = 21.81 - 21.81e^{-(kt)^d} \quad (70)$$

Where,

- $W(t)$ = magnitude of the normalized wavelength shift
- k = scale parameter, a constant
- d = shape parameter, a constant
- t = time

Weibull function consisted of two parameters, k and d . Parameter d described the length of the lag portion at the beginning of a sigmoidal curve while parameter k described the response rate of the sigmoidal curve. The resulting model parameters obtained from the curve fitting were listed in Tables 14 to 16.

Table 14. Parameters and AIC_C obtained when Weibull function was fitted the wavelength shift of Sample 1

Day	k (95% CI)	d (95% CI)	SSE	AIC _C
1	0.021 (0.015, 0.027)	0.890 (0.679, 1.102)	23.260	5.339
2	0.010 (0.006, 0.014)	2.018 (1.514, 2.522)	0.413	-95.443
3	0.004 (6.0×10^{-4} , 0.007)	1.328 (0.921, 1.735)	0.326	-101.343
4	5.2×10^{-4} (-3.8×10^{-4} , 0.001)	0.712 (0.456, 0.968)	0.545	-88.490
5	4.8×10^{-6} (-2.7×10^{-5} , 3.7×10^{-5})	0.423 (0.129, 0.717)	0.380	-97.492
6	0.028 (0.026, 0.029)	2.755 (2.465, 3.046)	1.655	-60.734
7	0.047 (0.046, 0.048)	1.263 (1.208, 1.317)	2.155	-54.134

Table 15. Parameters and AIC_C obtained when Weibull function was fitted the wavelength shift of Sample 2

Day	k (95% CI)	d (95% CI)	SSE	AIC _C
1	0.070 (0.067, 0.072)	1.932 (1.741, 2.124)	18.110	-0.917
2	3.99×10^{-5} (-8.0×10^{-4} , 2.0×10^{-4})	0.250 (0.144, 0.357)	4.561	-35.390
3	2.00×10^{-5} (-1.0×10^{-5} , 4.0×10^{-5})	0.345 (0.283, 0.407)	2.225	-53.335
4	0.004 (0.003, 0.006)	1.020 (0.884, 1.156)	0.416	-95.256
5	0.054 (0.054, 0.054)	2.920 (2.853, 2.987)	0.809	-78.628

Table 16. Parameters and AIC_C obtained when Weibull function was fitted to the wavelength shift of Sample 3

Day	k (95% CI)	d (95% CI)	SSE	AIC _C
1	0.142 (0.139, 0.145)	2.790 (2.588, 2.992)	4.210	-37.390
2	0.282 (0.274, 0.290)	1.827 (1.694, 1.960)	2.983	-46.006
3	0.310 (0.286, 0.334)	1.585 (1.303, 1.867)	18.020	-1.042
4	0.198 (0.187, 0.208)	2.012 (1.691, 2.332)	18.800	0.017
5	0.128 (0.112, 0.143)	1.195 (0.922, 1.478)	84.120	37.477
6	0.002 (0.001, 0.002)	0.551 (0.505, 0.600)	0.362	-98.730
7	0.012 (0.008, 0.017)	1.496 (1.110, 1.882)	3.606	-41.264
8	0.084 (0.082, 0.086)	3.160 (2.819, 3.500)	14.180	-7.033

More ciprofloxacin resistant mutants were present in day 6, day 4, and day 7 of Sample 1, Sample 2, and Sample 3 respectively (Figure 71). The scale parameter, k , was seen to correlate with the presence of mutants since the parameter increased at those days after a consecutive decrease over the previous days. A more erratic trend was observed for the shape parameter, d , which increased as early as day 4 after a decreasing trend over the previous days. Weibull function, in general, did not manage to describe the sigmoidal trend of the wavelength shift data faithfully.

The descriptions of the generated Weibull function and the experimental data were provided in Figures 91-93. A large deviation of the fitted curve from the experimental data could be observed in Figure 91 and Figure 93 (day 1 of Sample 1 and day 5 of Sample 3). The function was not capable of fitting of the early and final phase of the data. The wavelength shift at the start of the day was sometimes overestimated.

Figure 91. Curve fitting of Weibull function on the magnitude of wavelength shift of Sample 1

Figure 92. Curve fitting of Weibull function on the magnitude of wavelength shift of Sample 2

Figure 93. Curve fitting of Weibull function on the magnitude of wavelength shift of Sample 3

9.1.3.4. The Fitting of Logistic Function to the Normalized Wavelength Shift Data

A logistic function was also fitted to the magnitude of the wavelength shift data. A modified form of logistic function had been previously used to describe the development of viable bacterial count [185]. The saturation of the logistic function was set to 21.81 nm. The function was described as:

$$W(t) = 21.81 - \frac{21.81}{1 + \left(\frac{t}{m}\right)^p} \quad (71)$$

$W(t)$ = magnitude of the normalized wavelength shift

m = the center of the graph in terms of x-axis, a constant

p = the power, a constant

t = time

The results of the fittings were described in Figures 94-96. The Figures show that the logistic function could describe the sigmoidal trend of the wavelength shift quite well except day 1 data of Sample 1 (Figure 94). Nevertheless, the function underestimated the lag portion of days 1 and 8 data of Sample 3 (Figure 96). Underestimation of the shift was also observed in the curve fitting of the stationary stage of days 3 and 4 data of Sample 3. On the other hand, an overestimation at the final portion of the experimental data (15th-24th hour) was seen in the data from day 5.

Figure 94. Curve fitting of Logistic function on the magnitude of wavelength shift of Sample 1

Figure 95. Curve fitting of Logistic function on the magnitude of wavelength shift of Sample 2

Figure 96. Curve fitting of Logistic function on the magnitude of wavelength shift of Sample 3

The model parameters resulted from the curve fitting with the logistic function were provided in Tables 17 to 19. No observable trend could be derived from the

resulting parameters. Therefore, it was difficult to use the parameters to determine the presence of mutants in the sample.

Table 17. Parameters and AIC_C obtained when Logistic function was fitted to the magnitude of wavelength shift of Sample 1

Day	m (95% CI)	p (95% CI)	SSE	AIC_C
1	33.570 (26.870, 40.260)	1.044 (0.814, 1.275)	20.480	2.157
2	96.010 (58.160, 133.90)	2.050 (1.540, 2.559)	0.409	-95.662
3	265.40 (48.370, 482.40)	1.343 (0.933, 1.754)	0.324	-101.466
4	1790.0 (-1265.0, 4844.0)	0.720 (0.460, 0.980)	0.548	-88.371
5	1.0×10^5 $(-4.9 \times 10^5, 6.9 \times 10^5)$	0.457 (0.152, 0.762)	0.381	-97.440
6	33.540 (31.490, 35.580)	2.972 (2.632, 3.313)	1.918	-57.047
7	15.610 (15.410, 15.810)	1.633 (1.581, 1.684)	1.102	-70.901

Table 18. Parameters and AIC_C obtained when Logistic function was fitted to the magnitude of wavelength shift of Sample 2

Day	m (95% CI)	p (95% CI)	SSE	AIC_C
1	11.410 (11.230, 11.600)	2.900 (2.678, 3.029)	3.331	-43.247
2	1.100×10^{-4} $(-0.0002, 0.0004)$	0.270 (0.157, 0.382)	4.505	-35.699
3	1.590×10^{-4} $(-3651, 0.0003)$	0.396 (0.328, 0.465)	0.242	-108.799
4	198.800 (132.500, 265.100)	1.050 (0.908, 1.192)	0.428	-93.967
5	15.970 (15.720, 16.220)	4.058 (3.781, 4.335)	6.330	-27.196

Table 19. Parameters and AIC_C obtained when Logistic function was fitted to the magnitude of wavelength shift of Sample 3

Day	<i>m</i> (95% CI)	<i>p</i> (95% CI)	SSE	AIC _C
1	6.030 (5.826, 6.324)	4.463 (3.893, 5.033)	12.760	-9.671
2	2.818 (2.684, 2.952)	3.032 (2.678, 3.386)	7.762	-22.098
3	2.483 (2.266, 2.700)	2.667 (2.182, 3.172)	20.000	1.564
4	4.103 (3.819, 4.386)	3.448 (2.770, 4.125)	27.470	9.498
5	5.639 (5.028, 6.250)	1.990 (1.630, 2.350)	49.840	24.391
6	412.1 (297.5, 526.7)	0.582 (0.534, 0.630)	0.357	-99.080
7	73.41 (46.25, 100.6)	1.553 (1.143, 1.964)	3.734	-40.392
8	10.34 (10.080, 10.600)	4.715 (4.233, 5.197)	12.770	-9.652

9.1.3.5. The Fitting of Gompertz Function to the Normalized Wavelength Shift Data

One of the earliest sigmoidal functions used in microbiology was Gompertz function. It had been used to model bacterial kinetics [242, 243]. Therefore, it was included for comparison in this study. Since the original Gompertz function could not be fitted to graphs which started at (0,0), the Gompertz function used in this study was modified into Equation (72).

$$W(t) = 21.81 * e^{-e^{(-k(t-m))}} - 21.81 * e^{-e^{(km)}} \quad (72)$$

Where,

$W(t)$ = magnitude of the normalized wavelength shift

k = constant

m = constant

t = time

The curve fitting results is presented in Figures 97-99. In general, the curve fitting shows that Gompertz function also gave a good description of the trend of the wavelength shift. However, the saturation of the response at day 1 of Sample 1 and day 2 of Sample 2 (Figure 71) could not be fitted by the function. Overestimation and underestimation of the experimental data is observed in those Figures.

Figure 97. Curve fitting of Gompertz function on the magnitude of wavelength shift of Sample 1

Figure 98. Curve fitting of Gompertz function on the magnitude of wavelength shift of Sample 2

Figure 99. Curve fitting of Gompertz function on the magnitude of wavelength shift of Sample 3

The model parameters produced during the fitting of Gompertz function to the magnitude of the wavelength shift is presented in Tables 20-22. No observable

trend was found in the development of the model parameters over time. Hence, the resulting parameters could not be used to determine the presence of mutants in the sample. Similar to Logistic function and Weibull function, Gompertz function tend to underestimate the early part of the sigmoidal curves since they are not derived to fit wavelength shift data. However, those models were used here since they represent some of the commonly used model in fitting bacterial viable count data [176, 242, 244]

Table 20. Parameters and AIC_C obtained when Gompertz function was fitted to the magnitude of wavelength shift of Sample 1

Day	k (95% CI)	m (95% CI)	SSE	AIC _C
1	0.061 (0.05549, 0.06629)	0 (fixed at bound)	22.070	4.026
2	0.008 (-0.014, 0.030)	123.00 (48.840, 197.20)	0.917	-75.484
3	0.006 (-0.010, 0.023)	144.30 (98.670, 190.00)	0.371	-98.090
4	5.4×10^{-3} (4.8×10^{-3} , 6.0×10^{-3})	0 (fixed at bound)	0.619	-85.320
5	3.0×10^{-3} (2.4×10^{-3} , 3.5×10^{-3})	0 (fixed at bound)	0.535	-88.957
6	0.070 (0.063, 0.076)	27.850 (27.000, 28.710)	1.604	-61.516
7	0.094 (0.092, 0.096)	7.755 (6.887, 8.623)	2.000	-56.000

Table 21. Parameters and AIC_C obtained when Gompertz function was fitted to the magnitude of wavelength shift of Sample 2

Day	k (95% CI)	m (95% CI)	SSE	AIC _C
1	0.178 (0.165, 0.180)	9.555 (9.185, 9.924)	10.410	-14.750
2	NA	NA	NA	NA
3	0.010 (0.009, 0.0112)	0 (fixed at bound)	3.035	-45.374
4	0.012 (0.007, 0.016)	35.55 (-42.750, 113.900)	0.404	-95.988
5	0.175 (0.164, 0.185)	13.9 (13.660, 14.140)	5.651	-30.033

Table 22. Parameters and AIC_C obtained when Gompertz function was fitted to the magnitude of wavelength shift of Sample 3

Day	k (95% CI)	m (95% CI)	SSE	AIC _C
1	0.474 (0.423, 0.524)	5.233 (5.042, 5.424)	9.871	-11.089
2	0.710 (0.659, 0.762)	2.355 (2.241, 2.470)	4.472	-35.883
3	0.772 (0.649, 0.894)	2.112 (1.867, 2.355)	22.250	4.230
4	0.536 (0.452, 0.620)	3.470 (3.194, 3.749)	21.210	3.033
5	0.275 (0.250, 0.300)	3.112 (2.464, 3.759)	35.340	15.796
6	0.022 (0.020, 0.024)	0.000 (NA)	5.397	-31.183
7	0.033 (0.022, 0.044)	40.44 (35.610, 45.270)	3.041	-45.524
8	0.305 (0.272, 0.338)	9.119 (8.820, 9.418)	15.660	-4.551

9.1.4. Comparison of Model Wv-2 and Other Models for the Fitting of the Wavelength Shift Data

The normalized wavelength shift was fitted with Logistics, Gompertz, and Weibull functions. All models have been earlier shown to describe the trend of the wavelength shift well. A more complex model would produce better fitting and hence, lower SSE. However, the model might not produce large improvement over a simpler model unless it was assessed according to the difference in the number of parameters. Therefore, in order to judge the performance of the models, corrected Akaike's Information Criterion (AIC_C), was used. The criterion gave information on how likely a model was correct. Described in Equation (63), the method balanced the assessment of a model from the SSE and the number of parameters in the model.

Table 23. Comparison of the AIC_C obtained from the fitting of the wavelength shift data of Sample 1 with Model Wv-1, Model Wv-2, Weibull function, Logistic function, and Gompertz function

Model	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Model Wv-1	-80.272	-102.073	-108.234	-85.199	-91.359	-84.369	-57.741
Model Wv-2	-80.378	-101.83	-102.81	-79.423	-88.392	-84.365	-57.741
Weibull	5.339	-95.443	-101.343	-88.490	-97.492	-60.734	-54.134
Logistic	2.157	-95.662	-101.466	-88.371	-97.440	-57.047	-70.901
Gompertz	4.026	-75.484	-98.090	-85.320	-88.957	-61.516	-56.000

Table 24. Comparison of the AIC_C obtained from the fitting of the wavelength shift data of Sample 2 with Model Wv-1, Model Wv-2, Weibull function, Logistic function, and Gompertz function

Model	Day 1	Day 2	Day 3	Day 4	Day 5
Model Wv-1	-41.385	-62.078	-97.584	-93.130	-70.372
Model Wv-2	-42.753	-70.732	-97.584	-93.130	-80.760
Weibull	-0.917	-35.390	-53.335	-95.256	-78.628
Logistic	-43.247	-35.699	-108.799	-93.967	-27.198
Gompertz	-14.750	NA	-45.574	-95.988	-30.033

Table 25. Comparison of the AIC_C obtained from the fitting of the wavelength shift data of Sample 3 with Model Wv-1, Model Wv-2, Weibull function, Logistic function, and Gompertz function

Model	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Model Wv-1	-62.730	-53.420	-5.497	-27.555	-12.047	-73.970	-45.864	-37.355
Model Wv-2	-62.730	-53.420	-5.497	-27.555	-66.831	-73.970	-45.864	-36.157
Weibull	-37.390	-46.006	-1.042	0.017	37.477	-98.730	-41.264	-7.033
Logistic	-9.671	-22.098	1.564	9.498	24.391	-99.080	-40.392	-9.652
Gompertz	-11.089	-35.883	4.230	3.033	15.716	-31.183	-45.524	-4.551

Tables 23-25 listed the AIC_C of all the functions fitted to the wavelength shift data. The AIC_C values were then used to calculate the evidence ratio (ER) to determine the relative likelihood of model with lower AIC_C being more correct than the other compared models. In this study, Model Wv-2 was considered the best model. Hence, it would be compared with the other fitted functions to assess its relative suitability to describe the wavelength shift trend obtained using SMMIP.

Table 26. Evidence ratio of Model Wv-2 with respect to other models in describing the wavelength shift data of Sample 1

Model	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Model Wv-1	1.05	1.12*	15.06*	17.96*	4.41*	1.00	1.00
Weibull	4.1×10^{18}	24.37	2.08	93.08*	94.63*	1.4×10^5	6.1
Logistic	8.3×10^{17}	21.84	1.96	87.71*	92.20*	8.6×10^4	720.54*
Gompertz	2.1×10^{18}	5.26	10.59	19.08*	1.33*	9.2×10^4	2.39

*The compared model was of lower AIC_C value. Otherwise, Model Wv-2 was of lower AIC_C value.

Table 27. Evidence ratio of Model Wv-2 with respect to other models in describing the wavelength shift data of Sample 2

Model	Day 1	Day 2	Day 3	Day 4	Day 5
Model Wv-1	1.98*	75.72*	1.00	1.00	180.19
Weibull	1.21×10^9	4.73×10^7	4.06×10^9	2.90*	2.90
Logistic	1.28*	4.05×10^7	272.46*	1.52*	4.27×10^{11}
Gompertz	1.20×10^6	NA	1.97×10^{11}	4.17*	1.04×10^{11}

*The compared model was of lower AIC_C value. Otherwise, Model Wv-2 was of lower AIC_C value.

Table 28. Evidence ratio of Model Wv-2 with respect to other models in describing the wavelength shift data of Sample 3

Model	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Model Wv-1	1.00	1.00	1.00	1.00	7.87×10^{11}	1.00	1.00	1.82
Weibull	3.18×10^5	40.73	9.28	9.71×10^5	4.47×10^{22}	2.38×10^5 *	9.97	2.11×10^6
Logistic	3.32×10^{11}	6.33×10^6	34.14	1.11×10^8	6.44×10^{19}	2.84×10^5 *	15.43	5.69×10^5
Gompertz	1.64×10^{11}	6428.52	129.48	4.39×10^6	8.41×10^{17}	1.95×10^9	1.19	7.30×10^6

*The compared model was of lower AIC_C value. Otherwise, Model Wv-2 was of lower AIC_C value.

In using the ER comparison, only ER scores of more than 100 were considered to be of an overwhelming support in favor of the model with the lower AIC_C [241]. Tables 26-28 show that Model Wv-2 was up to $\sim 10^{22}$ times more likely to be correct in representing the data than the other models. It was earlier mentioned that Model Wv-2, which turned to Model Wv-1 at $k < 0.0001$, was not fitting wavelength shift data obtained at day 7 of Sample 3 well. Yet, the ER still shows Model Wv-2 was better than other models when the AIC_C value was compared.

Weibull and logistic function outperformed Model Wv-2 in fitting day 4 and 5 data of Sample 1; and day 6 data of Sample 3. The established functions generally fit such nearly zero response better while Model Wv-1 and Wv-2 provided weaker fits. Model Wv-2 underestimated the shift experienced at the early part of the day for day 6 data of Sample 3 (Figure 90) while Weibull and Logistics function could fit the portion with less error (Figures 93 and 96). Nevertheless, of all fittings performed, it was established that Model Wv-2 was far more likely to be correct in 41.25% of the fittings. Only in 5% of the total fittings that Model Wv-2 fitted the response curves far worse than other functions. Therefore, Model Wv-2 should be used to fit the wavelength shifts obtained by SMMIP.

Besides the good performance of Model Wv-2 in describing the general trend of the wavelength shift, the model was also capable of describing several aspects of the sample empirically. It was explained earlier that the physiological parameter, Q_0 , correctly described the physiological state of the sample. A better adapted sample at

the beginning of the data acquisition was seen to possess higher physiological parameter.

The generation rate could also be correlated with the wavelength shift. Steeper sigmoidal curves were observed to be of higher generation rates (Figures 88-90). After getting used to the new antibiotic environment, it was seen how day 2 data was fitted with a higher generation rate before the rate then declined consecutively over the next few days. The increase of the generation rate could be use as an indicator of the presence of more ciprofloxacin mutants. Nevertheless, for Samples 1 and 3, the increase in the generation rate was experienced at the day where the PAP analysis done at the beginning of the day pointed the presence of mutants in the sample (compare Table 6 and Figure 76). Therefore, the mutants were present in the sample before the medium experienced detectable wavelength shift. Yet, the result of the PAP analysis was obtained 48 hours after the plates was put into the incubator while SMMIP results were real time.

The presence of mutants could be observed by comparing the response curves between days 5 and 6 data of Sample 1, days 3 and 4 data of Sample 2, and days 6 and 7 of Sample 3 (Figure 71). The latter days produced a steeper trend of the normalized wavelength shift and the shifts were of a higher magnitude. Figure 71 clearly shows the steeper trend of the wavelength shift at day 6 compared to day 5. The first 15 hours of day 7 data of Sample 3 shows a slower development of wavelength shift compared to the development of wavelength shift at day 6. The development of wavelength shift was seen to be of steeper trend at the last 7 hours of day 7 to indicate the suspected emergence of mutant.

Figure 100. Development of wavelength shift data near the emergence of mutant for Sample 2 and Sample 3

The determination of the presence of resistant mutants by the development of generation rate produced by Model Wv-1 and Model Wv-2 was later by one day. Yet, combined with the analysis of parameter D , it was shown that the comparison of data points at three time points allowed a determination of mutation at the day similar to the result of the viable plate count. Hence, Model Wv-2 was more appropriate to fit the resulting wavelength shift data and the model was able to help the determination of mutation in progress. The model theoretically could be fitted to the data in real time. Therefore, a prediction algorithm utilizing the model would enable the prediction of the presence of mutants before 24-hour incubation cycle finished. An increase of the parameters prescribed in the algorithm would then allow the system to alert the user to extract the sample for further study.

9.2. Modeling the Intensity Ratio Data of the Emergence of Antibiotic Resistance as Monitored by the SMMIP

There was currently no published data on the change of IR over time in a mutation monitoring study. As a result of bacterial growth, the turbidity of the growth medium would increase since more bacteria were present in the medium. The increase of the turbidity was reflected as an increase of IR value. However, in this study, it was found that the growth medium became less turbid when *Staphylococcus aureus* experienced lysis during exposure to ciprofloxacin. The normalized value of IR became more positive instead of negative. The data was then fitted with a non linear regression model called Model IR-1. Another model was designed specifically to fit the experimental IR data. This model was called Model IR-2.

9.2.1. Model IR-1 to Explain the Normalized IR Data Produced by SMMIP

Model IR-1 was taken from a nonlinear regression function [239]. The function was described as follows

$$y = \frac{a + bt}{1 + ct + dt^2} - a \quad (73)$$

The equation consisted of four parameters. The nominator was a linear function which increased or decreased depending upon the sign of parameter b . On the other hand, the denominator was a parabolic function. The function was mainly used to describe exponential and parabolic curves.

9.2.2. Model IR-2 to Explain the Normalized IR Data Produced by SMMIP

Figure 75 shows that there are data with sharp gradient changes. Hence, the data might be better fitted with double section curves. Therefore, the model, called Model IR-2, should also contain 2 functions. Model IR-2 could be described as follows:

$$IR_{600} = U(t)f(t) + V(t)g(t) \quad (74)$$

$$\text{Where } U(t)_{t < a} = 1; U(t)_{t \geq a} = 0 \text{ and } V(t)_{t < a} = 0; V(t)_{t \geq a} = 1$$

$$\text{While } 0 \leq t \leq 24$$

The fitting of the model would be performed on the IR value at 600 nm. Unit function $U(t)$ and $V(t)$ controlled the activation of function $f(t)$ and $g(t)$ respectively. At any time, only one of the two components in Equation (74) would be functional. Function $f(t)$ was active from the starting time and was used to explain the data before $t = a$. At time above $t = a$, function $f(t)$ would become 0 since $U(t)_{t \geq a} = 0$ and function $g(t)$ would then be used to fit the remaining data points since $V(t)_{t \geq a} = 1$. Function $g(t)$ might not be active if $a > 24$.

Let $IR_{600,i}$ be the value of IR at time $t = i$. For function $f(t)$ in Equation (74) to stay active, the condition that $IR_{600,i} > IR_{600,i+1}$ was checked for correctness at all time. When $IR_{600,i} < IR_{600,i+1}$, the condition of $IR_{600,i+1} \leq IR_{600,i+2}$ was checked. If the

condition was correct, $a = i$ and hence, $U(t)_{t \geq a} = 0$ and $V(t)_{t \geq a} = 1$. The function $g(t)$ was activated and used for the fitting of the subsequent data. If $IR_{600,i+1} \geq IR_{600,i+2}$, the fitting with function $f(t)$ was continued. The entire data would be fitted with only function $f(t)$ if $a = i = 5$ and the IR change at $0 \leq t = a \leq 5$ did not exceed -0.01.

9.2.2.1. First Component of Model IR-2

Function $f(t)$ was formulated to account of the increase of medium's turbidity due to bacterial growth. On the other hand, the function $g(t)$ was designed to explain the lysis process. The derivative of function $f(t)$ (Equation (74)) was defined as follows:

$$\frac{df(t)}{dt} = \frac{dI(t)}{dt} = a(Q)(C)(L_{A-IR})I(t) \quad (75)$$

Where,

- $I(t)$ = change of IR
- a = rate of increase of the turbidity of medium
- (Q) = physiological adaptation
- (C) = antibiotic effect
- (L_{A-IR}) = limiting function A for Model IR-2

The rate of increase of the turbidity of the medium, a , was the main modifier of the change of IR. Such rate was considered to explain the speed of the increase in the turbidity of the medium when certain inoculum was present in the antibiotic-containing medium. The change of physiological condition of bacteria due to different medium state slowed down the contribution of the rate to the change of turbidity at the beginning of the incubation. Function (Q) was used to describe the lag phase resulted from the adaptation to the new medium. The same function had

been previously used in Model Wv-1 and Model Wv-2. Consequently, the function was as follows

$$(Q) = \left(\frac{Q_0}{Q_0 + e^{at}} \right) \quad (76)$$

Where,

Q_0 = the physiological value of the culture at time 0

In Equation (76), the denominator used e^{at} instead of e^{-at} seen in Equation (45) since the value of a was expected to be negative. Therefore, e^{at} would be the term to produce a sigmoidal function of between 0 and 1 for Equation (76).

In addition to the physiological adaptation, the turbidity was also affected by the ciprofloxacin concentration in the medium. However, since the antibiotic concentration was constant in this study, (C) was set to 1. Since the IR always started at the value of 1 (Equation (39)) and the value could not go lower than 0, the normalization of IR resulted in a maximum value of -1 for $I(t)$. A limiting function, (L_{A-IR}) , was then included to account for such factor. The function would diminish the rate as the $I(t)$ was closer to -1. The limiting function could be described as follows

$$(L_{A-IR}) = \left(\frac{DI(t)}{I_{\max}} - 1 \right) \quad (77)$$

Where,

$I_{\max}$ = maximum possible $I(t)$, which is -1

D = adjustment constant for non maximum saturation

Equation (77) shows that the contribution of a diminished as the change of IR get closer to $I_{\max}$. The final value of (L_{A-IR}) was 0 which was to indicate no further change of turbidity was possible since $I_{\max}$ had been reached. The adjustment constant helped to account for earlier saturation when not enough bacteria were

present to affect further increase of turbidity. The use of such limiting function was again a mirror of what had been used previously for Model Wv-1 and Model Wv-2.

The general form of the first component of Model IR-2 could be described as follows

$$\frac{df(t)}{dt} = \frac{dI(t)}{dt} = a \left(\frac{Q_0}{Q_0 + \exp(at)} \right) \left(\frac{DI(t)}{I_{\max}} - 1 \right) I(t) \quad (78)$$

Equation (78) was integrated analytically with Mathematica software and the result was as follows

$$I(t) = \frac{I_{\max} (Q + e^{at})}{D(Q + e^{at}) + e^{at} e^{-AI_{\max}}} \quad (79)$$

A = integration constant

It can be seen that Equation (79) could not be fitted to IR data, whose origin was (0, 0). Therefore, the integration constant was solved by creating an origin of (0, I_{st}). The shifted equation was as follows

$$I^*(t) = \frac{I_{\max}^* (Q + e^{at})}{D(Q + e^{at}) + e^{at} \frac{(I_{\max}^* - I_{st} D)(1 + Q)}{I_{st}}} \quad (80)$$

Where,

I_{st} = the shifted IR at time 0

The equation was then shifted back to obtain the following equation

$$f(t) = I(t) = \frac{I_{\max}^* (Q + e^{at})}{D(Q + e^{at}) + e^{at} \frac{(I_{\max}^* - I_{st} D)(1 + Q)}{I_{st}}} - I_{st} \quad (81)$$

9.2.2.2. Second Component of Model IR-2

The increase of the turbidity due to the presence of persisting bacteria in the presence of ciprofloxacin was lessened by the clearing of the medium due to bacterial lysis. The lysis produced when the susceptible bacteria was killed by ciprofloxacin. Therefore, the second component of Model IR-2 was intended to account for the clearing of the medium as a result of the lysis, which resulted in a reduction of IR change over time. The derivative of function $g(t)$ (Equation (74)) was defined as follows:

$$\frac{dg(t)}{dt} = \frac{dI(t)}{dt} = b(Q)(C)(L_{B-IR})I(t) \quad (82)$$

Where,

$$\begin{aligned} I(t) &= \text{change of IR} \\ b &= \text{rate of the clearing of medium} \\ (C_{ab}) &= \text{competition function} \\ (C) &= \text{antibiotic effect} \\ (L_{B-IR}) &= \text{limiting function B for Model IR-2} \end{aligned}$$

The rate of the clearing due to lysis was denoted by b , which was the main modifier of the reduction of the turbidity. The contribution of the rate was modified by (C_{ab}) , (C) , and (L_{B-IR}) . (C_{ab}) was used to explain that there would be a lag phase, which reduced the affect of b in reducing the turbidity. The lag phase was a result of a competition between the increase of the turbidity by a and reduction of turbidity by b . Consequently, the competition function would be similar to the physiological adaptation function, which was sigmoidal in nature to signal a gradual adaptation. Since the value of b was expected to be a positive, (C_{ab}) used e^{-bt} in the denominator. The function was as follows

$$(C_{ab}) = \left(\frac{C_{ab_0}}{C_{ab_0} + e^{-bt}} \right) \quad (83)$$

Where,

C_{ab_0} = competition value of the culture

It could be seen from Equation (83) that the function was a sigmoidal curve which saturated at 1. A higher competition value would allow a full contribution of b as early as possible. The higher b value would also shorten the time required by C_{ab_0} to reach the value of 1 and hence, a faster full contribution of b to the reduction of turbidity.

The antibiotic effect, (C) , was here again set at 1. It was expected that higher antibiotic concentration would produce a higher (C) value to enhance the contribution of b since more bacteria were lysed. On the other hand, the limiting function (L_{B-IR}) was used to limit the clearing of the medium gradually. The clearing of the medium could not go indefinitely since the bacteria number could fall below the level where any lysis would not produce detectable change of turbidity. The gradual reduction of the lysis effect on the turbidity of the medium was expected to be gradual and therefore the (L_{B-IR}) function would be progressing from 1 to 0 in sigmoidal manner. The function was as follows

$$(L_{B-IR}) = \left(\frac{e^{-bt}}{S + e^{-bt}} \right) \quad (84)$$

Where,

S = the lysis value of the culture

Higher S would cause Equation (84) to reach 0 faster and hence, indicated a faster rate of bacterial number reduction to saturate the function. The saturation of (L_{B-IR})

at 0 resulted in the saturation of the $g(t)$ function which saturated the IR change as well.

The general form of the second component was then as follows

$$g(t) = \frac{dI(t)}{dt} = b \left(\frac{C_{ab_0}}{C_{ab_0} + e^{-bt}} \right) \left(\frac{e^{-bt}}{S + e^{-bt}} \right) I(t) \quad (85)$$

Mathematica was used again to solve the equation above and the following equation was produced

$$I(t) = Ae^{\frac{C_{ab_0}(\ln(1+e^{bt}C_{ab_0}) - \ln(1+e^{bt}S))}{C_{ab_0} - S}} \quad (86)$$

Where,

A = integration constant

The end point of the fitted curve of $f(t)$ would serve as the starting point of the function in $g(t)$. Hence, the start point of the activation of Equation (86) was set to (t_0, I_0) . Using the boundary value, the integration constant was solved to result in Equation (87).

$$g(t) = I(t) = \frac{I_0 e^{\frac{C_{ab_0}(\ln(1+e^{bt}C_{ab_0}) - \ln(1+e^{bt}S))}{C_{ab_0} - S}}}{e^{\frac{C_{ab_0}(\ln(1+e^{bt_0}C_{ab_0}) - \ln(1+e^{bt_0}S))}{C_{ab_0} - S}}} \quad (87)$$

The parameters of Equation (87) were expected to be of positive values while the boundary value of equation (I_0) was of negative values. A careful observation of Equation (86) shows that those values would not allow the equation to give an increasing response curve to describe a reduction of IR change over time. Hence, a shift was performed such that the boundary value was at $(t_0, 1)$ to ensure that the

graphs produced by Equation (87) was of an increasing trend. The resulting equation was then shifted back to the starting I_0 to allow the fitting to be conducted to the original data. The final equation used for the study was therefore,

$$g(t) = I(t) = \frac{e^{\frac{C_{ab0}(\ln(1+e^{bt}C_{ab0}) - \ln(1+e^{bt}S))}{C_{ab0}-S}}}{e^{\frac{C_{ab0}(\ln(1+e^{bt_0}C_{ab0}) - \ln(1+e^{bt_0}S))}{C_{ab0}-S}}} - (1 + I_0) \quad (88)$$

9.2.2.3. General Form of Model IR-2

The general form of Model IR-2 was previously stated in Equation (74) as

$$IR_{600} = U(t)f(t) + V(t)g(t)$$

$$\text{Where } U(t)_{t < a} = 1; U(t)_{t \geq a} = 0 \text{ and } V(t)_{t < a} = 0; V(t)_{t \geq a} = 1$$

$$\text{While } 0 \leq t \leq 24$$

Including Equations (78) and (85), the final general form of Model IR-2 which would be used to explain the IR data was as follows

$$IR_{600} = U(t) \left(\frac{I_{\max}^* (Q + e^{at})}{D(Q + e^{at}) + e^{at} \frac{(I_{\max}^* - I_{st} D)(1 + Q)}{I_{st}}} - I_{st} \right) + V(t) \left(\frac{e^{\frac{C_{ab0}(\ln(1+e^{bt}C_{ab0}) - \ln(1+e^{bt}S))}{C_{ab0}-S}}}{e^{\frac{C_{ab0}(\ln(1+e^{bt_0}C_{ab0}) - \ln(1+e^{bt_0}S))}{C_{ab0}-S}}} - (1 + I_0) \right) \quad (89)$$

$$\text{Where } U(t)_{t < a} = 1; U(t)_{t \geq a} = 0 \text{ and } V(t)_{t < a} = 0; V(t)_{t \geq a} = 1$$

$$\text{While } 0 \leq t \leq 24, a \geq 0, b \leq 0, 0 \leq Q, S \leq 1$$

9.2.3. The Fitting of the Normalized IR Data with the Models

Models IR-1 and IR-2 were fitted to the normalized IR data obtained with SMMIP. Akaike's Information Criterion (AIC) was used to compare the two models. The

comparison method was similar to the one used in the data fitting of the normalized wavelength shift. The equations are provided in Equations (61) to (65).

9.2.3.1. The Fitting of Normalized IR with Model IR-1

The fitting of Model IR-1 (Equation (73)) to the change of IR experienced by Samples 1, 2, and 3 produced graphs shown in Figures 101-103. The fitting to the trend of the data was quite well. The model, however, was unable to follow the sharp turn experienced by some experimental data. Model IR-1 also overestimated the IR changes at the beginning of each day's data.

Figure 101. Curve fitting of Model IR-1 on the normalized intensity ratio of Sample 1

Figure 102. Curve fitting of Model IR-1 on the normalized intensity ratio of Sample 2

Figure 103. Curve fitting of Model IR-1 on the normalized intensity ratio of Sample 3

The fitting of Model IR-1 produced parameters shown in Tables 29-31. In general, no trend can be observed from all the parameters. A large 95% confidence interval

was produced for most fittings. The IR data were more scattered compared to the wavelength shift data. Hence, a wide range of parameters would produce almost identical curves. However, although the trend of the data could be followed by the model, no obvious trend could be derived from the parameters to indicate the emergence of mutants. The non linear regression model used in this case was not derived specifically for this study. Consequently, it was difficult to relate any of the parameters with changes observed in the samples.

Table 29. Parameters and AIC_C obtained when Model IR-1 was fitted to the normalized IR of Sample 1

Day	<i>a</i> (95% CI)	<i>b</i> (95% CI)	<i>c</i> (95% CI)	<i>d</i> (95% CI)	SSE	AIC _C
1	0.0412 (0.0279, 0.0544)	-0.0114 (-0.0130, -0.0097)	-0.2122 (-0.2378, -0.1865)	0.0162 (0.0132, 0.0192)	0.0025	-217.34
2	0.4724 (-2.6430, 3.5870)	0.0267 (-0.0321, 0.0855)	0.0597 (-0.2549, 0.3742)	-7.6 x 10 ⁻⁴ (-0.0072, 0.0057)	3.0 x 10 ⁻⁴	-270.27
3	0.2337 (-5.9310, 6.3990)	0.1263 (-2.8960, 3.1480)	0.5687 (-2.1020, 3.2390)	-2.0 x 10 ⁻³ (-0.0604, 0.0563)	2.6 x 10 ⁻⁴	-273.32
4	0.2923 (-36.440, 37.020)	0.1185 (-13.990, 14.220)	0.3716 (-1.4140, 2.1580)	8.9 x 10 ⁻⁴ (-0.1070, 0.1088)	9.1 x 10 ⁻⁴	-242.32
5	0.2599 (-117.80, 118.40)	0.1211 (-54.090, 54.330)	0.4484 (-5.2100, 6.1070)	5.8 x 10 ⁻⁴ (-0.2603, 0.2615)	1.0 x 10 ⁻³	-238.86
6	0.2416 (-264.70, 265.20)	0.1245 (-141.90, 142.20)	0.5111 (-31.400, 32.430)	-3.6 x 10 ⁻⁴ (-0.3688, 0.3681)	5.9 x 10 ⁻⁴	-253.09
7	0.0166 (0.0021, 0.0312)	-0.0068 (-0.0099, -0.0037)	-0.2992 (-0.4534, -0.1450)	0.0348 (0.0111, 0.0586)	4.2 x 10 ⁻³	-204.28

Table 30. Parameters and AIC_C obtained when Model IR-1 was fitted to the normalized IR of Sample 2

Day	<i>a</i> (95% CI)	<i>b</i> (95% CI)	<i>c</i> (95% CI)	<i>d</i> (95% CI)	SSE	AIC _C
1	0.1687 (0.1315, 0.2059)	-0.0272 (-0.0311, -0.0234)	-0.1793 (-0.2177, -0.1408)	0.0171 (0.0106, 0.0236)	6.6 x 10 ⁻³	-192.64
2	0.6060 (-12.420, 13.630)	0.1166 (-2.1850, 2.4180)	0.2078 (-0.5764, 0.9919)	-9.7 x 10 ⁻⁴ (-0.0231, 0.0212)	1.0 x 10 ⁻³	-239.66
3	0.1955 (-3.0390, 3.4300)	0.0156 (-0.2424, 0.2737)	0.0974 (-0.2399, 0.4348)	-7.8 x 10 ⁻⁴ (-0.0143, 0.0128)	1.4 x 10 ⁻⁴	-288.60
4	0.0401 (-3.9820, 4.0620)	-6.4 x 10 ⁻⁴ (-0.1296, 0.1283)	-0.0193 (-1.3350, 1.2960)	-3.0 x 10 ⁻⁴ (-0.0413, 0.0407)	3.9 x 10 ⁻⁴	-263.32
5	1.9920 (-152.60, 156.60)	0.0542 (-5.1300, 5.2380)	0.0354 (-0.1382, 0.2089)	-2.0 x 10 ⁻⁴ (-0.0167, 0.0163)	5.9 x 10 ⁻³	-195.54

Table 31. Parameters and AIC_C obtained when Model IR-1 was fitted to the normalized IR of Sample 3

Day	a (95% CI)	b (95% CI)	c (95% CI)	d (95% CI)	SSE	AIC _C
1	0.8300 (0.5696, 1.0900)	-0.0028 (-0.0589, 0.0532)	-0.0101 (-0.0661, 0.0459)	0.0065 (0.0035, 0.0096)	4.0×10^{-3}	-205.38
2	2.0000 (-1.4590, 5.4590)	-0.0205 (-0.0737, 0.0328)	0.0183 (-0.0485, 0.0852)	-0.0013 (-0.0040, 0.0014)	2.5×10^{-2}	-159.37
3	0.2110 (0.1444, 0.2776)	-0.0661 (-0.0695, -0.0626)	-0.0800 (-0.1073, -0.0524)	0.0132 (0.0093, 0.0170)	2.8×10^{-3}	-106.21
4	24.170 (-200.50, 248.90)	-0.0737 (-1.0690, 0.9218)	7.9×10^{-4} (-0.0426, 0.0427)	-1.6×10^{-4} (-0.0017, 0.0014)	2.3×10^{-2}	-161.90
5	14.550 (-317.60, 346.70)	1.9850 (-43.750, 47.720)	0.1459 (-0.0672, 0.3590)	-3.9×10^{-4} (-0.0094, 0.0087)	3.5×10^{-2}	-151.16
6	2.3070 (-102.20, 106.80)	-5.2×10^{-4} (-0.4834, 0.4824)	1.6×10^{-4} (-0.2179, 0.2182)	-1.1×10^{-4} (-0.0054, 0.0052)	9.8×10^{-4}	-240.54
7	0.7762 (-73.040, 74.590)	1.5080 (-129.80, 132.80)	2.0000 (-28.120, 32.120)	-0.0041 (-0.4303, 0.4221)	8.0×10^{-3}	-188.12
8	7.0540 (-515.40, 529.50)	1.0400 (-77.510, 79.590)	0.1547 (-0.2112, 0.5205)	-2.5×10^{-4} (-0.0190, 0.0185)	1.2×10^{-2}	-176.78

9.2.3.2. The Fitting of Normalized IR with Model IR-2

Model IR-2 was stated in Equation (89). The shifting of the original data by -1 resulted in the value of $I_0 = -1$ and $I_{\max}^* = -2$. Entering the boundary value for component one to Equation (89) resulted in the following equation

$$IR_{600} = U(t)f(t) + V(t)g(t)$$

$$\Leftrightarrow IR_{600} = U(t) \left(\frac{-2(Q + e^{at})}{D(Q + e^{at}) + e^{at} \frac{(-2-D)(1+Q)}{1}} + 1 \right) + V(t) \left(\frac{e^{\frac{C_{ab0}(\ln(1+e^{bt}C_{ab0}) - \ln(1+e^{bt}S))}{C_{ab0}-S}}}{e^{\frac{C_{ab0}(\ln(1+e^{bt0}C_{ab0}) - \ln(1+e^{bt0}S))}{C_{ab0}-S}}} - (1 + I_0) \right) \quad (90)$$

Where $U(t)_{t < a} = 1; U(t)_{t \geq a} = 0$ and $V(t)_{t < a} = 0; V(t)_{t \geq a} = 1$

While $0 \leq t \leq 24$, $a \geq 0$, $b \leq 0$, $0 \leq Q_0, S \leq 1$

The response of the $f(t)$ at $t = t_0$ would be used to determine the start point of $g(t)$. In order to determine the value of t_0 , condition stated in section 9.2.2 was tested. The condition allowed a fitting of the first component of Equation (90) to the response at $0 \leq t \leq t_0$ and the fitting of the second component of the equation to the

response at $t_0 \leq t \leq 24$. The result of the determination of t_0 was shown in Table 32. The value of t_0 was then substituted into Equation (90) along with $I_0 = f(t_0)$. The resulting equation was used to fit each data section accordingly.

Table 32. List of t_0 used in fitting normalized IR data with Model IR-2

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Sample 1	8	0	0	0	0	0	7	NA
Sample 2	17	6	0	0	9	NA	NA	NA
Sample 3	24	10	10	11	8	0	9	9

* NA = not available

Table 33. Parameters of $f(t)$ when the function was fitted to Sample 1

Day	D (95% CI)	Q_0 (95% CI)	a (95% CI)	SSE
1	1.6910 (1.6740, 1.7080)	0.0015 (-0.0005, 0.0036)	-1.3340 (-1.6230, -1.0450)	1.4×10^{-4}
7	0.7593 (-185.10, 186.60)	0.1738 (-17.400, 17.750)	-0.0924 (-5.8020, 5.6170)	5.8×10^{-4}

Table 34. Parameters of $g(t)$ when the function was fitted to Sample 1

Day	C_{ab} (95% CI)	S (95% CI)	b (95% CI)	SSE
1	3.5×10^{-4} (-0.0009, 0.0016)	0.0108 (-0.0225, 0.0441)	0.3788 (0.1604, 0.5972)	8.0×10^{-4}
2	8.5×10^{-4} (-7.1×10^{-5} , 0.0018)	0.0337 (0.0054, 0.0620)	0.2980 (0.2128, 0.3832)	2.7×10^{-4}
3	0.9375 (-3168.0, 3170.0)	0 (fixed at bound)	2.8×10^{-4} (-0.4948, 0.4953)	7.0×10^{-4}
4	1 (-2.5×10^4 , 2.5×10^4)	2.2×10^{-4} (-2.4×10^4 , 2.4×10^4)	0.0014 (-53.170, 53.170)	2.2×10^{-3}
5	1 (fixed at bound)	1.5×10^{-5} (-1763.0, 1763.0)	4.5×10^{-4} (-0.8018, 0.8027)	1.3×10^{-3}
6	1 (fixed at bound)	1.6×10^{-5} (-896.00, 896.00)	5.3×10^{-4} (-0.4733, 0.4743)	6.0×10^{-4}
7	1 (fixed at bound)	3.4670 (-0.5490, 7.4830)	0.2273 (0.0006, 0.4541)	0.0043

Table 35. Parameters of $f(t)$ when the function was fitted to Sample 2

Day	D (95% CI)	Q_0 (95% CI)	a (95% CI)	SSE
1	1.5610 (1.5560, 1.5660)	0.0110 (0.0060, 0.0159)	-0.8520 (-0.9306, -0.7734)	4.0×10^{-4}
2	1.1890 (-36.460, 38.840)	0.0095 (-0.3868, 0.4058)	-0.3733 (-1.6830, 0.9364)	1.6×10^{-5}
5	1.7360 (1.6660, 1.8050)	0.0459 (0.0040, 0.0878)	-0.5141 (-0.7310, -0.2971)	1.3×10^{-4}

Table 36. Parameters of $g(t)$ when the function was fitted to Sample 2

Day	C_{ab} (95% CI)	S (95% CI)	b (95% CI)	SSE
1	2.0×10^{-9} (fixed at bound)	3.8×10^{-8} (fixed at bound)	0.7788 (0.6352, 0.6611)	4.8×10^{-4}
2	0.0209 (-0.1192, 0.1610)	0.9999 (-3.8190, 5.8180)	0.3017 (-0.0650, 0.6683)	2.4×10^{-4}
3	0.0093 (-1.8×10^5 , 1.8×10^5)	0.9674 (-5.1×10^5 , 5.1×10^5)	4.7×10^{-5} (-900.90, 900.90)	1.4×10^{-3}
4	5.6×10^{-4} (-0.0018, 0.0029)	0.0124 (-0.0573, 0.0821)	0.1771 (-0.1179, 0.4721)	3.8×10^{-4}
5	1 (fixed at bound)	0.7713 (-0.7363, 2.279)	0.0397 (-0.4928, 0.5721)	1.8×10^{-3}

Table 37. Parameters of $f(t)$ when the function was fitted to Sample 3

Day	D (95% CI)	Q_0 (95% CI)	a (95% CI)	SSE
1	1.1920 (1.1670, 1.2170)	0.1770 (0.0976, 0.2565)	-0.2297 (-0.2678, -0.1916)	7.6×10^{-3}
2	1.4310 (1.3380, 1.5240)	0.2373 (-0.0020, 0.4766)	-0.3795 (-0.5713, -0.1877)	1.1×10^{-3}
3	1.2610 (1.1990, 1.3230)	0.7945 (-0.0951, 1.6840)	-0.3489 (-0.4976, -0.2002)	1.3×10^{-3}
4	1.4160 (1.3910, 1.4400)	1 (fixed at bound)	-0.3720 (-0.4135, -0.3306)	1.5×10^{-3}
5	1.4230 (1.4000, 1.4460)	0.1628 (0.0328, 0.2928)	-0.8342 (-1.0630, -0.6054)	6.8×10^{-4}
7	1 (fixed at bound)	0.7552 (-32.720, 34.230)	-0.0169 (-0.4191, 0.3853)	8.2×10^{-4}
8	1.6890 (1.5490, 1.8290)	0.9999 (-3.8620, 5.8620)	-0.3371 (-0.9908, 0.3166)	1.2×10^{-3}

Table 38. Parameters of $g(t)$ when the function was fitted to Sample 3

Day	C_{ab} (95% CI)	S (95% CI)	b (95% CI)	SSE
2	7.0×10^{-4} (-0.0014, 0.0028)	0.0018 (-0.0020, 0.0055)	0.3167 (0.1552, 0.4781)	6.0×10^{-3}
3	0.5972 (-35.570, 36.770)	0.9995 (-2.3280, 4.3270)	0.0404 (-1.1990, 1.2800)	7.3×10^{-4}
4	0.0911 (0.0027, 0.1796)	5.5×10^{-9} (fixed at bound)	0.1180 (0.0870, 0.1491)	0.01534
5	0.3953 (-1.3740, 2.1640)	0.5397 (-0.3657, 1.4450)	0.1272 (-0.0028, 0.2572)	1.5×10^{-3}
6	0.0011 (-2.4×10^{-4} , 0.0024)	0.0208 (0.0022, 0.0394)	0.2813 (0.1911, 0.3716)	7.9×10^{-4}
7	1 (fixed at bound)	0.0027 (-0.0454, 0.0509)	0.9999 (-0.9752, 2.9750)	4.6×10^{-3}
8	0.7441 (-138.60, 140.10)	0.9990 (-5.5300, 7.5280)	0.0369 (-3.4000, 3.4740)	9.4×10^{-3}

Tables 33-38 show the parameters produced when Model IR-2 was fitted to the normalized IR data. In general, the SSE was $< 10^{-3}$, which indicated a good fit since

the error was one thousand times less than the response. The fitting was better for the section utilizing function $f(t)$ since the parameters were of narrower confidence intervals than the parameters for the section utilizing function $g(t)$. Hence, less uncertainty was associated with the sections of data indicating the increase of the medium's turbidity. On the other hand, the latter section of the IR data, which was related to the clearing of the medium, shows trends ranging from exponential to linear curves. Hence, it was more difficult to fit and the parameters of $g(t)$ was produced with wide range of 95% confidence interval.

Figure 104. Curve fitting of Model IR-2 on the normalized intensity ratio of Sample 1

Figure 105. Curve fitting of Model IR-2 on the normalized intensity ratio of Sample 2

Figure 106. Curve fitting of Model IR-2 on the normalized intensity ratio of Sample 3

The illustration of the fitting of the data established that Model IR-2 could describe the trend of the IR data very well (Figures 104-106). The increase of the turbidity of

the medium was of exponential curves while the declining turbidity was fitted with various trends. Function $g(t)$ was more difficult to fit than function $f(t)$ since small parameter changes did not change the response curve very much. This explains the wide confidence interval. Nevertheless, Model IR-2 was seen to be robust enough to accommodate the various data trend.

Although the model could depict the trend of the experimental data well, only the fitted parameters from Sample 2 produced a discernible pattern to be used in the monitoring of mutation in progress. The activation of function $g(t)$ was related to the clearing of medium. However, when the function was invoked at time $t_0 = 0$, the growth medium no longer experienced increase in the turbidity. Hence, at that condition, bacterial lysis was more prevalent. Such phenomenon was seen in day 3 and 4 data of Sample 2. Nevertheless, function $f(t)$ was used for the subsequent day 5 data.

In Sample 1, a period of near zero response was observed before the turbidity of the sample increased in day 7. Function $f(t)$ was not used in the near zero responses since the activations of $g(t)$ in those days were less than 5 hours. Consequently, the day data was fitted using function $g(t)$ only. In Sample 1, the activation of $f(t)$ at day 7 after a subsequent inactivation period could be used to indicate the presence of mutants in this study.

Such activation and inactivation of function $f(t)$ also occurred at day 6 and day 7 of Sample 3. However, the turbidity increase rate was small. The activation of $f(t)$ was confirmed at the next day and higher rate (a) was produced. The use of the function after a string of inactivation indicated the increase of the medium's turbidity after a period of medium's clearing. Therefore, more bacterial mass were present at the activation of $f(t)$. The increase in the turbidity in this case could then be associated the emergence of resistant mutants. The activation of function $f(t)$ after a period of inactivation could then be used in the confirmation of the presence

of mutant in the sample although it was stated earlier to be one day later than the normalized wavelength shift result.

9.2.4. Comparison of Model IR-1 and Model IR-2 for the Fitting of the Intensity Ratio Data

Model IR-1 and Model IR-2 were fitted to the normalized intensity ratio data and the results show a good fitting for both models. The SSE was then used again to calculate the corrected Akaike's Information Criterion (AIC_C) (Equation (63)). Based on the AIC_C value, evidence ratio (Equation (65)) was then determined to provide an understanding on how more correct Model IR-2 was in fitting the experimental IR data.

Table 39. Comparison of the AIC_C obtained from the fitting of the intensity ratio data of Sample 1 with Model IR-1 and Model IR-2

Model	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Model IR-1	-217.34	-270.27	-273.32	-242.32	-238.86	-253.09	-204.28
Model IR-2	-234.28	-275.82	-255.11	-241.97	-240.05	-258.83	-196.84

Table 40. Comparison of the AIC_C obtained from the fitting of the intensity ratio data of Sample 2 with Model IR-1 and Model IR-2

Model	Day 1	Day 2	Day 3	Day 4	Day 5
Model IR-1	-192.65	-239.66	-288.60	-263.32	-195.54
Model IR-2	-243.11	-265.79	-235.64	-267.02	-222.82

Table 41. Comparison of the AIC_C obtained from the fitting of the intensity ratio data of Sample 3 with Model IR-1 and Model IR-2

Model	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Model IR-1	-205.38	-159.37	-106.21	-161.90	-151.16	-240.54	-188.12	-176.78
Model IR-2	-192.48	-183.43	-215.14	-169.37	-213.04	-249.12	-197.93	-173.72

Table 42. Evidence ratio of Model IR-2 with respect to Model IR-1 in describing the intensity ratio data of Sample 2 and Sample 3

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Sample 1	4.7×10^3	16.0	$9 \times 10^3^*$	1.19 [*]	1.81	17.6	43.1 [*]	NA
Sample 2	9.06×10^{10}	4.72×10^5	$3.16 \times 10^{11}^*$	6.36	8.39×10^5	NA	NA	NA
Sample 3	633 [*]	1.68×10^5	4.51×10^{23}	41.9	2.74×10^{13}	73	135	4.62 [*]

*Model IR-1 fitted the data better. Otherwise, Model IR-2 was better.

Model IR-2 was up to 10^{23} times more likely to be correct than Model IR-1 in fitting the resulting IR data. The designed model gave lower AIC_C value for ~70% of the data. Model IR-2 was seen to perform worse than Model IR-1 in some days data of Sample 1. Both Model IR-1 and IR-2 tried to fit a trend to the response which hovered closely to zero. Figures 104-106 show that the data at that day was less than $\pm \sim 0.015$. Therefore, the data fluctuation was most likely a result of system's uncertainty. A linear $y = 0$ curve would have sufficed for such graphs. Since the Model was derived from a derivative function, it was difficult to ensure that the analytical solution of the derivative function could fit a $y=0$ into the data. Nevertheless, the theorized Model IR-2 was also capable of determining the occurrence of mutation in progress despite the fact that change in the IR data was lagging by one day compared to the medium's colour change. Hence, by fitting the IR data in real time, parameters trending can be obtained before 24 hours of incubation period is achieved.

In the real time fitting for predictive modeling of the experimental data, value of t_0 is continuously determined using Model IR-2. The activation of $g(t)$ after t_0 indicates the clearing of the growth medium. The use of the function $f(t)$ after a string of inactivation of the function indicated the increase of the medium's turbidity after a period of medium's clearing. This increase of turbidity denotes the emergence of resistant mutants. Due to the need to determine t_0 , the fitting ability of Model IR-2 is not exactly real time but rather lag by at least 2 hours.

Chapter 10

CONCLUSIONS

In this study, an *in vitro* system for monitoring mutation-in-progress was successfully built and studied. The system is called “System for Monitoring of Mutation in Progress (SMMIP)”. The system consists of Detection System (DS), Controlled Environmental Chamber (CEC), Replenishing System (RS), pH Control System (pHCS), and Extraction System (ES), and Control Unit (CU). The DS is housed inside the CEC while all the other components are connected to the DS via sterile tubing. The details of every component are provided in Chapter 4.

The SMMIP was utilized to study the emergence of antibiotic resistance as one of the examples of mutation-in-progress. *Staphylococcus aureus* was chosen as the model organism while ciprofloxacin was chosen as the model drug. It was shown that the emergence of ciprofloxacin resistant *Staphylococcus aureus* could be monitored via the change of the normalized wavelength shift and normalized intensity ratio of the growth media. The study was conducted by inoculating MSSA ATCC 29213 into MSBCip-0.5 and MSBCip-1.

During the constant concentration exposure at MSBCip-0.5, mutants were determined by SMMIP to emerge at day 6 (120-144 hours), day 5 (96-120 hours), and day 7 (144-168 hours) for samples with initial inoculum of 6.0×10^6 CFU/ml (Sample 1), 1.3×10^7 CFU/ml (Sample 2) and 1.3×10^8 CFU/ml (Sample 3) respectively. The mutants were not present in the starting culture. On the other hand, the study utilizing increasing ciprofloxacin concentration in MSBCip-1 produced no mutants. Hence, incubating MSSA ATCC 29213 at 1x MIC produced mutants but not at 2x MIC. The study also confirmed the randomness of mutation since larger initial inoculum did not correlate to earlier emergence of mutants. Some of the mutants produced in the study using SMMIP were then subjected to passages on antibiotic free medium to assess the stability of the mutation. Based on the changes in MIC value, the mutants tested were considered to have a stable antibiotic resistance [203-205].

The monitoring results were confirmed with viable plate count, Population Analysis Profile (PAP), and a genomic study. Both the viable plate count and PAP technique confirmed the emergence of the mutants established by SMMIP. Sequencing work of the quinolone resistance determining region (QRDR) of GyrA, GyrB, GrlA, and GrlB subunit of DNA gyrase and DNA topoisomerase IV along with NorA promoter showed unaltered coding sequence of the *gyrA*, *gyrB*, and *grlA* gene sections. Nevertheless, a single mutation was found to change codon 470 of GrlB subunit from Asparagine to Isoleucine for some mutants and codon 396 of GrlB subunit from Alanine to Proline for a mutant (Figure 82 and Figure 84). Mutation at codon 470, which conferred ciprofloxacin resistance, was reported few years ago [133]. Nevertheless, in that study, the Asparagine residue was substituted by Asparatate due to difference in the location of nucleic acid substitution. On the other hand, mutation at codon 396 was novel.

During the study, extended lysis of *Staphylococcus aureus* after being exposed to ciprofloxacin was observed by electron microscopy (Figure 66 and Figure 68). Nevertheless, evidence of bacteria with normal shape existing in the culture was also found. Earlier trial had also encountered normal shape bacteria amidst lysed bacteria even at MSBCip-2 (Figure 68). It was hypothesized that these bacteria with normal shape could have been specialized survivor cells called persisters which was found to occur in some bacterial culture [225-229]. The study on such persisters was beyond the scope of the current investigation.

The results obtained with MSSA ATCC 29213 established that SMMIP was indeed capable of monitoring the emergence of ciprofloxacin resistant mutants in *Staphylococcus aureus* culture. The presence of the mutants was confirmed with two conventional monitoring method, i.e. viable count technique and PAP analysis. Significant reduction of resources was achieved by using SMMIP instead of the conventional viable count technique in monitoring mutation in progress. A five days study using SMMIP required only 21 ml of broth while a 5 days study using viable plate count data required 1440 pieces of agar plates or 28.8 liters of agar solution if viable plate count was performed every hour. PAP analysis needed at

least 466 pieces of agar plates or 9.32 liters of agar solution when six different antibiotic concentrations were investigated. The results provided by SMMIP were in real time and hence, were obtained earlier than the results of viable count and PAP analysis.

The results provided by SMMIP could also reveal whether extended lysis occurred or not via the alkalization and clearing of the growth medium. SMMIP could be scaled to allow multiple monitoring of various drug regimes with different bacterial strains and therefore a large scale monitoring work could be performed. Such scale of work would be taxing to perform if viable plate count and PAP analysis method were used.

Mathematical models were designed to explain the monitoring result provided by SMMIP. The evidence ratio (ER) calculated shows that Model Wv-2 was appropriate and more likely to be correct in fitting the magnitude of the normalized wavelength shifts compared to the conventional models. The generation rate, g , is found to correlate with wavelength shift. Higher generation rate can be seen to fit into a steeper sigmoidal curve (Figures 88-90). Hence, the change in wavelength shift rate which corresponds to the emergence of mutation should be able to translate into a change in the generation rate.

It is found that monitoring the development of generation rate of Model Wv-2, presence of resistant mutants can be determined. The determined day, however, is 1 day later than obtained from visual observation of experimental data (Figure 100). The determination ability is improved when parameter D , the adjustment constant, is included in the model interpretation. At the presence of maximum saturation, the adjustment constant is expected to produce value ~ 1 . However, when the antibiotic inhibition is great enough to force the wavelength shift to achieve non-maximum inhibition, the constant will produce value > 1 . Hence, once D exceeds 1, the next day's D value is expected to increase if no resistant mutants are present. The increase is expected since without resistant mutants, metabolism activity of the culture will diminish and greater inhibition of wavelength shift will be experienced.

Nevertheless, if the D value is fitted with value < 1 after a period of $D > 1$, resistant mutant is present since the wavelength shift data is no longer saturating below 21.81 nm if the sigmoidal curve is completed.

By monitoring both the values of g and D , Model Wv-2 can be used to monitor the emergence of resistant mutants. The development of g and D can be monitored if continuous fitting is performed on the experimental data in real time. Hence, theoretically, with the models running in background, the emergence of resistant mutants can be determined at any incubation point. Culture extraction can then be performed automatically and further studies on the mutants can be done.

Despite the understanding that IR data provides slower determination of the presence of resistant mutants, a model, Model IR-2, was also developed to understand the IR experimental data. Model IR-2 consists of two functions. The second function, $g(t)$, is invoked only when a certain condition detailed in Section 9.2.2 is fulfilled. The activation of function $g(t)$ is related to the clearing of growth medium.

In Model IR-2, activation of function $f(t)$ after a period of inactivation denotes the emergence of resistant mutants. The activation indicates the increase in medium's turbidity after a string of medium's clearing. However, the activation lags behind the positive presence of resistant mutants indicated by the wavelength shift data. Nevertheless, using the comparison method based on ER, Model IR-2 was found to be more likely correct than a nonlinear regression model in fitting the normalized IR data. Real time fitting of experimental data with Model IR-2 can also be performed while continuously determining the value of t_0 in order to determine the activation of $g(t)$. Due to the need to determine t_0 , the fitting ability of Model IR-2 is not exactly real time but rather lag by at least 2 hours.

This study thus shows that SMMIP can be used to monitor mutation in progress in terms of antibiotic resistance. The emergence of ciprofloxacin resistant mutants in

MSSA ATCC 29213 is shown by monitoring the wavelength shift and IR of MSBCip-0.5. Some mathematical models were also successfully developed to complement the SMMIP. In further studies, the models could be tested whether they indeed can serve as real time predictive models.

The results of this study have been published in the following publications:

1. Sutandar, A., Lim, C. S., and Hsu, L.Y., *System for Real-Time Monitoring of Mutation-in-Progress*. Journal of Applied Microbiology, 2008. 104(5): 1400-1407.
2. Sutandar, A. and Lim, C. S., Non-Molecular Rapid Discrimination of Genetically Mutated Bacteria in the Proceedings of the International Conference on Optics Within Life Sciences 8. 28 November – 1 December 2004. Melbourne, Australia.

Award:

Travel grant from Conference on Optics Within Life Sciences 8, 28 November – 1 December 2004, Melbourne, Australia.

Chapter 11

FUTURE WORK

The project reported in this thesis was not yet reaching its full potential. Some possible development could be considered in few areas such as the system development, mathematical model, and genetic study. Elaboration of possible further works in each area is provided below:

1. Development of SMMIP

1.1. Replenishing System (RS)

The current Replenishing System (RS) reduced the amount of bacteria present in the system to 20% of the amount before fresh sterile nutrient was fed into the sample. Therefore, there were fewer bacteria at the start of a new 24-hours interval compared to the amount of bacteria before nutrient replenishing was conducted. Fewer bacteria meant that there were fewer bacteria which might mutate. Therefore, ideally, there should not be any dilution during nutrient replenishment.

To prevent dilution, the nutrient replenishing should be conducted such that no sample was extracted at the end of the replenishing exercise. One possible method to do so is to have separate sterile nutrient and sample compartments. The separation can be provided by a membrane that allows active nutrient transfer to occur between compartments without bacterial transfer. The nutrient transfer process should ideally finish in less than 30 minutes so that the spectral reading process is not disrupted. The optimization of the nutrient transfer might be determined by performing mannitol and protein quantification tests on the sample.

1.2. Controlled Environmental Chamber (CEC)

At the time of writing (January 2007), the setting of incubation temperature was performed by burning a program written in Assembly language into the microprocessor chip. To ease the setting of the temperature, several buttons could be incorporated to allow user to adjust the temperature any time. More functions including temperature ramping and temperature variation could also be included either by direct burning of program or button pressing.

2. Mathematical model

Model Wv-2 and IR-2 presented in this study has been shown to fit the experimental result better compared to the conventional models. The models, however, could not be used to fit several types of data well. Some other functions could be used to replace the current modification and limiting functions in order to improve the data fitting.

The mathematical models could be used to fit the monitoring result in real time. The adjustment of the fitted parameters as the wavelength shift and IR data were obtained would allow a faster determination of the presence of mutants in the sample according to the trend of the parameters. A program would be written to obtain the results from the data acquisition software and then fitted

3. Genetic study

A mutation in *griB* gene is reported in this study. However, no published explanation on the function of the gene was found at the time of writing (January 2007). Therefore, a genetic study could be performed in order to understand the role of the mutation in *griB* gene. However, since there were changes of MIC during the passaging of the mutants on antibiotic-free agar, there might be other mutations in non-QRDR regions. It would be interesting to investigate which other genome regions contribute towards the observed ciprofloxacin resistance.

4. Persisters

The presence of non-lysed bacteria in MSBCip was reported. Those bacteria might be persisters. A study should be conducted to ascertain whether those bacteria were indeed persisters or not. Furthermore, it was not yet known whether persisters were the parent of the resistant subpopulations or not. Although it was known that persisters were specialized cells, how the persister cell emerged was not yet understood. No study was yet found to grow a single bacterial cell in order to monitor how persister subpopulation emerged from that single cell. Nevertheless, such a study would require quite an extensive genetic study.

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APPENDIX

APPENDIX A

Ingredients of Mannitol Salt Broth (MSB):

Phenol Red = 0.025 g

Meat extract = 1 g

Polypeptone = 10 g

D-Mannitol = 10 g

Sodium Chloride = 75 g

Deionized water = 1 L

Ingredients of Luria Bertani Broth (LB Broth):

Tryptone = 10 g

Yeast extract = 5 g

Sodium Chloride = 10 g

Deionized water = 1 L

APPENDIX B

Specifications for S2000 Spectrometer:

Detector	:	2048-element linear silicon CCD array
Detection range	:	350-1000 nm
Resolution	:	1.5 nm FWHM
Sensitivity	:	86 photons/count, 2.9×10^{-17} joule/count
S/N ratio	:	250 : 1 (at full signal)
Dark noise	:	2.5 – 4.0 (RMS)

APPENDIX C-1

Normalized wavelength shift experienced by blank MSBOxa-4

Normalized wavelength shift experienced by blank MSBOxa-6

Normalized wavelength shift experienced by blank MSBOxa-8

Normalized wavelength shift experienced by blank MSBOxa-12

Normalized wavelength shift experienced by blank broth of MSBCip-1.25

APPENDIX C-2

Spectral reading of MSB spiked with 5.0×10^4 CFU/ml of *Staphylococcus aureus*

Intensity ratio of MSB spiked with 5.0×10^4 CFU/ml of *Staphylococcus aureus*

Spectral reading of MSB spiked with 4.0×10^5 CFU/ml of *Staphylococcus aureus* Intensity ratio of MSB spiked with 4.0×10^5 CFU/ml of *Staphylococcus aureus*

Spectral reading of MSB spiked with 8.9×10^6 CFU/ml of *Staphylococcus aureus*

Intensity ratio of MSB spiked with 8.9×10^6 CFU/ml of *Staphylococcus aureus*

APPENDIX C-3

Normalized wavelength shift experienced by MSBOxa-4 spiked with MSSA ATCC 29213

Normalized intensity ratio at 600 nm experienced by MSBOxa-4 spiked with MSSA ATCC 29213

Normalized wavelength shift experienced by MSBOxa-4 spiked with MRSA ATCC 43300

Normalized intensity ratio at 600 nm experienced by MSBOxa-4 spiked with MRSA ATCC 43300

Normalized wavelength shift experienced by MSBOxa-6 spiked with MSSA ATCC 29213

Normalized intensity ratio at 600 nm experienced by MSBOxa-6 spiked with MSSA ATCC 29213

Normalized wavelength shift experienced by MSBOxa-6 spiked with MRSA ATCC 43300

Normalized intensity ratio at 600 nm experienced by MSBOxa-6 spiked with MRSA ATCC 43300

Normalized wavelength shift experienced by MSBOxa-8 spiked with MSSA ATCC 29213

Normalized intensity ratio at 600 nm experienced by MSBOxa-8 spiked with MSSA ATCC 29213

Normalized wavelength shift experienced by MSBOxa-8 spiked with MRSA ATCC 43300

Normalized intensity ratio at 600 nm experienced by MSBOxa-8 spiked with MRSA ATCC 43300

Normalized intensity ratio at 600 nm experienced by MSBOxa-12 spiked with MSSA ATCC 29213

Normalized wavelength shift experienced by MSBOxa-12 spiked with MSSA ATCC 29213

Normalized wavelength shift experienced by MSBOxa-12 spiked with MRSA ATCC 43300

Normalized intensity ratio at 600 nm experienced by MSBOxa-12 spiked with MRSA ATCC 43300

APPENDIX D

The exploded view for shaker assembly in Controlled Environment Chamber (CEC)

APPENDIX E-1

Schematic diagram for the LED control circuit

APPENDIX E-2

294

Schematic diagram for the temperature control circuit

The schematic diagram illustrates a temperature control system. It features a power supply section where a 230V AC input is transformed and rectified to a 12V DC output. This output is then regulated by an LM358C (U1) to provide a stable 5V supply. The microcontroller (AT89C51, U2) is connected to this 5V supply and ground. It controls two relays (RELAY1, RELAY2) through its P0 and P1 ports. The relays are connected to two 12V lamps (Lamp1, Lamp2). The circuit also includes a temperature sensor (DS18B20, U3) connected to the microcontroller's P1 port. The microcontroller's P0 port is connected to a 10K pull-up resistor (R1) and a 10K feedback resistor (R2). The circuit is powered by a 5V supply and ground.

File	Number	Revision
B		A
Date	9/30/2016	Sheet 1 of 1
7/4/16	© Program P0/P1 Temp. circuit 8: HD-C1	Drawn by: jayco Schuler

APPENDIX F

Spectral output for LS-1 tungsten halogen lamp:

APPENDIX G

Single letter database codes of 20 amino acids

Full Name	Abbreviation (3 letters)	Abbreviation (1 letter)
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

APPENDIX H-1

Nucleotide Multiple Sequence Alignment of *grlA* gene sections

APPENDIX H-2

Protein Multiple Sequence Alignment of Gr1A protein sections

APPENDIX H-3

Nucleotide Multiple Sequence Alignment of *grlB* gene sections

APPENDIX H-4

Protein Multiple Sequence Alignment of Gr1B protein sections

Nucleotide Multiple Sequence Alignment of *gyrA* gene sections

System for Monitoring Mutation-in-Progress	301
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APPENDIX H-6

Protein Multiple Sequence Alignment of GyrA protein sections

Nucleotide Multiple Sequence Alignment of *gyrB* gene sections

APPENDIX H-8

Protein Multiple Sequence Alignment of GyrB protein sections

APPENDIX H-9

Nucleotide Multiple Sequence Alignment of *norA* promoter gene sections

APPENDIX H-10

Protein Multiple Sequence Alignment of NorA promoter protein sections

GLOSSARY

ATCC	American Type Culture Collection
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
DNA	DeoxyriboNucleic Acid
FDA	Food and Drugs Administration
kb	kilo base pairs
MIC	Minimal Inhibitory Concentration
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
MSA	Mannitol Salt Agar
MSB	Mannitol Salt Broth
MSBCip-0.5	Mannitol Salt Broth containing 0.5 µg of ciprofloxacin per ml of broth
MSBCip-1	Mannitol Salt Broth containing 1 µg of ciprofloxacin per ml of broth
MSBOxa-4	Mannitol Salt Broth containing 4 µg of oxacillin per ml of broth
MSBOxa-6	Mannitol Salt Broth containing 6 µg of oxacillin per ml of broth
MSBOxa-8	Mannitol Salt Broth containing 8 µg of oxacillin per ml of broth
MSBOxa-12	Mannitol Salt Broth containing 12 µg of oxacillin per ml of broth
MSSA	Methicillin Susceptible <i>Staphylococcus aureus</i>
MSW	Mutant Selection Window
NCCLS	National Committee for Clinical Laboratory Standards
PCR	Polymerase Chain Reaction
RT PCR	Real-Time Polymerase Chain Reaction