INVESTIGATION OF PYRIMIDINE SALVAGE PATHWAYS TO CATEGORIZE
INDIGENOUS SOIL BACTERIA OF AGRICULTURAL AND MEDICAL
IMPORTANCE AND ANALYSIS OF THE PYRIMIDINE BIOSYNTHETIC
PATHWAY'S ENZYME PROPERTIES FOR CORRELATING CELL MORPHOLOGY
TO FUNCTION IN ALL PHASES OF GROWTH

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Dissertation Prepared for the Degree of

DOCTOR OF PHILOSOPHY

UNIVERSITY OF NORTH TEXAS

May 2003

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Meixner, Jeffery Andrew, Investigation of Pyrimidine Salvage Pathways to Categorize Indigenous Soil Bacteria of Agricultural and Medical Importance and Analysis of the Pyrimidine Biosynthetic Pathway’s Enzyme Properties for Correlating Cell Morphology to Function in All Phases of Growth. Doctor of Philosophy (Biology), May 2003, 265 pp., 6 tables, 178 illustrations, references, 56 titles.

This dissertation comprises three parts and presented in two chapters. Chapter 1 concerns Arthrobacter, a bacterium with an intriguing growth cycle. Whereas most bacteria exist as either a rod or coccus, this bacterium shares the rod/coccus lifestyle. It therefore seemed important to examine the growth regulatory pathways from the rod and coccus. The committed step, that catalyzed by aspartate transcarbamoylase (ATCase), in the pyrimidine biosynthetic pathway was chosen. The ATCase in Arthrobacter is like the well known Pseudomonas enzyme except that it has an active dihydroorotase (DHOase) associated.

Included in Chapter 1 is the description of a microorganism, Burkholderia cepacia, whose ATCase has characteristics that are at once reminiscent of bacteria, mammals, and fungi. It differs in size or aggregation based on environmental conditions. In addition, it has an active DHOase associated with the ATCase, like Arthrobacter. B. cepacia is important both medically and for bioremediation. Since B. cepacia is resistant to most antibiotics, its unique ATCase is a prime target for inhibition.

Whereas the first chapter deals with the de novo pathway to making pyrimidines, which is found mainly in the lag and log phase, Chapter 2 addresses the salvage pathway,
which comes more into play during the stationary phase. This section focuses on the isolation, identification, and grouping of a number of natural soil bacteria from various soil locations. These organisms are important agriculturally, medically, and industrially. Addition of these soil isolates to poor soils has been found to improve the soil. In a previous study by D.A. Beck, the salvage schemes for a number of laboratory strains of microorganisms were determined. Nine separate classes of salvage were designated by determining the salvage enzymes present. In this study emphasis has been placed on soil bacteria, which had not previously been analyzed. A number of species of soil bacteria were identified using the MIDI. The salvage enzymes were then determined for these organisms and a comparison of these isolates to the previous study was performed in order to group the new organisms into 19 salvage schemes, that is 10 more than in the previous study.
ACKNOWLEDGMENTS

I would like to thank my friends and family who have supported me over the years. I was inspired by my family, as my father worked his way through his Ph.D. program at Iowa State University. I would like to thank Linsey for all of her help and support during the research and preparation of this dissertation. Also, to Dr. Gerard O'Donovan for accepting me into his laboratory, pointing me in the correct direction and being a friend and mentor to me. Last of all I would like to thank Orotate for always being a friend.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS ................................................................. iii

LIST OF TABLES ........................................................................... v

LIST OF ILLUSTRATIONS .......................................................... vi

Chapter I

1. INTRODUCTION ............................................................... 1

2. MATERIALS AND METHODS .............................................. 28

3. RESULTS ............................................................................. 37

4. DISCUSSION ........................................................................ 52

Chapter II

1. INTRODUCTION ............................................................... 62

2. MATERIALS AND METHODS .............................................. 93

3. RESULTS ............................................................................. 102

4. DISCUSSION ........................................................................ 238

APPENDICES ............................................................................ 249

REFERENCES ........................................................................... 262
LIST OF TABLES

TABLE                                       Page

1. Organisms assorted by salvage groups from earlier study. ...................... 103
2. Organisms assorted by salvage groups from this study........................... 104
3. Pyrimidine salvage enzymes present in each salvage group. .................... 105
4. Results of enzyme assays for each microorganism................................. 106
5. Results of enzyme assays for additional microorganisms....................... 107
6. List of bacterial strains used in this study............................................ 250
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>6</td>
</tr>
<tr>
<td>Electron micrograph of <em>Arthrobacter globiformis</em>.</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>7</td>
</tr>
<tr>
<td>Chromophore production in stationary phase <em>Arthrobacter globiformis</em>.</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>14</td>
</tr>
<tr>
<td>Pyrimidine biosynthetic and salvage pathways in <em>Escherichia coli</em>.</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>15</td>
</tr>
<tr>
<td>Pyrimidine biosynthetic (<em>de novo</em>) pathway.</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>21</td>
</tr>
<tr>
<td>Oxidative pathway of the breakdown of uracil.</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>22</td>
</tr>
<tr>
<td>Reductive pathway of the breakdown of uracil.</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>24</td>
</tr>
<tr>
<td>Classes of the ATCase enzyme.</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>38</td>
</tr>
<tr>
<td>Native gradient PAGE of <em>Arthrobacter globiformis</em> cells in lag and log phase.</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>39</td>
</tr>
<tr>
<td><em>Arthrobacter globiformis</em> growth cycle.</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>40</td>
</tr>
<tr>
<td>A Michaelis-Menten graph illustrating the kinetics of the ATCase enzyme from lag phase of <em>Arthrobacter globiformis</em>.</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>41</td>
</tr>
<tr>
<td>A Lineweaver-Burk graph illustrating the kinetics of the ATCase enzyme from lag phase of <em>Arthrobacter globiformis</em>.</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>42</td>
</tr>
<tr>
<td>A Michaelis-Menten graph illustrating the kinetics of the ATCase enzyme from early log phase of <em>Arthrobacter globiformis</em>.</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>43</td>
</tr>
<tr>
<td>A Lineweaver-Burk graph illustrating the kinetics of the ATCase enzyme from early log phase of <em>Arthrobacter globiformis</em>.</td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>44</td>
</tr>
<tr>
<td>A Michaelis-Menten graph illustrating the kinetics of the ATCase enzyme from middle log phase of <em>Arthrobacter globiformis</em>.</td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>45</td>
</tr>
<tr>
<td>A Lineweaver-Burk graph illustrating the kinetics of the ATCase enzyme from middle log phase of <em>Arthrobacter globiformis</em>.</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>46</td>
</tr>
<tr>
<td>A Michaelis-Menten graph illustrating the kinetics of the ATCase enzyme from late log phase of <em>Arthrobacter globiformis</em>.</td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td>47</td>
</tr>
<tr>
<td>A Lineweaver-Burk graph illustrating the kinetics of the ATCase enzyme from late log phase of <em>Arthrobacter globiformis</em>.</td>
<td></td>
</tr>
</tbody>
</table>
18. A Michaelis-Menten graph illustrating the kinetics of the ATCase enzyme from stationary phase of *Arthrobacter globiformis* ..........48

19. A Lineweaver-Burk graph illustrating the kinetics of the ATCase enzyme from stationary phase of *Arthrobacter globiformis* ..........49

20. $K_M$ at various stages of the growth cycle of *Arthrobacter globiformis* ....50

21. Thermostability of ATCase from various points in the growth cycle. .....51

22. *Arthrobacter* cellular morphology at four stages of the growth cycle. .....57

23. Computer analysis and identification resulting from the gas chromatogram for *Acinetobacter lwoffii* ........................................67

24. Electron micrograph of *Aeromonas* ........................................68

25. Computer analysis and identification resulting from the gas chromatogram for *Aeromonas hydrophila* ........................................69

26. Computer analysis and identification resulting from the gas chromatogram for *Aeromonas sobria* ........................................70

27. Computer analysis and identification resulting from the gas chromatogram for *Aeromonas trota* ........................................71

28. Electron micrograph of *Agrobacterium* ........................................73

29. Computer analysis and identification resulting from the gas chromatogram for *Agrobacterium radiobacter* ........................................73

30. Electron micrograph of *Bacillus cereus* ........................................76

31. Computer analysis and identification resulting from the gas chromatogram for *Bacillus cereus* ........................................76

32. Computer analysis and identification resulting from the gas chromatogram for *Bacillus marinus* ........................................77

33. Electron micrograph of *Bacillus subtilis* ........................................78

34. Computer analysis and identification resulting from the gas chromatogram for *Bacillus subtilis* ........................................78

35. Computer analysis and identification resulting from the gas
chromatogram for *Brevibacillus agri*. .................................................. 79

36. Electron micrograph of *Brevibacterium linens*. ........................................... 80

37. Computer analysis and identification resulting from the gas chromatogram for *Brevibacterium iodinum*.......................................................... 81

38. Electron micrograph of *Deinococcus*. .......................................................... 82

39. Computer analysis and identification resulting from the gas chromatogram for *Deinococcus erythromyxa*.............................................................. 82

40. Electron micrograph of *Flavobacterium psychrophilum*. ............................... 83

41. Computer analysis and identification resulting from the gas chromatogram for *Flavobacterium johnsoniae*............................................................. 84

42. Gram stain of *Klebsiella pneumoniae*. .......................................................... 85

43. Computer analysis and identification resulting from the gas chromatogram for *Microbacterium saperdae*. .............................................................. 87

44. Computer analysis and identification resulting from the gas chromatogram for *Microbacterium liquefaciens*............................................................. 87

45. Gas chromatogram for *Paenibacillus macerans*. ........................................... 89

46. Electron micrograph of *Rhodococcus*. .......................................................... 90

47. Computer analysis and identification resulting from the gas chromatogram for *Rhodococcus luteus*................................................................. 91

48. Electron micrograph of *Shigella*................................................................. 92

49. HPLC chromatogram of a mixture of the standards cytosine, uracil, cytidine, and uridine................................................................. 108

50. Pyrimidine salvage pathway of Group I. ................................................. 109

51. HPLC chromatograms of *Flavobacterium johnsoniae* cytidine deaminase and cytidine hydrolase assays......................................................... 110

52. HPLC chromatograms of *Flavobacterium johnsoniae* cytidine kinase and cytosine deaminase assays......................................................... 111

53. HPLC chromatograms of *Flavobacterium johnsoniae*
orotate decarboxylase and uridine hydrolase assays. ......................... 112

54. HPLC chromatograms of *Flavobacterium johnsoniae*
    uridine kinase and uridine phosphorylase assays. ................................ 113

55. HPLC chromatograms of *Flavobacterium johnsoniae*
cytidine phosphorylase assay and filtered cell extract
    (control) assays. ........................................................................... 114

56. HPLC chromatograms of *Flavobacterium johnsoniae*
    CMP glycosylase and 5′-nucleotidase assays. ........................................ 115

57. HPLC chromatograms of *Aeromonas trota*
cytidine deaminase and cytidine hydrolase assays. .......................... 116

58. HPLC chromatograms of *Aeromonas trota*
cytidine kinase and cytosine deaminase assays. ............................ 117

59. HPLC chromatograms of *Aeromonas trota*
orotate decarboxylase and uridine hydrolase assays. .................... 118

60. HPLC chromatograms of *Aeromonas trota*
    uridine kinase and uridine phosphorylase assays. ....................... 119

61. HPLC chromatograms of *Aeromonas trota*
cytidine phosphorylase assay and filtered cell extract
    (control) assays. ........................................................................... 120

62. HPLC chromatograms of *Aeromonas trota*
    CMP glycosylase and 5′-nucleotidase assays. ........................................ 121

63. Pyrimidine salvage pathway of Group II. ........................................ 122

64. Pyrimidine salvage pathway of Group III.................................... 123

65. Pyrimidine salvage pathway of Group IV. .................................... 124

66. Pyrimidine salvage pathway of Group V.................................... 125

67. Pyrimidine salvage pathway of Group VI. .................................... 126

68. Pyrimidine salvage pathway of Group VII. .................................... 127

69. Pyrimidine salvage pathway of Group VIII. ............................... 128

70. Pyrimidine salvage pathway of Group IX. .................................... 129
71. Pyrimidine salvage pathway of Group X ........................................ 130

72. HPLC chromatograms of Microbacterium saperdae cytidine deaminase and cytidine hydrolase assays ...................... 131

73. HPLC chromatograms of *Microbacterium saperdae* cytidine kinase and cytosine deaminase assays ................................. 132

74. HPLC chromatograms of *Microbacterium saperdae* orotate decarboxylase and uridine hydrolase assays ...................... 133

75. HPLC chromatograms of *Microbacterium saperdae* uridine kinase and uridine phosphorylase assays ............................ 134

76. HPLC chromatograms of *Microbacterium saperdae* cytidine phosphorylase and filtered cell extract (control) assays .................. 135

77. HPLC chromatograms of *Microbacterium saperdae* CMP glycosylase and 5’-nucleotidase assays ................................. 136

78. Pyrimidine salvage pathway of Group XI .................................. 137

79. HPLC chromatograms of Klebsiella oxytoca cytidine deaminase and cytidine hydrolase assays ................................. 138

80. HPLC chromatograms of Klebsiella oxytoca cytidine kinase and cytosine deaminase assays ................................. 139

81. HPLC chromatograms of Klebsiella oxytoca orotate decarboxylase and uridine hydrolase assays ...................... 140

82. HPLC chromatograms of Klebsiella oxytoca uridine kinase and uridine phosphorylase assays ............................ 141

83. HPLC chromatograms of Klebsiella oxytoca cytidine phosphorylase assay and filtered cell extract (control) assays ................................. 142

84. HPLC chromatograms of Klebsiella oxytoca CMP glycosylase and 5’-nucleotidase assays ................................. 143

85. Pyrimidine salvage pathway of Group XII .................................. 144

86. HPLC chromatograms of Bacillus marinus
cytidine deaminase and cytidine hydrolase assays..............................145

87. HPLC chromatograms of Bacillus marinus
cytidine kinase and cytosine deaminase assays..............................146

88. HPLC chromatograms of Bacillus marinus
orotate decarboxylase and uridine hydrolase assays......................147

89. HPLC chromatograms of Bacillus marinus
uridine kinase and uridine phosphorylase assays..............................148

90. HPLC chromatograms of Bacillus marinus
cytidine phosphorylase assay and filtered cell extract
(control) assays. ................................................................................149

91. HPLC chromatograms of Bacillus marinus
CMP glycosylase and 5’-nucleotidase assays..............................150

92. Pyrimidine salvage pathway of Group XIII..............................151

93. HPLC chromatograms of Brevibacillus agri
cytidine deaminase and cytidine hydrolase assays..............................152

94. HPLC chromatograms of Brevibacillus agri
cytidine kinase and cytosine deaminase assays..............................153

95. HPLC chromatograms of Brevibacillus agri
orotate decarboxylase and uridine hydrolase assays......................154

96. HPLC chromatograms of Brevibacillus agri
uridine kinase and uridine phosphorylase assays..............................155

97. HPLC chromatograms of Brevibacillus agri
cytidine phosphorylase assay and filtered cell extract
(control) assays. ................................................................................156

98. HPLC chromatograms of Brevibacillus agri
CMP glycosylase and 5’-nucleotidase assays..............................157

99. Pyrimidine salvage pathway of Group XIV..............................158

100. HPLC chromatograms of Morganella morganii
cytidine deaminase and cytidine hydrolase assays..............................159

101. HPLC chromatograms of Morganella morganii
cytidine kinase and cytosine deaminase assays..............................160
102. HPLC chromatograms of *Morganella morganii* orotate decarboxylase and uridine hydrolase assays. .......................... 161
103. HPLC chromatograms of *Morganella morganii* uridine kinase and uridine phosphorylase assays. .............................. 162
104. HPLC chromatograms of *Morganella morganii* cytidine phosphorylase and filtered cell extract (control) assays. ................................................................. 163
105. HPLC chromatograms of *Morganella morganii* CMP glycosylase and 5’-nucleotidase assays. ............................. 164
106. Pyrimidine salvage pathway of Group XV. .................................................. 165
107. HPLC chromatograms of *Brevibacterium iodinum* cytidine deaminase and cytidine hydrolase assays ......................... 166
108. HPLC chromatograms of *Brevibacterium iodinum* cytidine kinase and cytosine deaminase assays ............................. 167
109. HPLC chromatograms of *Brevibacterium iodinum* orotate decarboxylase and uridine hydrolase assays. ...................... 168
110. HPLC chromatograms of *Brevibacterium iodinum* uridine kinase and uridine phosphorylase assays. .............. 169
111. HPLC chromatograms of *Brevibacterium iodinum* cytidine phosphorylase and filtered cell extract (control) assays. ................................................................. 170
112. HPLC chromatograms of *Brevibacterium iodinum* CMP glycosylase and 5’-nucleotidase assays. ............................. 171
113. Pyrimidine salvage pathway of Group XVI .................................................. 172
114. HPLC chromatograms of *Microbacterium liquefaciens* cytidine deaminase and cytidine hydrolase assays ........................... 173
115. HPLC chromatograms of *Microbacterium liquefaciens* cytidine kinase and cytosine deaminase assays ......................... 174
116. HPLC chromatograms of *Microbacterium liquefaciens* orotate decarboxylase and uridine hydrolase assays. .................. 175
117. HPLC chromatograms of *Microbacterium liquefaciens* uridine kinase and uridine phosphorylase assays .........................176

118. HPLC chromatograms of *Microbacterium liquefaciens* cytidine phosphorylase and filtered cell extract (control) assays. .................................................................177

119. HPLC chromatograms of *Microbacterium liquefaciens* CMP glycosylase and 5’-nucleotidase assays ..............178

120. Pyrimidine salvage pathway of Group XVII .....................179

121. HPLC chromatograms of *Aeromonas sobria* cytidine deaminase and cytidine hydrolase assays ..................180

122. HPLC chromatograms of *Aeromonas sobria* cytidine kinase and cytosine deaminase assays ..................181

123. HPLC chromatograms of *Aeromonas sobria* orotate decarboxylase and uridine hydrolase assays ............182

124. HPLC chromatograms of *Aeromonas sobria* uridine kinase and uridine phosphorylase assays ..................183

125. HPLC chromatograms of *Aeromonas sobria* cytidine phosphorylase and filtered cell extract (control) assays. .................................................................184

126. HPLC chromatograms of *Aeromonas sobria* CMP glycosylase and 5’-nucleotidase assays ......................185

127. Pyrimidine salvage pathway of Group XVIII ....................186

128. HPLC chromatograms of *Bacillus cereus* cytidine deaminase and cytidine hydrolase assays ..................187

129. HPLC chromatograms of *Bacillus cereus* cytidine kinase and cytosine deaminase assays ..................188

130. HPLC chromatograms of *Bacillus cereus* orotate decarboxylase and uridine hydrolase assays ............189

131. HPLC chromatograms of *Bacillus cereus* uridine kinase and uridine phosphorylase assays ..................190
132. HPLC chromatograms of *Bacillus cereus* cytidine phosphorylase and filtered cell extract (control) assays ............................................................... 191

133. HPLC chromatograms of *Bacillus cereus* CMP glycosylase and 5’-nucleotidase assays ......................................................... 192

134. Pyrimidine salvage pathway of Group XIX ...................................... 193

135. HPLC chromatograms of *Acinetobacter lwoffii* cytidine deaminase and cytidine hydrolase assays ........................................... 194

136. HPLC chromatograms of *Acinetobacter lwoffii* cytidine kinase and cytosine deaminase assays ........................................... 195

137. HPLC chromatograms of *Acinetobacter lwoffii* orotate decarboxylase and uridine hydrolase assays ........................................ 196

138. HPLC chromatograms of *Acinetobacter lwoffii* uridine kinase and uridine phosphorylase assays ........................................ 197

139. HPLC chromatograms of *Acinetobacter lwoffii* cytidine phosphorylase and filtered cell extract (control) assays ................................. 198

140. HPLC chromatograms of *Acinetobacter lwoffii* CMP glycosylase and 5’-nucleotidase assays ......................................................... 199

141. HPLC chromatograms of *Klebsiella pneumoniae* cytidine deaminase and cytidine hydrolase assays ........................................... 200

142. HPLC chromatograms of *Klebsiella pneumoniae* cytidine kinase and cytosine deaminase assays ........................................... 201

143. HPLC chromatograms of *Klebsiella pneumoniae* orotate decarboxylase and uridine hydrolase assays ........................................ 202

144. HPLC chromatograms of *Klebsiella pneumoniae* uridine kinase and filtered cell extract (control) assays........................................ 203

145. HPLC chromatograms of *Deinococcus erythromyxa* cytidine deaminase and cytidine hydrolase assays ........................................... 204

146. HPLC chromatograms of *Deinococcus erythromyxa* cytidine kinase and cytosine deaminase assays ........................................... 205
147. HPLC chromatograms of Deinococcus erythromyxa
   orotate decarboxylase and uridine hydrolase assays. .......................206

148. HPLC chromatograms of Deinococcus erythromyxa
   uridine kinase and filtered cell extract (control) assays................207

149. HPLC chromatograms of Rhodococcus luteus
   cytidine deaminase and cytidine hydrolase assays. ........................208

150. HPLC chromatograms of Rhodococcus luteus
   cytidine kinase and cytosine deaminase assays.............................209

151. HPLC chromatograms of Rhodococcus luteus
   orotate decarboxylase and uridine hydrolase assays. ......................210

152. HPLC chromatograms of Rhodococcus luteus
   uridine kinase and filtered cell extract (control) assays..............211

153. HPLC chromatograms of Bacillus subtilis
   cytidine deaminase and cytidine hydrolase assays. ........................212

154. HPLC chromatograms of Bacillus subtilis
   cytidine kinase and cytosine deaminase assays.............................213

155. HPLC chromatograms of Bacillus subtilis
   orotate decarboxylase and uridine hydrolase assays. ......................214

156. HPLC chromatograms of Bacillus subtilis
   uridine kinase and filtered cell extract (control) assays..............215

157. HPLC chromatograms of Paenibacillus macerans
   cytidine deaminase and cytidine hydrolase assays........................216

158. HPLC chromatograms of Paenibacillus macerans
   cytidine kinase and cytosine deaminase assays.............................217

159. HPLC chromatograms of Paenibacillus macerans
   orotate decarboxylase and cytidine phosphorylase assays...............218

160. HPLC chromatogram of Paenibacillus macerans
    filtered cell extract (control) assay. ........................................219

161. HPLC chromatograms of Aeromonas hydrophila
    cytidine deaminase and cytidine hydrolase assays........................220

162. HPLC chromatograms of Aeromonas hydrophila
cytidine kinase and cytosine deaminase assays..........................221

163. HPLC chromatograms of *Aeromonas hydrophila*
orotate decarboxylase and uridine hydrolase assays..................222

164. HPLC chromatograms of *Aeromonas hydrophila*
uridine kinase and filtered cell extract (control) assays..........223

165. HPLC chromatograms of *Shigella sonnei*
cytidine deaminase and cytidine hydrolase assays...............224

166. HPLC chromatograms of *Shigella sonnei*
cytidine kinase and cytosine deaminase assays.......................225

167. HPLC chromatograms of *Shigella sonnei*
orotate decarboxylase and uridine hydrolase assays...........226

168. HPLC chromatograms of *Shigella sonnei*
uridine kinase and filtered cell extract (control) assays........227

169. HPLC chromatograms of *Arthrobacter globiformis*
cytidine deaminase and cytidine hydrolase assays.............228

170. HPLC chromatograms of *Arthrobacter globiformis*
cytidine kinase and cytosine deaminase assays....................229

171. HPLC chromatograms of *Arthrobacter globiformis*
orotate decarboxylase and uridine hydrolase assays........230

172. HPLC chromatograms of *Arthrobacter globiformis*
uridine kinase and uridine phosphorylase assays...............231

173. HPLC chromatograms of *Agrobacterium radiobacter*
cytidine deaminase and cytidine hydrolase assays...............232

174. HPLC chromatograms of *Agrobacterium radiobacter*
cytidine kinase and cytosine deaminase assays...............233

175. HPLC chromatograms of *Agrobacterium radiobacter*
orotate decarboxylase and uridine hydrolase assays...........234

176. HPLC chromatograms of *Agrobacterium radiobacter*
uridine kinase and uridine phosphorylase assays...............235

177. HPLC chromatograms of *Agrobacterium radiobacter*
cytidine phosphorylase and filtered cell extract (control) assays......236

178. HPLC chromatograms of Agrobacterium radiobacter CMP glycosylase and 5'-nucleotidase assays. ........................................237
CHAPTER I

Whatever our accomplishments, our sophistication, our artistic pretension, we owe our very existence to a six-inch layer of topsoil-and the fact that it rains.

Anonymous, *The Cockle Bur*

INTRODUCTION

This six inch layer of top soil is where the majority of life-maintaining food springs from. Without soil microorganisms, this would not be possible. The soil is a complex society, resembling a “battle field”, containing Gram positive and Gram negative bacteria, archaebacteria, organisms without cell walls and eukaryotes. These organisms must coexist in a very stressful, limiting, ever-changing environment. These minimalist survivors cast a wanting eye on the feast and famine lifestyle of the coliforms. What is a famine for a coliform would be considered a feast in the soil. Soil bacteria contend with a variety of environmental conditions, such as fluctuations in temperature, varying concentration of nutrients, pH, osmolarity, and soil moisture. The introduction of toxic compounds of both synthetic and natural origins to the soil poses as an additional threat. As one author states, “Any one biological system is… a reflection of the other biological systems functioning in association or in opposition and of the physical and chemical characteristics of the environment” (Alexander, 1961). The conditions in a gram of soil can be vastly different from the environment in a gram of soil a foot away.

The first question that comes to mind is: how do they do it? Adaptation throughout time has bestowed these soil bacteria with creative ways to beat the odds and prevail. For example, organisms such as *Bacillus* produce hearty endospores that can endure for hundreds, if not thousands, of years. Even *Arthrobacter*, one of the most
common soil microorganisms, produces “arthrospores” (coci) presumably for survival purposes. In addition, the ability to exist for long periods of time in stationary phase increases the chance for survival.

One type of adaptation with perhaps the most profound ecological relevance is how bacteria have developed symbiotic and commensal relationships between different species and individual cells. An analogy to help describe the magnitude of a symbiotic relationship is following the destiny of a cow in a slaughter house. The body of a single cow ultimately provides a number of products such as leather, meat, milk, airplane de-icer, hydraulic fluid to name a few. Just as each worker in the slaughter house removes a needed part, the products of decomposition of compounds by one organism are often a required substance for a symbiont. For example, one microorganism will perform the initial cleavage of a ring which will be further degraded by the next microorganism. In the microbial world, growth factors synthesized by certain organisms allow for the establishment of nutritionally diverse soil inhabitants. In addition, microbial decomposition of metabolic products that are inhibitory to the growth of other soil residents also exemplifies a beneficial relationship. This is seen when one bacterium living close to an antibiotic producing microorganism excretes an enzyme, such as â-lactamase, to break down the antibiotic, thus sparing other bacteria in the region. Another example is aerobic microorganisms allowing for the proliferation of obligate anaerobes by consuming the oxygen in the environment. As can be seen, soil microorganisms interact in a way that can not be re-enacted with pure cultures in a laboratory (Alexander, 1961). This is the reason only about one percent of the bacteria in a gram of soil can be cultured in a laboratory (A.J. McCarthy, personal
communication). The populace of soil microorganisms in any habitat is directed by the biological equilibrium established through relationships and interactions of all individuals of the populace (Alexander, 1961). This concept applies to life in general, where:

To survive and flourish in the long run, we usually need to develop mutualistic relationships. Nonmutualistic members will eventually disappear or lose influence in the community.

J.M. Trappe

Two Coryneforms: Arthrobacter and Brevibacterium

Arthrobacter is a genus of Gram positive, high G + C bacteria (59-66% Tm) with lysine in its peptidoglycan (Sneath et al., 1986). Initially classified as Bacterium globiforme by Conn (1928), this genus was almost placed into Corynebacterium by Jensen (1934). Ultimately the genus Arthrobacter was created by Conn (1947) and A. globiformis was named the type species. The name Arthrobacter is derived from the Greek noun arthrus, meaning a joint, and the Latin bacter, meaning a rod. Globiformis is from the Latin noun globis, meaning globe or ball and the word forma means shape. The literal translation of this would be a jointed rod in the shape of a ball, which becomes quite clear when the cellular morphology throughout the growth cycle is considered. It is a member of the Order Actinomycetales and Family Arthrobacteriaceae. The order Actinomycetales contains many important soil microorganisms, such as Streptomyces. Streptomyces produces geosmin, which gives soil its earthy odor as well as many antibiotics. Also included in Actinomycetales is Corynebacterium (one species is the causative agent of diphtheria), Micrococcus, Mycobacterium (two species cause leprosy and tuberculosis), Nocardia, and Propionibacterium (produces propionic acid, acetic
acid, and is implicated in acne) to name a few (Sneath *et al*., 1986). *Mycobacterium* can be used to produce methane from the breakdown of aliphatic hydrocarbons as simple as ethane to complex paraffins. *Micrococcus* breaks down urea to CO$_2$ and NH$_3$ and can reduce nitrate to molecular N$_2$. *Nocardia* converts oximes of organic acids to nitrite, a compound used by plants (Alexander, 1961).

*Arthrobacter* is a catalase-positive obligate aerobe that undergoes respiratory metabolism, never fermentation. It is nonmotile, or occasionally motile by one sub-polar flagellum. Its’ resistance to desiccation (Boylen, 1973) and starvation (Boylen and Ensign, 1970) along with its nutritional versatility (Hagedorn and Holt, 1975a) allows *Arthrobacter* to be the most common genus in the majority of soils (Hagedorn and Holt, 1975b). It comprises 30-60% of the bacterial flora, with *Bacillus* being the prevailing soil bacterium in fallow soil. In the presence of wheat roots, the number of *Arthrobacter* drops and the number of pseudomonads, also ubiquitous in soil, increases. *Arthrobacter* produces a cytokinin-like growth regulator which induces an increase in the root hairs in colza seedlings (Sneath *et al*., 1986). Some strains of *Arthrobacter* produce chitinase and are able to lyse yeast cells as well as control *Fusarium roseum*, a fungus that infects carnation roots (Szajer and Koths, 1973). *Arthrobacter* is a prodigious degrader of recalcitrant compounds. This ability to degrade toxic compounds, such as polychlorinated biphenols (PCB) and polychlorinated phenol (PCP), makes the organism commercially important. Both PCB and PCP are toxic carcinogens that are the result of extensive herbicidal use. *Arthrobacter* also has medical importance as it causes rare human infections.
The most remarkable characteristic of *Arthrobacter* is its unusual growth cycle. Typically, most bacteria are either rods or cocci in nature. Not so for *A. globiformis* cells, which grow out from short rods and cocci in lag phase to long, slender rods, including some V-shaped rods, in log phase (Sneath *et al.*, 1986). The rod to coccus growth cycle is very uncommon, being seen in *Brevibacterium, Pimelobacter* and some *Rhodococcus*. In addition, there are a few genera such as *Microbacterium* that come very close to the rod to coccus growth cycle (Sneath *et al.*, 1986). The V-shaped rods are produced by snap division. Snap division occurs in bacteria with two-layered cell walls. A transverse wall, or septum, that divides the two daughter cells is produced by growth of the inner layer. The septum puts tension on the outer wall layer, which then ruptures asymmetrically in a snapping movement. The remaining portion of the outer layer acts as a hinge to hold the two daughter cells at an angle to one another (Prescott, Harley and Klein 2002). The log stage for *Arthrobacter* is brief, but the stationary phase is indefinite and the cells exhibit a return to the more stable coccus form, analogous to the Y-M shift seen in pathogenic fungi. These coccoid forms have been named “arthrospores” and “cystites.” Arthrospores, although not endospores, have properties outside the realm of a vegetative cell (Sneath *et al.*, 1986). *Arthrobacter* may be thought to segue between sporeformers and non-sporeformers. Figure 1 is an electron micrograph of *A. globiformis* provided by Hani Teal. Note the cocci, pleomorphic rods and V-shaped rods.
A. globiformis is capable of sustained growth at temperatures as low as -5°C. When exposed to cold temperatures, A. globiformis synthesizes new peptides and increases the synthesis of some existing peptides. These new peptides may act as anti-freeze enzymes, a concept not unique to A. globiformis, but the peptide synthesis continues longer than in other organisms. This ability to withstand cold temperatures most likely contributes to its abundance in soils (Berger et al., 1996).

A culture of A. globiformis is white when grown in Arthrobacter minimal medium until it reaches stationary phase. In stationary phase, A. globiformis produces a chromophore which turns the medium a peach color, as shown in Figure 2.

Figure 1: Electron micrograph of Arthrobacter globiformis
Figure 2: Chromophore production in stationary phase *Arthrobacter globiformis*

*Brevibacterium*, another corynebacterium, is a nonmotile, Gram positive, obligate aerobe that has a high G+C content, typically falling between 60-64% ($T_m$). *B. linens* is found in many types of cheese and is a vital part of the aging of some types. This organism, and other species of *Brevibacterium*, are capable of surviving in as much as 15% NaCl, and in some cases the addition of NaCl stimulates growth. Light is commonly required for pigment production in bacteria, however, in most strains of *B. linens*, there is an orange, carotenoid pigment that is clearly visible in isolated colonies on nutrient agar plates grown in the dark (Sneath *et al.*, 1986). The pyrimidine biosynthetic pathway has until now not been investigated in an organism with this type of growth cycle.

*Burkholderia cepacia*

*Burkholderia cepacia*, originally isolated from infected onions (Burkholder, 1950), is a common soil microorganism that has shown great potential as a biotechnology agent for use in bioremediation (Holmes *et al.*, 1998). This is mainly due to the organism’s wide metabolic range, especially the ability to degrade complex hydrocarbons...
and aromatic compounds. *Burkholderia* is named after W.H. Burkholder, and *cepacia* is from the Latin word *cepa* meaning of or like an onion. Its ability to suppress the growth of other bacterial species also makes it a candidate for a biological control agent in agriculture (Holmes *et al*., 1998). On the other hand, *B. cepacia* is a significant pathogen in cystic fibrosis (CF) patients. It is also a problem in patients with chronic granulomatous disease and in those requiring mechanical ventilation (Govan *et al*., 1996). The organism is capable of causing a fatal respiratory pneumonia, and in CF patients this is highly communicable, resulting in a 50% decrease in patient survival (Pujol *et al*., 1992; Govan & Vandamme, 1998). *B. cepacia*’s bioremediation abilities, unfortunately, give it the capability to break down most antibiotics. Because of both the potential technological benefits of studying *B. cepacia* and its medical significance, the genomes of many different species of *Burkholderia*, including *B. cepacia*, are currently being sequenced. A lack of information about the pyrimidine biosynthetic pathway in this organism has led to this investigation of the enzyme aspartate transcarbamoylase (ATCase), as this is the committed step in the pyrimidine pathway in prokaryotes.

**Additional Useful Soil Bacteria**

The majority of bacteria used in this study serve functions that make soil such a valuable and life-sustaining substance. For instance, soil bacteria with the ability to fix nitrogen sustain balance in the nitrogen cycle (Alexander, 1961). Nitrogen fixation is a process in which atmospheric nitrogen (N\(_2\)) is converted into ammonia (NH\(_4^+\)). Ammonia can then be converted by other soil microorganisms into nitrite and nitrate, two compounds utilized by plants. Even with expansion of the use of nitrogenous fertilizer, microbes are still required in order to convert components of the fertilizer into usable
forms. Organisms in the soil, such as Azotobacter, also fix nitrogen but only in low ammonia concentrations. Indeed the use of chemical fertilizers creates an ironic situation in which the steady use of fertilizers becomes vital in order to continue returning nitrogen to the soil. The addition of these fertilizers influences the indigenous microorganisms to stop fixing nitrogen from the atmosphere. The result is the need to add more nitrogen to the soil than if no fertilizer had been utilized in the first place. In other words, “…when a soil loses fertility we pour on fertilizer, or at best alter its tame flora and fauna, without considering the fact that its wild flora and fauna, which built the soil to begin with, may likewise be important to its maintenance” (Aldo Leopold). Another soil microorganism which fixes nitrogen but is traditionally thought of as a pathogen is Klebsiella pneumoniae, a diazotroph that inhabits the rhizosphere.

Bioremediation is another example of a positive attribute of some soil microorganisms. Pseudomonas putida, for example, has the ability to break down toluene and P. fluorescens can decompose cyanide. P. aeruginosa has a surfactant glycolipid emulsifier (EM) that improves the removal of crude oil from solid surfaces (Prescott, Harley and Klein 1990). P. aeruginosa is resistant to high levels of heavy metal pollutants, so much so that in some polluted areas, the pollutants act as a selective agent for this potential pathogenic organism (Dirk van Elsas, 1997). Flavobacterium is able to decompose biphenols and Rhodococcus is also known for its ability to break down lignin-related compounds and humic acid. In addition, some rhodococci can degrade cyanide-substituted compounds, such as the acrylamide monomer, and camphor (Sneath et al., 1986).
Several Actinomycetes are useful for the degradation of recalcitrant herbicides. For instance, *Pseudomonas* breaks down 3-(p-chlorophenol)-1,1-dimethylurea (Monuron) and trichloroacetic acid (TCA). Monuron persists in the soil for approximately 4-12 months, while TCA remains 2-9 weeks. *Flavobacterium* degrades 4-chlorophenoxyacetic acid (4-CPA). Finally, both *Corynebacterium* and *Flavobacterium* break down 2,4-dichlorophenoxyacetic acid (2,4-D), a herbicide that takes two to eight weeks to degrade in the soil (Alexander, 1961).

*Acinetobacter* is a common soil bacterium which readily takes up DNA and can act as a “phosphate wrangler” by removing high levels of phosphate from contaminated bodies of water. In aerobic situations, *Acinetobacter* can bioaccumulate large amounts of phosphorus, in the form of granules of up to 30% of the dry weight of the cell. This phosphate can then be released in a lower volume by growing the *Acinetobacter* anaerobically (Ingraham & Ingraham, 2000).

*Alcaligenes eutrophus* is a hydrogen-oxidizing soil bacterium that stores carbon as a type of plastic, called polyester, in much the same way we store carbon as glycogen. The name of this plastic is polyhydroxyalkanoate (PHA). *A. eutrophus* is also able to produce an extracellular enzyme, a depolymerase, capable of degrading PHA into â-hydroxy acid. This compound can then be utilized by other microorganisms. Due to obvious reasons, this plastic is now being used for commercial purposes. One advantage of such a plastic is a decreased number of pollutants created as byproducts in contrast to normal plastic production. In addition, the conversion of man-made to microorganism-made products will bring about a significant reduction in land fill utilization due to the easily remediated new plastics (Ingraham & Ingraham, 2000).
One relatively new application with some microorganisms is their use as probiotics. *Bacillus subtilis* has been used as a probiotic in chicken feed because it causes an increase in the speed of weight gain, while decreasing the number of coliforms, such as the poultry borne pathogen *Campylobacter jejuni*. *Lactobacillus* spp. are being used in humans to mitigate the effects of *Helicobacter pylori*, which is implicated in a number of health problems such as stomach ulcers and migraines.

Not surprisingly, many of the soil organisms possess other intriguing qualities: *Deinococcus erythromyxa* is resistant to radiation due to its ability to survive desiccation. *Bacillus marinus* is found in marine sediments. *B. cereus* produces proteolytic enzymes that lyse bacterial cells. *P. fluorescens* fluoresces under UV radiation. Also, *Flavobacterium* selectively stimulates root exudates (Sneath *et al.*, 1986).

**Importance of Pyrimidines**

Organic chemistry is the chemistry of carbon compounds. Biochemistry is the study of carbon compounds that crawl.

Anonymous

In order for organisms to compete and survive in nature, the pyrimidine bases uracil, thymine, and cytosine must be synthesized by the biosynthetic (*de novo*) pathway and recycled by salvage. These pyrimidine bases are the precursors of DNA and RNA. However, pyrimidines are required for numerous other activities. The balance of these precursors is maintained by a combination of enzyme inhibition/activation and gene repression/derepression of the pyrimidine biosynthetic and salvage pathways. The pyrimidine salvage pathway is the other pathway working alongside the biosynthetic pathway for the production of RNA and DNA precursors. The salvage pathway becomes
extremely important when the biosynthetic pathway is blocked or inoperable. New
evidence suggests that it is also important during stationary phase of bacterial growth, but
is active at all phases of growth. In most organisms, addition of exogenous pyrimidines
represses the biosynthetic pathway. The biosynthetic pathway has traditionally been
considered invariant from organism to organism, while the salvage pathway differs from
species to species and has a high degree of variability among organisms. Whereas some
organisms may lack a de novo pathway, all organisms have some element of salvage as
part of their repertoire.

First, the pyrimidine biosynthetic pathway converts bicarbonate, ammonia and
ATP to carbamoylphosphate by the enzyme carbamoylphosphate synthetase (CPSase),
encoded by pyrA. The carbamoylphosphate is then converted into N-carbamoyl-l-
aspartate by joining it with L-aspartate. In this reaction, the phosphate is cleaved to
generate the energy required to unite the two molecules. This reaction is carried out by
aspartate transcarbamoylase (ATCase), produced by the gene pyrB. The next enzyme in
the pathway is dihydrooratase (DHOase), produced by the expression of the pyrC gene,
which converts N-carbamoyl-L-aspartate into the ring structure dihydroorotate.
Dihydroorotate is then dehydrogenated to orotate by dihydroorotate dehydrogenase,
encoded by pyrD. The first pyrimidine nucleotide is made by orotate
phosphoribosyltransferase (pyrE), which converts orotate into orotidine 5’-
monophosphate (OMP) by combining it with 5’-phosphoribosyl-1’-pyrophosphate
(PRPP). The decarboxylation of OMP by OMP decarboxylase (pyrF) produces UMP.
The next three steps are shared by the biosynthetic and salvage pathways, as shown in
Figure 3. The first of these, uridine 5’-monophosphate kinase, converts UMP to UDP by
converting ATP to ADP. UTP is next produced from the addition of a phosphate to UDP
by the enzyme nucleoside diphosphokinase (ndk), which converts an ATP to ADP. The
third shared step is catalyzed by cytidine 5’-triphosphate synthetase, encoded by pyrG,
which aminates UTP to produce CTP. Figure 4 outlines the entire pyrimidine
biosynthetic pathway.
Figure 3: Pyrimidine Biosynthetic and Salvage Pathways in *Escherichia coli* (provided by Debrah Beck, 1995)
Figure 4: Pyrimidine biosynthetic (de novo) pathway (provided by Heidi Hammerstein)
Figure 4 continued
Figure 4 continued
Figure 4 continued
The de novo pathway has evolutionary constraints that keep the steps constant in most organisms that possess the pathway. This is probably due to a normally low concentration of pyrimidine de novo pathway intermediates in the environment. In addition, typically orotate is the only de novo pathway intermediate which will enter the cell. Even orotate gets in with some difficulty; therefore, the de novo pathway is under little influence from exogenous intermediates. The salvage pathway is not under the same constraints because there is a myriad of possible salvage schemes and most cells can take up uracil, uridine, cytosine and cytidine. Therefore, salvage is influenced by the concentration and type of bases commonly found in the particular microorganism’s environment. The intermediates in salvage pathways are more abundant because they are produced by the breakdown of DNA and RNA. The salvage pathway can give the organism greater metabolic flexibility. For instance, in a situation where the organism is starved for aspartate and UTP, an organism that possesses only the de novo pathway would not be able to rectify the low concentration of UTP. On the other hand, an organism with the proper salvage enzymes would be able to convert CMP to UTP using a 5’-nucleotidase to convert CMP to cytidine. Then, cytidine could be deaminated to uridine, provided cytidine deaminase were present. Uridine can then be phosphorylated to UMP, UDP, and UTP via kinases. An alternative may be CMP glycosylase acting on the CMP to produce cytosine and ribose-5-P. If cytosine deaminase is present, then the cytosine is deaminated to uracil. Uracil can then be converted to UMP by uracil phosphoribosyltransferase. UMP is converted to UTP by a series of kinases. This illustrates one of the many possibilities of salvage.
In addition to their importance in DNA and RNA, pyrimidines are used in cell envelope biosynthesis in the form of UDP galactose as well as dTDP rhamnose. The enzyme galactokinase uses ATP to phosphorylate galactose to galactose-1-phosphate. The galactose of galactose-1-phosphate is then exchanged with the glucose of UDP-glucose by the enzyme phosphogalactose uridyld transferase. The glucose-1-phosphate can then be utilized by the cell in a number of ways. The UDP-galactose can be used in cell wall biosynthesis or can undergo epimerization at the number 4 carbon to produce UDP-glucose. The enzyme that performs this epimerization is UDP-glucose epimerase (Zubay, 1998).

Pyrimidines are also involved in the breakdown of lactose, beginning with its hydrolysis, by α-galactosidase into galactose and glucose. The glucose can be used immediately by a number of different pathways in the cell. The galactose is joined to the carrier UDP to form UDP-galactose. The UDP-galactose is acted upon by an epimerase and converted into UDP-glucose. The α-lactalbumin of milk catalyzes the reaction:

\[ \text{UDP-galactose + D-glucose } \rightarrow \text{ UDP + lactose for the production of lactose.} \]

UDP is also used in glycogen formation. Glycogen synthase uses the UDP-glucose to add another glucose residue to glycogen (in animals). Bacteria utilize ADP-glucose (Zubay, 1998).

There are many other examples of how vital pyrimidines are for life. First, CTP is used in the production of phosphatidylserine and phosphatidylethanolamine. Second, amino acids are added to UDP-N-acetylmuramic acid (NAM) for use in peptidoglycan synthesis. The amino acids added are then acted upon by transpeptidase to form the cross links. UDP-N-acetylglucosamine (NAG) is used to add NAG to the NAM-pentapeptide. This comprises the repeating unit of peptidoglycan (Prescott et al., 1999). Third,
Barbituric acid is made by the oxidation of uracil (see Figure 5). Barbituric acid is then broken down into urea and malonate, and the malonate can then be converted into malonyl-CoA.

\[
\begin{align*}
\text{Uracil} & \xrightarrow{1} \text{H}_2\text{O} \\
\text{Barbituric acid} & \xrightarrow{2} 2\text{H}_2\text{O} \\
\text{Urea} + \text{Malonate} &
\end{align*}
\]

**Figure 5:** Oxidative pathway of the breakdown of uracil

(1) uracil dehydrogenase EC 1.2.99.1  
(2) barbiturase EC 3.5.2.1

The reductive degradation of uracil produces â-alanine, which combines with pantothenic acid to produce CoA (Vogels and Van der Drift, 1976), see Figure 6.
Figure 6: Reductive pathway of the breakdown of uracil
(1) dihydouracil dehydrogenase EC 1.3.1.1
(2) dihydropyrimidinase EC 3.5.2.2
(3) α-ureidopropionase EC 3.5.1.6
Aspartate transcarbamoylase (ATCase)

The pyrimidine biosynthetic pathway is required for the synthesis of UTP and CTP for RNA and of dCTP and dTTP for DNA. The pathway is nearly ubiquitous, with the same order of enzymes being found in most organisms (Jones, 1980). The enzymes that catalyze individual steps in pyrimidine biosynthesis are highly conserved in bacteria, yet the regulation of the pyrimidine pathway and the organization of the pyrimidine genes in different organisms are quite diverse. The enzyme aspartate transcarbamoylase, EC 2.1.3.2, also known as aspartate carbamoyltransferase, carbamylaspartotranskinase and carbamoyl-phosphate L-aspartate carbamoyltransferase, catalyzes the reaction between the substrates carbamoylphosphate and l-aspartate to yield the products N-carbamoyl-L-aspartate and orthophosphate (Webb, 1992). This is the committed step in the pyrimidine biosynthetic pathway. N-carbamoyl-L-aspartate is then converted to UMP in four steps. This reaction has been shown to be highly regulated in most bacteria, at both the levels of enzyme activity and gene expression. The clearly defined biochemical properties and metabolic significance of this enzyme have not been lost on a number of research laboratories, and consequently over the years it has become one of the more highly characterized enzymes from bacteria. While the fundamental catalytic aspects of the enzyme have been conserved across almost all organisms, the regulatory properties of bacterial ATCases do differ, and along with size, have been used by Bethell & Jones (1969) to categorize ATCase enzymes into three different classes that essentially follow phylogenetic lines (see Figure 7).
<table>
<thead>
<tr>
<th>ATCase Classification</th>
<th>Holoenzyme</th>
<th>Represented Organisms</th>
</tr>
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| Class A1              | ![Class A1 holoenzyme](image) | Mycobacterium tuberculosis  
Mycobacterium smegmatis  
Streptomyces coelicolor  
Burkholderia cepacia |
| Class A2              | ![Class A2 holoenzyme](image) | Pseudomonas aeruginosa  
Pseudomonas putida  
Pseudomonas fluorescens  
Arthrobacter globiformis  
Brevibacterium linens |
| Inactive DHOase       | ![Inactive DHOase](image) | |
| Class B               | ![Class B holoenzyme](image) | Escherichia coli  
Salmonella typhimurium  
Proteus vulgaris |
| Class C               | ![Class C holoenzyme](image) | Bacillus subtilis  
Enterococcus faecalis  
Stenotrophomonas maltophilia  
Streptococcus pyogenes |

Legend:
- **ATCase monomer (34 kDa)**
- **Inactive DHOase (45 kDa)**
- **DHOase (45 kDa)**
- **Regulatory Subunit (17 kDa)**

**Figure 7**: Classes of the ATCase Enzyme
The class C enzyme is 100 kDa in size and is typified by the *Bacillus subtilis* enzyme. The *Bacillus* pyrimidine biosynthetic pathway enzymes are encoded by an operon. This enzyme class is found in all low G+C Gram positive organisms studied to date including *Lactobacillus* (Becker and Brendel, 1996), and as evidenced by the deduced amino acid sequences in *Lactococcus* (GenBank) and *Streptococcus* (GenBank). Like the enteric catalytic trimer, the *Bacillus* ATCase contains three identical catalytic polypeptide chains, each with a molecular mass of 34 kDa. This ATCase is distinct from class B enzymes in that it is devoid of any associated regulatory (*pyrI*) encoded polypeptides, and accordingly is unregulated by nucleotide effectors (Brabson and Switzer, 1975).

The ATCase from *E. coli* is the archetypal class B enzyme which forms a dodecamer of 310 kDa (Weber, 1968). The holoenzyme is comprised of six identical catalytic polypeptides (c) encoded by *pyrB*, organized into two enzymatically functional catalytic trimers (c$_3$) of 100 kDa, and six identical regulatory peptides (r) encoded by *pyrI*, organized into three regulatory dimers (r$_2$), each with a mass of 34 kDa. The *pyrB* and *pyrI* genes are contiguous on the chromosome. The *E. coli* ATCase is active as a trimer, but does not exhibit regulation. As a dodecamer, allosteric inhibition and activation are seen. This 2c$_3$:3r$_2$ dodecameric arrangement is conserved among all enteric and class B ATCases (Wild *et al*., 1980).

ATCases belonging to class A are dodecameric, like class B, yet have a larger molecular mass, typically 450-500 kDa. The genes for this class of enzyme were first cloned and sequenced from *Pseudomonas putida* (Schurr *et al*., 1995) and *P. aeruginosa* (Vickrey, 1993), but subsequently have been found in a wide range of organisms (Kenny
et al., 1996; Linscott, 1996). *Pseudomonas* ATCases are active only as dodecameric holoenzymes comprised of six pyrB-encoded (37 kDa) polypeptides complexed with six pyrC’-encoded (45 kDa) polypeptide chains. The pyrB gene and the pyrC’ genes overlap by three base pairs. The PyrC’ subunits have no discernible DHOase activity and appear to play a scaffold-like role in the assembly of the active ATCase holoenzyme. However, recently, class A ATCases that have active DHOase subunits have been described for *Thermus* (Van de Casteele et al., 1997), *Deinococcus* (Kenny and Shepherdson, 1996), *Streptomyces*, where the pyrimidine pathway exists as an operon (Hughes, 1998), and *B. cepacia* (Linscott, 1996). An organism whose enzyme complex contains an active DHOase makes it more similar to the mammalian CAD, where the first three enzymes (CPSase, ATCase, and DHOase) are encoded in a single polypeptide.

The enzyme which follows ATCase in the pyrimidine biosynthetic pathway is dihydroorotase (DHOase). DHOase, E.C. 3.5.2.3, is also known as carbamoylaspartic dehydrase and (S)- Dihydroorotate amidohydrolase or (S)- Dihydroorotate to N-carbamoyl-l-aspartate + H₂O (Webb, 1992).

Studies using gradient activity gel analysis of native *B. cepacia* cell extracts showed the ATCase activity resided in two molecular forms, namely 550 kDa and 150 kDa (Linscott, 1996). The pyrB gene encoding ATCase from *Burkholderia cepacia* (ATCC 25416) is 1296-bp long, encoding a polypeptide of 432 amino acids with a theoretical molecular mass of 48 kDa. The cloned gene product expressed in *E. coli* had a molecular mass of approximately 144 kDa by ATCase activity gradient gel analysis (Kim, unpublished data, 2002). The ATCase from *B. cepacia* is produced as a 48 kDa monomer that then forms a trimer of 144 kDa and a dodecamer of 550 kDa. It has been
shown (Kim and O’Donovan, 2002) that the enzyme can undergo autocleavage to form a 40 kDa monomer. The *Bordetella pertussis pyrB* sequence is nearly identical in size (431 amino acids) to the *B. cepacia* polypeptide (432 amino acids) with an 86% overall sequence similarity and 76% identity.

The *B. cepacia* ATCase polypeptide is the largest ATCase polypeptide to date. When compared with the catalytic polypeptide of *E. coli*, the *B. cepacia* PyrB is longer at the N-terminus by 86 amino acids and at the C-terminus by 26 amino acids. Despite the unusual size of the *pyrB* polypeptide, the encoded protein maintains significant sequence homology, via NCBI-BLAST analysis, with representative ATCases from the three domains of life. These include the ATCase of plant *Pisum sativum*, the CAD-like complex in fungi, the prokaryotic ATCases in *Bordetella* and *Shewanella*, and in the archaea, *Pyrococcus*. Such similarities may have major implications with respect to the evolution of ATCase and the related CAD-like enzymes.

Because the stationary coccoid forms of *Arthrobacter* and *Brevibacterium* have been referred to as “arthrospores” or “cystites” and because ATCase is an important growth regulating enzyme, its kinetics were studied throughout the cell cycle. Moreover, *Arthrobacter* is nutritionally versatile and highly resistant to most elements, making it analogous to the well-studied *Bacillus subtilis*, where ATCase is degraded at the onset of sporulation (Switzer and Quinn, 1993). This raised the question of an alternate structure of ATCase in each form of the cell, whether coccus, coccobacillus, or bacillus.
MATERIALS AND METHODS

Bacterial Strains

*Burkholderia cepacia* ATCC25416 was obtained from the American Type Culture Collection (Manassas, VA). The strain was stored frozen at -80°C in Nutrient Broth (Difco Laboratories) in the presence of 8% DMSO (Sigma Chemicals). *B. cepacia* was subcultured from frozen stocks in *Pseudomonas* minimal medium (PMM) (Ornston & Stanier, 1996) with 0.2% succinate, shaking at 200 RPM at 30°C or on PMM Agar plates containing 1.5% agar (Difco). *B. cepacia* was also grown in Luria Bertani (LB) broth (Difco), shaking at 200 RPM at 25°C and 37°C, as well as LB agar plates containing 1.5% agar (Difco).

Preparation of Growth Medium for *Arthrobacter*

Minimal medium for culturing *Arthrobacter globiformis* was made by adding the following to a total of one liter: 10 ml 5% MgSO₄, 10 ml of 2.5 M Potassium phosphate buffer, pH 7, 10 ml Trace salts, 10 ml 10% (NH₄)₂SO₄, 25 ml 20% Glucose, and 935 ml distilled H₂O.

The 5% MgSO₄ was made by adding 5 g MgSO₄ to ultra pure water until a final volume of 100 ml was reached, and then autoclaved separately. The 2.5 M potassium phosphate, pH 7, was made by adding 23.12 g of dibasic potassium phosphate to 13.94 g of monobasic potassium phosphate, bringing to a total volume of 100 ml using ultra pure water, and autoclaving separately. Trace salts were made by bringing the following to a total volume of 0.1 L and then autoclaving the resulting solution separately: 1.5 g nitrilotriacetic acid, 3.0 g MgSO₄, 0.5 g MnSO₄, 1.0 g NaCl, 0.1 g FeSO₄, 0.1 g CaCl₂, 0.1 g CoCl₂, 0.1 g ZnSO₄, 0.01 g CuSO₄, 0.01 g AlK(SO₄)₂, 0.01 g H₃BO₃, and 0.01 g
Na$_2$MoO$_4$. Finally, the 10% (NH$_4$)$_2$SO$_4$ is made by adding 10 g (NH$_4$)$_2$SO$_4$ to ultra pure water until a final volume of 100 ml was reached.

ATCase and DHOase Assays

ATCase activity was assayed by measuring the amount of carbamoylaspartate produced in 20 minutes at 30°C according to the method of Gerhart and Pardee (1962), with the modified color development procedure of Prescott and Jones (1969). CAA was measured by using a CAA standard curve at an absorbance of 466 nm. DHOase activity was assayed in degradative direction with 2mM dihydroorotate in a 20 minute incubation at 30°C. The reaction was terminated with the same stop mix as for the ATCase assay, and the amount of CAA produced was measured as described above. Protein concentration was determined by the method of Bradford (1976).

Disruption of Cells

Five liters of sterile Arthrobacter minimal medium with glucose was inoculated with Arthrobacter globiformis. The A. globiformis was grown at 30°C, shaking at 250 rpm, and harvested at the appropriate stage of the growth cycle. Cells were harvested by centrifugation at 4000 x g for 10 minutes at 4°C and the pellets were washed in ATCase buffer (50 mM TrisHCl, pH 8.0, 2 mM â-mercaptoethanol, 20 mM ZnSO$_4$ and 20% glycerol) for a final concentration of 0.5 g of cells per ml. The cells were broken by a lysozyme enzymatic digestion. This was performed by adding lysozyme to a final concentration of 2 mg per ml. The solution was allowed to incubate at 25°C for 1 hour. One half volume of 0.1 mm glass beads were added to the solution and vortexed for five minutes. Cell debris was removed by centrifugation at 10,000 x g for 30 minutes at 4°C. The supernatant was used for all assays and activity gels.
Analysis of ATCase Bands from *B. cepacia* Grown Under Different Environmental Conditions

One liter of LB was inoculated with *B. cepacia* and grown to stationary phase at 25°C. A second liter of LB was inoculated with *B. cepacia* and grown to stationary phase at 37°C. Cells were harvested by centrifugation at 4000 x g for 10 minutes at 4°C and the pellets were washed in ATCase buffer and then resuspended in ATCase buffer for a final concentration of 0.5 g of cells per ml. The cells were broken by explosive decompression as described previously. Cell debris was removed by centrifugation at 10,000 x g for 30 minutes at 4°C. The two products were then electrophoresed under native polyacrylamide gel conditions.

Native Polyacrylamide Gel Electrophoresis Analysis

ATCase enzyme size was estimated using gradient non-denaturing PAGE (BioRad precast gels, 4-20%) using ATCases of known size as markers. The enzyme was visualized with a modified ATCase activity gel procedure of Bothwell (1975) using a histidine buffer as modified by Kedzie (1987). ATCase activity gel analysis entails placing the non-denaturing PAGE gel in 50 ml of ice-cold 50 mM histidine, pH 7.0 for 5 minutes. Next, 1 ml of 1.0 M aspartate and 2 ml of 0.1 M carbamoylphosphate were added and allowed to react for 20 minutes. This reaction took place at room temperature, a modification that allowed for the visualization of bands that are normally too weak to see. The gel is then rinsed three times with ice-cold distilled water to remove reactants. The enzymatically released P_i, trapped in the gel is precipitated by the addition of 250 ml of 3 mM lead nitrate in ice-cold 50 mM histidine, pH 7.0, for 10 minutes. The soluble lead ions are removed with three changes of ice-cold water. Areas containing ATCase
enzyme appear as white bands from the lead phosphate precipitate. These white bands can be counterstained into dark brown, lead sulfide bands with the addition of 300 ml of 5% ammonium sulfide (Kedzie, 1987).

Growth Curve

A sterile 250 ml Klett flask with 100 ml of Arthrobacter minimal growth medium was inoculated with 1 ml of a 2 day old culture of Arthrobacter globiformis, which had been grown on Arthrobacter minimal medium at 30 C. The starting optical absorbance was determined using a Klett-Summerson photoelectric colorimeter. This flask was allowed to shake at 250 rpm in a 30C incubator for the entire length of the growth curve. Absorbance was read every hour using the Klett meter.

Gel Electrophoresis

Preparation of Activity Gel. A PAGE clamp assembly with two glass plates, one short and one long, and two 0.75 mm spacers was assembled and placed into a casting stand. The separating gel was prepared by adding 2.67 ml Solution A (30% Acrylamide, 0.8% Bis-acrylamide- see Appendix), 2.5 ml Solution B (see Appendix), 4.83 ml ultra pure H2O, 0.02 g ammonium persulfate, and 5 µl TEMED for a final volume of 10 ml. The separating gel was then poured into the cassette until the level hit the line 2 cm below the top of the short plate. The solution was then topped off with enough N-butanol to cover the top of the gel, and the gel was allowed to polymerize for 15-30 minutes. The N-butanol was poured off and the top of the gel was rinsed with ultra pure water. The top of the gel was blotted with paper towels.

Next, a stacking gel was prepared by adding 4.6 ml ultra pure H2O, 1.34 ml Solution A, 2 ml Solution C (see Appendix), 0.02 g ammonium persulfate, and 5 µl
TEMED. The gel was poured to the top of the short glass plate and a comb was inserted into top of the gel until the top of the teeth reached the top of the front plate. After allowing the gel to polymerize for 15-30 minutes, the clamp assembly was removed from the casting stand, followed by removal of the comb. Two clamp assemblies were inserted into an electrode assembly, which was then placed in a buffer tank. A non-denaturing PAGE buffer, comprised of 30 g Tris, 144 g glycine, and enough water to give a final volume of 1 L at pH 8.8, was made and poured into the inner and outer chambers.

The sample of the protein to be analyzed was mixed with 5x loading dye (see Appendix) so that the final concentration of the loading dye was 1x. 25 µl of each sample-dye mixture was then loaded into the wells and the cell lid with power cables was placed on top of the running chamber. The leads were connected to a power supply and electrophoresed at 150 V until the dye ran off the end of the gel (about 1.5 hours). After electrophoresis, the lid was removed and clamp assembly removed from the electrode assembly. The glass plates were removed, followed by gentle removal of the gel.

*ATCase Activity Stain*

The polyacrylamide gel from above was then placed in a large Petri plate and equilibrated for 5 minutes in ice cold 50 mM histidine (0.388 g histidine/50 ml water), pH 7.0. 1 ml of 1.0 M L-aspartate potassium salt (0.17 g of aspartate into 1 ml of water) and 2 ml of 0.1 M carbamoylphosphate dilithium salt (0.03g of carbamoylphosphate/ 2.0 ml water) was added to the solution. The enzymatic reaction was allowed to occur for 20 minutes at 25°C. The gel was then rinsed 3X with ice cold distilled water to remove reactants. The enzymatic release of orthophosphate trapped in the gel precipitated after adding ice cold 3 mM lead nitrate in 50 mM histidine, pH 7.0. The 3 mM lead nitrate
was made by combining 0.05 g lead nitrate to 50 ml of 50 mM histidine, pH 7. After 10 minutes, the lead nitrate mixture was removed with 3 changes of ice cold water. The gel was then stored overnight at 4°C in water.

General Assays

**ATCase.** The following ingredients were added for a total reaction volume of 900 µl: 800 µl dd H₂O, 40 µl Tribuffer, pH 9.5 (see Appendix), 50 µl 20x aspartate, pH 9.5, and 10 µl partially purified ATCase from *A. globiformis* with H₂O added in place of enzyme. Only L-aspartate potassium salt was used because D-aspartate and the racemer are not utilized. Sodium inhibits ATCase. The tubes were placed at in 30°C water bath for 5 minutes. Next, 100 µl carbamoylphosphate was added to start the reaction, and incubated for 20 minutes in a 30°C water bath. The reaction was stopped by adding an equal volume (1 ml) of Stop Mix (2 parts antipyrine/1 part monoxime; mix must be pre-made in a dark flask) to each tube and covered with a marble. Next, the samples were incubated at 65°C in a water bath for 1 hour in the light. The tubes were removed from the water bath and the absorbance at 466 nm was read. Finally, the specific activity was then calculated using a CAA standard curve. Each assay was performed in triplicate and values given as the average of these trials.

**DHOase.** The following ingredients were added for a total reaction volume of 900 µl: 690 µl ddH₂O, 100 µl Tris, 1M, pH 8.6, 100 µl 10 mM EDTA, and 10 µl partially purified ATCase from *A. globiformis* with blank tube prepared with H₂O in place of enzyme. The tubes were placed at in 30°C water bath for 5 minutes. Next, 100 µl dihydroorotate was added to start the reaction, and incubated for 20 minutes in a 30°C
water bath. The reaction was stopped and the color developed as described above for ATCase.

Kinetics Assays

ATCase Assay to Determine Optimal pH Conditions for the Arthrobacter globiformis Enzyme. The following ingredients were added for a total reaction volume of 900 µl: 800 µl ddH2O, 40 µl Tribuffer with varying pH’s, 50 µl 20x aspartate pH 9.5, and 10 µl partially purified ATCase from A. globiformis. Twelve tubes were prepared as described above with Tribuffer varying in pH from 5.0 to 10.0, in 0.5 pH unit increments. A blank with dd H2O instead of Tribuffer was included. All tubes were placed in a water bath at 30ºC for 5 minutes. Next, 100 µL of carbamoylphosphate was added to each tube in 10 second increments to start the reaction and incubated for 20 minutes in a 30ºC water bath. The reaction was stopped by adding an equal volume (1 ml) of Stop Mix to each tube in 10 second increments. The same order of tubes was followed as when the carbamoylphosphate was added. The tubes were further incubated to develop color, removed, cooled to room temperature, and the absorbance read on a spectrophotometer at 466 nm. A graph was plotted to determine the enzyme’s optimal pH. The pH was graphed on the X-axis and absorbance on the Y-axis. The pH giving the highest enzyme activity was designated the optimal pH. The experiment was repeated two additional times.

ATCase Assay to Determine Optimal Enzyme Concentration for Assays. The following ingredients were combined for a total reaction volume of 900 µl: X µl dd H2O, 40 µl Tribuffer, at optimal pH, 50 µl 20x aspartate pH 9.5, and X µl partially purified ATCase from A. globiformis with concentrations varying from 1 µl of enzyme to 45 µl. The amounts of H2O added were adjusted when necessary. Eleven tubes with enzyme amount varying from 1µl - 45µl in 5 µl increments were prepared. A blank with dd H2O instead of enzyme was also prepared and all tubes were placed in a water bath at 30ºC for 5 minutes. Next, 100 µl of carbamoylphosphate was added to each tube in 10 second
increments to start the reaction and incubated for 20 minutes per tube in a 30°C water bath. The reaction was stopped by adding an equal volume (1 ml) of Stop Mix to each tube in 10 second increments. The same order of tubes was followed as when the carbamoylphosphate was added. The tubes were removed and the absorbance read on a spectrophotometer at 466 nm. To determine the enzyme’s optimal concentration the volume was graphed on the X-axis and absorbance on the Y-axis. The experiment was repeated two additional times.

**ATCase Assay with Varying Aspartate Concentration.** The following ingredients were combined for a total reaction volume of 900 µl: 800 µl dd H₂O, 40 µl Tribuffer, at optimal pH, X µl 20x aspartate pH 9.5 (varying concentration 1 mM to 60 mM), and X µl partially purified ATCase from *A. globiformis*. Fourteen tubes with enzyme amount varying from 1mM – 60 mM in 5 mM increments were prepared. A blank with dd H₂O instead of enzyme was also prepared and all tubes were placed in a water bath at 30°C for 5 minutes. Next, 100 µL of carbamoylphosphate was added to each tube in 10 second increments to start the reaction and incubated for 20 minutes per tube in a 30°C water bath. The reaction was stopped by adding an equal volume (1 ml) of Stop Mix to each tube in 10 second increments. The same order of tubes was followed as when the carbamoylphosphate was added. The tubes were removed and the absorbance read on a spectrophotometer at 466 nm. The aspartate concentrations were then graphed on the X-axis and change in concentration per minute (velocity) on the Y-axis for Michaelis-Menten kinetics. The experiment was repeated two additional times.
Assay to Determine Protein Concentration

First, the Bradford Reagent was gently mixed in the bottle (Bradford, 1976). Standard protein solutions ranging from 50 µg/ml to 1,000 µg/ml were prepared using bovine serum albumin, with serial dilutions from the highest standard concentration being used. 100 µl of the standard protein solution was added to each appropriately labeled tube and a blank of 100 µl of distilled H₂O was made. 100 µl of the unknown samples were also added to their respective tubes. All standards, the blank and unknowns were prepared in duplicate. Next, 1 ml of Bradford Reagent was added to each tube and mixed. Prior to reading the absorbance of each sample, the spectrophotometer was auto-zeroed with the blank tube. The absorbance was read between 5-60 minutes at 595 nm using disposable cuvets. The absorbance vs. concentration of all trials of the standard protein solutions was first plotted on a graph, and the protein concentration of the unknown sample was determined by comparing the A₅₉₅ values against the standard curve. The experiment was repeated two additional times.
RESULTS

In chapter 1, results are organized as follows and seen in Figures 8-21. Figure 8 shows a non-denaturing polyacrylamide gel in which the *E. coli* and *Pseudomonas aeruginosa* ATCase are run as size markers. Also seen on this gel are the ATCases of *Arthrobacter globiformis* in lag and log phases and *Brevibacterium linens* in stationary phase. The growth cycle in figure 9 where the y-axis is expressed as log of the number of cells/ml and the x-axis as time(min). Figures 10-19 are alternating Michaelis-Menten and Lineweaver-Burk graphs illustrating the kinetics of *A. globiformis* ATCase from various phases of the growth cycle. A bar graph (figure 20) summarizes the data from Figures 10-19. The thermostability of *A. globiformis* ATCase from different stages of the growth cycle is shown in another bar graph as Figure 21.
**Figure 8:** Native gradient PAGE of *Arthrobacter globiformis* cells in lag and log phase and *Brevibacterium linens* in stationary phase

(a) *E. coli*, (b) *A. globiformis* in lag phase, (c) *P. aeruginosa*,
(d) *A. globiformis* in log phase, (e) *B. linens* in stationary phase
Figure 9: *Arthrobacter globiformis* growth cycle
**Figure 10:** A Michaelis-Menten graph illustrating the kinetics of the ATCase enzyme from lag phase of *Arthrobacter globiformis*
**Figure 11**: A Lineweaver-Burk graph illustrating the kinetics of the ATCase enzyme from lag phase of *Arthrobacter globiformis*
Figure 12: A Michaelis-Menten graph illustrating the kinetics of the ATCase enzyme from early log phase of *Arthrobacter globiformis*
Figure 13: A Lineweaver-Burk graph illustrating the kinetics of the ATCase enzyme from early log phase of *Arthrobacter globiformis*
Figure 14: A Michaelis-Menten graph illustrating the kinetics of the ATCase enzyme from mid-log phase of *Arthrobacter globiformis*
Figure 15: A Lineweaver-Burk graph illustrating the kinetics of the ATCase enzyme from mid-log phase of *Arthrobacter globiformis*
**Figure 16:** A Michaelis-Menten graph illustrating the kinetics of the ATCase enzyme from late log phase of *Arthrobacter globiformis*
Figure 17: A Lineweaver-Burk graph illustrating the kinetics of the ATCase enzyme from late log phase of *Arthrobacter globiformis*.
**Figure 18:** A Michaelis-Menten graph illustrating the kinetics of the ATCase enzyme from stationary phase of *Arthrobacter globiformis*
Figure 19: A Lineweaver-Burk graph illustrating the kinetics of the ATCase enzyme from stationary phase of *Arthrobacter globiformis*
Figure 20: Apparent $K_M$ of *Arthrobacter* ATCase at various stages of the growth cycle
**Figure 21:** Thermal stability of *Arthrobacter* ATCase at various stages of the growth cycle
DISCUSSION

The true biologist deals with life, with teeming, boisterous life, and learns something from it, learns that the first rule of life is living.

John Steinbeck

Burkholderia cepacia

Pseudomonas aeruginosa contains a pyrB gene which codes for an ATCase enzyme that is a dodecamer of 480 kDa. Previous studies on B. cepacia have found the ATCase dodecamer to be 550 kDa. However, when the genomic sequence became available, it was discovered that B. cepacia had two genes coding for ATCase. One was a very large gene, coding for the polypeptide that ultimately makes up the 550 kDa dodecamer. The second gene was similar in size and sequence to the Pseudomonas pyrB gene, which codes for a polypeptide that ultimately makes up the 480 kDa dodecamer. However, the 480 kDa band had not been seen previously. Therefore, an experiment was conducted to determine if this 480 kDa existed in B. cepacia. It was discovered that B. cepacia grown in rich medium at 25°C produced the 480 kDa size enzyme. In addition, using a modification of the activity gel devised for this work, four more bands were found. One band appeared at 144 kDa, one at 136 kDa, one at 128 kDa and the last at 120 kDa.

ATCase from B. cepacia was partially purified from wild type cell extracts as described in Materials and Methods. The purified protein samples were subjected to PAGE analysis. Non-denaturing gradient PAGE gels stained for ATCase activity showed that the ATCase from wild type B. cepacia was present in 5 forms at approximately 550
kDa and 144 kDa (Linscott, 1996), as well as the three smaller bands of 136 kDa, 128 kDa, and 120 kDa. The 144 kDa band appears to be a trimer of 48 kDa polypeptides, such that the 136 kDa band would then be made up of two of the 48 kDa polypeptides and one of the 40 kDa auto-cleaved polypeptides. The 128 kDa band is made up of two auto-cleaved and one uncleaved polypeptide, and the 120 kDa band would be a trimer of cleaved ATCase polypeptides. Physiological conditions would dictate which aggregate would apply.

*Arthrobacter globiformis*

There is a limited number of cell shapes utilized by the microbial world. A majority of bacteria exist only as one shape, whether a rod, coccus, or spirochete. There are few bacteria that are pleomorphic, one example being *Arthrobacter*. Bacterial shape is useful to the microbiologist as a taxonomic and identification tool. To the bacteria, shape is important for several reasons. Shape gives structural integrity to the organisms. An example of this is the spherical bacterial endospore. This shape, along with a number of other properties, allows the spore to survive in the harsh environmental conditions a bacterium may be exposed to during its lifetime. Surface to volume ratios also play a part in structural integrity. Cocci have a lower surface to volume ratio, meaning they have less cell wall to contend with. This gives coccus-shaped cells less area to defend. For example, an oblong castle would require more sentries to defend than a circular castle. In addition, a sphere can withstand greater pressure than a cylinder. Force applied to a spherical cell wall can be more evenly distributed, while the same force applied to the flat area of a rod is contained at that one point. In pleomorphic bacteria, the rod shape and size is not very uniform. The cell in this stage appears to
expend little energy in regulating the size and shape of the cell. Instead, this energy is directed towards growth. Finally, in most cases, structure dictates function. The structure of cocci dictates a propensity for survival under unfavorable conditions, while the rod shape dictates a function of fast growth.

In good conditions, *Arthrobacter* cocci develop into rods and V-shaped forms by either growing outward from the cocci or undergoing snapping division, respectively. The rod shape gives an advantage to the cell for rapid growth because the *Arthrobacter* rod is not the same size or shape for every cell. The organism seems to pay less attention to uniformity of shape, thereby allowing for speedy growth rather than precision. When conditions start to decline, i.e. nutrition is limiting, the cells return to the coccus form. This type of rod to coccus shift is also observed in *Bacillus subtilis* when the gene *mreB* is mutated. The MreB and Mbl proteins of *B. subtilis* are actin-like cytoskeletal structures responsible for determining cell shape (Thomas, 2001). It is likely that *Arthrobacter* evolved a molecular switch that would allow it to turn off this gene when it sensed the environment was becoming less hospitable. This shift would allow the organism to take advantage of the benefits that the coccus form imparts on *Arthrobacter* during difficult situations.

Contemporary microbiologists tend to think of bacterial growth as a curve with three phases. The lag phase begins the curve, a stage where time is required for enzymes and intermediates to be produced as cells turn on genes needed for pathways. The second phase is exponential growth, where cells are dividing in minimal amounts of time. The cells are dividing as rapidly as possible. The last stage is stationary phase, where there is neither an increase nor decrease in net cell number. Typically, the entire growth curve
analysis covers a maximum of 24 hours. However, there has been a revitalization of the study of bacterial stationary phase in recent years.

Earlier studies on bacterial growth, during the first half of the twentieth century, describe a five stage cycle with no beginning or end. In this growth cycle, the lag phase acted as a segue between the end of the last phase discussed below and the beginning of exponential growth. The exponential phase was described as a time in which the cells divided as quickly as they possibly could. Stationary phase followed, which was a time where the environmental conditions become unfavorable (in the laboratory this is normally marked by the depletion of nutrients). The stationary phase was where the net number of cells stayed the same because the exponential increase in cell number was negated by the exponential death of cells of the same population. The next phase was the exponential death phase, in which cells die logarithmically. This is followed by the period of slow decline of cell number. This growth cycle was not measured over a 24 hour period, but over a 2 year period (Storz et al., 2000). This version most accurately describes how soil bacteria survive because the primary phase of the growth cycle for soil microorganisms is the period of slow decline of cell number. This is the stage that allows soil organisms, including non-sporeformers, to survive for years in extreme, nutritionally sparse conditions. Of course, other adverse circumstances are possible, including fluctuations in temperature, soil moisture, pH, and osmolarity. These adverse circumstances are more easily overcome in conditions of slow growth or no growth.

The soil is a most inhospitable environment. Throughout the year, temperatures in the soil can range from 50°C + to far below freezing. In some desert areas, a drop from 50°C + to freezing can occur overnight. The plants and microorganisms are inextricably
linked to ensure the survival of both. The soil contains a vast array of prime examples of mutualism. At this time, only a small portion of soil bacteria are culturable in the lab. This is due to a number of factors, such as the use of media that are too high in certain nutrients. In addition, the lack of some nutrients and the absence of other factors, such as compounds produced from other organisms, contribute to the inability to culture some soil microorganisms. An example of this is that the medium used to grow *Azotobacter* requires a certain soil extract, one that comes only from soil surrounding an African violet.

Soil microbiology is an old division of microbiology, almost as old as microbiology itself, but one that has been neglected. The opening quote in the Introduction is meant to remind the reader of the importance of soil. The second, and less obvious, idea to be gleaned from this quote is that without soil microorganisms, food would not spring from the soil.

*Arthrobacter* is a successful member of microorganisms in the soil. It is able to overcome the challenging obstacles of life in the soil. *Arthrobacter* has done this by evolving useful traits such as the rod to coccus growth cycle and modifying the kinetics of important regulatory enzymes such as ATCase.

### Growth Cycle

The lag phase in *Arthrobacter*’s life cycle, see Figure 9, is protracted and the log phase is relatively short. The generation time is significantly longer than for microorganisms such as *E. coli*. The growth curve continues with an extensive stationary phase. It can be reasoned that in the soil, under such limiting conditions, *Arthrobacter* must spend vast stretches of time in a frugal stationary phase. At this phase of the growth
cycle, the bacterium is back in its resistant coccoid shape. This transformation back to the coccus shape is most probably due to a molecular switch turning off the production of a protein such as MreB. The coccus form is believed to be a more primitive morphology as well as being more recalcitrant. The size of the ATCase through the growth cycle was monitored using a gradient PAGE stain for activity, Figure 8. All the phases of the growth cycle yield a 480 kDa band, the same size in *Pseudomonas aeruginosa*, with the exception of the lag phase. Lag phase cells of *Arthrobacter* produce a band of ~ 900 kDa for ATCase. This correlates with the beginning of elongation of the cocci into rods, as seen in Figure 22 (provided by R.M. Keddie and D. Jones).

![Figure 22: Arthrobacter cellular morphology at four stages of the growth cycle](image)

*Figure 22: Arthrobacter* cellular morphology at four stages of the growth cycle (a) after 6 hours, (b) after 12 h, (c) after 24 hours, (d) after 3 days

*P. aeruginosa* ATCase is 480 kDa and is made up of an enzymatically active ATCase and inactive DHOase. *Streptomyces* produces an ATCase of 480 kDa, but is active for both ATCase and DHOase, as is *Arthrobacter*. The size of *Arthrobacter*
ATCase/DHOase is 480 kDa in all phases of growth except in lag phase. In the lag phase, the enzyme seems to aggregate to 960 kDa. With the exception of lag phase, *Arthrobacter* ATCase is more similar to *Streptomyces*. This correlates with the 16S phylogenetic grouping, which places the two as evolutionary neighbors.

**Enzyme Thermal Stability**

The enzyme stability of ATCase from various parts of the growth cycle is shown in Figure 21. This graph indicates that the point of the growth curve with the greatest thermal stability for ATCase is the stationary phase. This is due to the cells’ need to survive during stationary phase. There is most likely not a change in the actual enzyme itself. It is probable that a cofactor or polypeptide is produced by the cell at the start of stationary phase, which helps to stabilize the enzymes. An example of this type of stabilizing cofactor is dipicolinate, which is produced by the *Bacillus* species during sporulation. The dipicolinate is believed to stabilize the DNA of the spore. In a similar fashion, a cofactor or polypeptide is apparently produced to stabilize important enzymes in the coccoidal arthrospore produced by *Arthrobacter*.

**Enzyme Kinetics**

There have been few, if any, comprehensive studies of $K_M$ in different phases of growth. Lag phase of *Arthrobacter* is the beginning of two major changes in the cell. One is a rapid increase in growth rate, and the other is a marked change in cellular morphology. How are the enzymes affected by these two transformations?

In a chemical reaction, the reactant(s) interact to form the product(s). The study of the velocity and stoichiometry at which this phenomenon occurs is called kinetics. All living cells must be able to perform chemical reactions. It is important that cells are able
to control the rate of these reactions and make them proceed more efficiently and specifically than would occur outside a living system. This feat is performed by a specific type of protein which can carry out chemical reactions, termed enzymes. Life would not be possible if cells were unable to increase the rate of chemical reactions by many orders of magnitude. An example of this is seen for the enzyme catalase, which converts hydrogen peroxide to water and oxygen 40 million times per second (Zubay, 1998). This reaction occurs in the absence of catalase but at a very slow rate. In addition to velocity, enzymes increase the specificity of the reactions they catalyze. This means that only very specific molecules are catalyzed; an example of this specificity is that L-amino acids are used exclusively over D-amino acids, with the exception of the cell wall of bacteria. Furthermore, enzymes can keep side reactions from occurring, a major problem in non-enzymatic chemical reactions. Also, the amount of enzyme produced can be controlled genetically by the cell, as is evidenced for the \( lac \) operon. When lactose is not present, the cell does not express the genes required to catabolize lactose. The rate of enzymatic catalysis can be controlled by the conditions inside the cell. Examples of this are the activation or deactivation of enzymes by phosphorylation and end-product feedback inhibition. A final major benefit of enzymes is that an enzymatic pathway pushes the reaction to completion. In a pathway which involves enzymes A, B, and C, the products of enzyme A are drawn off by enzyme B, therefore, through Le Chatelier’s Principle, enzyme A is able to convert its reactants to products.

Enzyme kinetics is the study of how fast an enzyme catalyzes a reaction and under what conditions the reaction occurs at this rate. The maximum speed or velocity at which an enzyme catalyzes a reaction under specific conditions is termed maximum
velocity ($V_{\text{max}}$). The specific conditions that must be kept uniform are pH, temperature, pressure, and enzyme and cofactor concentration. The condition that varies is the concentration of one substrate. The substrate concentration which causes an enzyme to function at one-half of the maximum velocity is the $K_M$. Measuring the $K_M$ value gives information about the binding affinity that an enzyme has for the substrate. The higher the $K_M$, the more substrate required to get the enzyme to function at $\frac{1}{2} V_{\text{max}}$. The lower the $K_M$, the less substrate required to get the enzyme to catalyze at $\frac{1}{2} V_{\text{max}}$. The kinetics of an enzyme can be expressed visually by a Michaelis-Menten graph and a Lineweaver-Burk double-reciprocal plot. An asymptote drawn on the Michaelis-Menten graph shows $V_{\text{max}}$ and from this, $K_M$ can be determined by looking at the substrate concentration at $\frac{1}{2} V_{\text{max}}$. Also, by calculating $K_M$ using the Michaelis-Menten equation, $V_o = \frac{(V_{\text{max}} \times [S])}{(K_M + [S])}$, where $V_o$ is the velocity at $\frac{1}{2} V_{\text{max}}$ and [S] is the substrate concentration corresponding to $V_o$. $K_M$ is calculated using the Lineweaver-Burk plot by calculating the negative reciprocal of the X-intercept. The X-intercept is extrapolated using the line formula $y = mx + b$. The reciprocal of the Y-intercept of the Lineweaver-Burk plot is $V_{\text{max}}$.

The kinetics were examined for an enzyme chosen because of its importance in the regulated pathway of pyrimidine biosynthesis. The committed enzyme in this pathway is aspartate transcarbamoylase (ATCase) and accordingly this was the enzyme selected.

The apparent $K_M$ for aspartate from ATCase was measured from all phases of the growth cycle (Figure 20). With the exception of lag phase, all other phases exhibited an apparent $K_M$ of approximately 10 mM. This apparent $K_M$ is most likely in the range of
the physiological concentration for aspartate inside the cell. The exception was the lag phase, with a apparent $K_M$ of approximately 7 mM.

Lag phase cells are in an interesting situation in that they must completely alter their metabolism from a very slow, almost nonexistent rate, to one which is as fast as the cell can negotiate. Cells enter into lag phase when conditions become favorable, such as an infusion of nutrient broth (NB) or Luria-Bertani (LB). One problem for soil bacteria is that these favorable conditions are ephemeral. Therefore, the soil organism must extract what resources it can as quickly and efficiently as possible because the likelihood is that the resources won’t remain available for long. Competition for nutritional resources is a continuous occurrence for cells in lag phase.

It appears that the cell is able to alter the ATCase such that the $K_M$ drops in lag phase. Interestingly enough, this is the same stage of the growth curve that gives the ATCase enzyme a molecular weight of twice that at all the other phases. This appears to be an aggregate that somehow influences the kinetics of the enzyme. This is also the phase where the cells are still primarily cocci but are starting to elongate into rods and snap into V-shaped cells.

An explanation for this change in apparent $K_M$ could be justified by the fact that a soil bacterium offered fleeting, desirable conditions must take full advantage of these evanescent resources. By reducing its $K_M$, an enzyme can function at much lower substrate concentrations.
CHAPTER II

Nature works only in cycles, there are no straight lines. The forward movement is provided by time. Everything within it must revolve.

Anonymous

INTRODUCTION

The pyrimidine biosynthetic pathway, providing precursors for RNA and DNA, is traditionally thought to be nearly identical in all bacteria while the pyrimidine salvage differs from species to species. Whereas the biosynthetic pathway is not found in all bacteria, some aspects of salvage is seen in every bacterium, making the salvage pathway valuable as a taxonomic marker. The salvage pathway uses a set of enzymes to regenerate the trinucleotides and for the catabolism of RNA and DNA. For example, salvage can be used to break RNA down to uracil, which can then be converted to α-alanine and then to coenzyme A, or to barbituric acid and then to malonyl CoA. The following is a list of the salvage enzymes addressed in this dissertation. Cytidine deaminase (Cdd) catalyzes the conversion of cytidine to uridine by using water to remove ammonia. Cytidine hydrolase converts cytidine to cytosine by using water to release ribose. This reaction is also carried out by non-specific hydrolases. Cytidine kinase (Udk) produces CMP from cytidine utilizing ATP and this reaction is also carried out by uridine kinase. Cytosine deaminase (Cod) catalyzes the conversion of cytosine to uracil by using water to remove ammonia from cytosine. This enzyme is a homohexamer in E.coli, with a molecular mass of approximately 300 kDa. It has a subunit of approximately 426 amino acids with a molecular weight of 48 kDa. CMP glycosylase converts CMP to cytosine and ribose-5-phosphate. 5’-nucleotidase was found in this study to convert CMP to cytidine. Orotate
decarboxylase removes CO\textsubscript{2} from orotate to yield uracil. Uridine hydrolase (Udh), also known as uridine nucleosidase (EC 3.2.2.3), produces uracil from uridine by using water to cleave the ribose from uridine. Uridine kinase (Udk) utilizes ATP to convert uridine into UMP. Uridine phosphorylase (Udp) uses inorganic phosphate to convert uridine into uracil and ribose-1-phosphate. In \textit{Escherichia coli}, the active enzyme is a hexamer comprised of identical subunits. Each subunit contains 253 amino acids with a molecular mass of 165 kDa. Cytidine phosphorylase uses inorganic phosphate to convert cytidine to cytosine and ribose-1-phosphate. Uracil phosphoribosyltransferase (Upp) utilizes phosphoribosylpyrophosphate (PRPP) to convert uracil to UMP. In \textit{E. coli}, the enzyme is a homotrimer. In \textit{Ureaplasma urealyticum}, the monomers have a molecular mass of 23 kDa and these monomers are made of 208 amino acids, with an isoelectric point (pI) of 9.1.

The composition of the salvage pathway differs from one species to the next. Each group is comprised of organisms with an identical set of salvage enzymes. Organisms of the same genus cannot always be grouped together based on their salvage scheme. The probable reason for this is that the concentration of the bases, as well as the concentration of nucleotides and nucleosides inside the cell, differs from one species to the next of the same genus. This is due to the environment the organism normally finds itself in, which selects for the most efficient salvage scheme for that particular organism. For example, some organisms have greater concentrations of diphosphates because they break their RNA down to the diphosphate level. Most degrade their RNA hydrolytically to the monophosphate level. Also, uracil can be used as a carbon and energy source by certain bacteria, while others can utilize ribose for this purpose. DNA is readily taken up
by a number of prokaryotes though *Pseudomonas* and *E. coli* do not do so. Another example of an adapted salvage scheme is that parasitic organisms tend to have very little salvage, due to their reliance on the host for nutrition. Finally, a number of bacteria live in nutritionally poor conditions therefore must possess a highly efficient salvage pathway.

The salvage pathways are analogous to a spider’s web, in which there is more than one path leading to a common destination. An example of this variety of pathways to a common end is present in *Microbacterium liquefaciens*, classified in salvage Group XVI (See Figure 113). When cytidine is available and the organism needs to make pyrimidine triphosphates, two paths exist. The first pathway is to convert cytidine into uridine using cytidine deaminase (Cdd), which removes ammonia by hydrolysis. The uridine is then converted to uracil by one of two enzymes, uridine phosphorylase (Udp) that uses inorganic phosphates to remove the ribose as ribose-1-phosphate, or uridine hydrolase (Udh), in which the ribose is removed by water. Uracil, by the action of uracil phosphoribosyltransferase (Upp), is converted to UMP with the consumption of phosphoribosylpyrophosphate. The UMP is then converted to UDP by uridine 5’-monophosphate kinase, using one molecule of ATP. UTP is produced from UDP by the action of nucleoside diphosphokinase (NdK). CTP can be produced from UTP using the enzyme CTP synthetase, consuming 1 ATP and converting glutamine to glutamate in the process. The other option for cytidine in *M. liquefaciens* is for the cytidine to be converted to cytosine. The enzyme responsible for this is uridine hydrolase, again using water to remove the ribose. Cytosine is then deaminated to uracil and NH$_3$, by the enzyme cytosine deaminase. Uracil then follows the same path as the previous example to UTP or CTP.
In our studies to identify ubiquitous soil bacteria, pure cultures of bacteria were isolated from Texas soils and identified to the species level using the MIDI system. The MIDI system is a MIS (Microbial Identification System), which employs an HP 6890 series gas chromatograph in which the methyl esters of fatty acid (FAME) extracts of microorganisms are separated and analyzed to produce a chromatogram. This chromatogram is then compared to chromatograms of known bacteria from a database of over 2000 entries in order to identify the unknown isolate.

Each isolate was assayed for the following enzymes using High Performance Liquid Chromatography (HPLC): uridine phosphorylase, cytidine or nucleoside hydrolase, cytidine deaminase, cytosine deaminase, uridine/cytidine kinase, cytidine phosphorylase, CMP glycosylase, 5’-nucleotidase and orotate decarboxylase, using a LUNA 250 X 4.6 mm 5 micron C18 reverse phase HPLC column. This gave a salvage profile for each natural isolate which was compared to previously determined salvage schemes of known ATCC strains. In an earlier study in our laboratory (D.A. Beck PhD dissertation 1995) found nine different salvage schemes among 60 bacterial species. Some of the new isolates from this study fit into one of the nine schemes but others do not. It is proposed here to add an additional ten new salvage schemes. Studies with pyrimidine salvage schemes are important as identification tools for the understanding of the evolutionary stresses of soil microbiota.
Introduction to Microorganisms Used in This Study

The genus *Acinetobacter* encompasses Gram-negative rods 0.9-1.6 μm in diameter and 1.5-2.5 μm in length. The mol % G+C is 38-47 ($T_m$). The name *Acinetobacter* is derived from the Greek adjective *kinetos*, meaning unable to move. *Bacter* is a Latin noun meaning a rod. These species are non-motile except for twitching motility. The rods become spherical during the stationary phase of growth. Most strains have an optimal temperature of 33-35°C and are catalase positive. All strains are oxidase (cytochrome $c$ oxidase) negative but contain $a$ and $b$ type cytochromes. *Acinetobacter* is also aerobic with only oxygen as the final electron acceptor in metabolism.

*Acinetobacter* is a genus of non sporeformers commonly found in soil, water, and sewage. In fact, it is thought that at least 0.001% of the total heterotrophic aerobic population in soil and water are acinetobacters. The type species is *A. calcoaceticus*, which has, along with other members of the genus, been found to be naturally competent in taking up foreign DNA. *A. lwaffii* is responsible for most cases of meningitis caused by *Acinetobacter* (Sneath *et al.*, 1986). On the following page is data from the gas chromatogram (GC) print out identifying this organism.
Figure 23: Computer analysis and identification resulting from the gas chromatogram for *Acinetobacter lwaffii*

*Aeromonas* is a genus of both motile and nonmotile, Gram-negative, facultative anaerobes typically found in water, sewage, and sludge. *Aeromonas* comes from the Greek noun *aer*, meaning air or gas, and *monas* or *monad* which means unit. The cells show deviation in shape, from that of a straight rod with rounded ends to a coccus. The average dimensions are 0.3-1.0 µm in diameter and 1.0-3.5 µm in length. The mol% G+C content of the DNA is 57-63 (Tm) (Sneath et al., 1986). The genus *Aeromonas* is currently classified in the family *Vibrionaceae*, but molecular genetic comparisons have justified the proposal of a new family *Aeromonadaceae*. *Aeromonas* differs from *Vibrio* in a number of ways. *Aeromonas* is unable to grow in 6% NaCl, whereas *Vibrio* is known for its ability to grow at high NaCl concentrations. In addition, *Aeromonas* is unaffected by treatment with 2,4-diamino-6,7-diisopropylpteridine, a compound that inhibits the growth of *Vibrio*. *Aeromonas* is broken into two groups, one of which grows at low temperature and infects fish. The other is a motile mesophile that infects humans and includes *A. sobria* and *A. hydrophila*. *A. hydrophila*, along with *A. caviae*, and *A.*
*sobria* may cause gastroenteritis in healthy humans and septicemia in immunocompromised individuals. These infections are acquired through open wounds or ingestion of fish contaminated with a sufficient number of these organisms, as well as the fecal-oral route (Sneath *et al.*, 1986). *A. sobria* differs from *A. hydrophila* in that *A. sobria* does not hydrolyze esculin, produce acid from salicin, produce gas from arabinose, or produce elastase (Labbe and Garcia, 2001).

Aeromonads are responsible for “red-leg” disease in amphibians, and other diseases in reptiles, fishes and cows. Types of food that can be contaminated with aeromonads are crustaceans, bivalve mullosks, raw milk and fish (Labbe and Garcia, 2001). The following is a scanning electron micrograph of *Aeromonas* attached to a human intestinal epithelial cell line courtesy of Northwest Fisheries Science Center (NOAA). Figures 23, 24, and 25 are the GC printouts for the three species of *Aeromonas* examined in this work.

**Figure 24**: Electron micrograph of *Aeromonas*
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Figure 25: Computer analysis and identification resulting from the gas chromatogram for Aeromonas hydrophila
Figure 26: Computer analysis and identification resulting from the gas chromatogram for Aeromonas sobria

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TBD145 [Rev 4.16] Aeromonas

- A. sobria: 0.371
- A. caudae: 0.397
- A. canicola: 0.402
- A. c. salmonicida: 0.402
- A. c. chromogenes: 0.204
- Photobacterium: 0.391
- P. aeruginos: 0.391
- P. damselae: 0.280 (Listonella damselae, Vibrio damselae)
- P. leignathii: 0.217
- Vibrio: 0.370
- V. anguillarum: 0.395
- V. parahemolyticus: 0.290
- V. harveyi: 0.246
**Figure 27**: Computer analysis and identification resulting from the gas chromatogram for *Aeromonas trota*
The genus *Agrobacterium* is placed in the family *Rhizobiaceae* but is unlike *Rhizobium* in that it does not stimulate root nodule formation or fix nitrogen (Prescott *et al.*, 2002). The name *Agrobacterium* is derived from *agros*, a Greek noun meaning a field and *bakterion*, a Greek noun meaning a small rod. The species of genus *Agrobacterium* are Gram-negative, non-spore forming rods typically 0.6-1.0 by 1.5-3.0 µm in size. The mol G+C% of the DNA is 58-63 (T_m). These motile organisms are aerobic with oxygen commonly as the final electron acceptor, but some strains are capable of anaerobic respiration in the presence of nitrate. This genus is catalase positive, typically oxidase negative, and urease positive. Agrobacteria are soil inhabitants with an optimum growth temperature of 25-28°C. In particular, *A. radiobacter* is isolated from the plant rhizosphere. Copious extracellular polysaccharide slime is usually produced by *Agrobacterium* on carbohydrate-containing media. With the exception of *A. radiobacter*, Agrobacteria invade the crown, roots, and stems of many plant cells and induce transformation of the plant cells into tumor cells. *A. tumefaciens* causes crown gall disease by transferring the T_i (tumor inducing plasmid) to plant cells. This T_i plasmid has been very useful for molecular botany because the splicing of genes into plants is facilitated by using this system. *A. radiobacter* produces agrocin which is used to control the crown gall tumors produced by *A. tumefaciens* (Sneath *et al.*, 1986).

Below is an electron micrograph of *Agrobacterium* provided by SCIMAT, followed by the gas chromatograph print out for *A. radiobacter* (Figure 29).
Figure 28: Electron micrograph of Agrobacterium

Figure 29: Computer analysis and identification resulting from the gas chromatogram for Agrobacterium radiobacter
*Bacillus* spp. are Gram-positive rod shaped bacteria which move by peritrichous flagella and tend to swarm on solid media. The rods can occur singularly or in chains. The mol% G+C of the DNA varies between 32-69 ($T_m$). Under unfavorable conditions, such as UV rays, X-rays, extreme temperatures, lack of oxygen, toxic compounds and nutrition depletion, they are able to produce a resistant endospore. Furthermore, some *Bacillus* species are able to form parasporal bodies, which are crystals of toxins extruded during germination. *Bacillus* is a large and diverse genus which contains many soil organisms as well as medically important bacteria (Sneath *et al*., 1986). They are useful in the soil for bioremediation, such as the oxidation of 3-chlorobenzoate to 3-chloro-2,3-dihydroxybenzoate. This oxidation sets up the eventual degradation of this recalcitrant compound in the soil (Walker, 1975). They are tolerant to oxygen, typically producing catalase and are either aerobic or facultative anaerobes. The nutritional requirements group them as chemoorganotrophs. The majority of *Bacillus* species contain meso-diaminopimelic acid in their cell wall and are naturally competent for the transformation of DNA into the cell (Sneath *et al*., 1986). This makes the investigation of their pyrimidine salvage very interesting.

*Bacillus cereus* is an aerobic rod, 1.0-1.2 µm in diameter and 3.0-5.0 µm in length, possessing peritrichous flagella. The murein consists of meso-DAP direct. The optimal growth temperature is 30-40°C, but can grow from 10 –50°C, and has a pH range of 4.9-9.3 (Sneath *et al*., 1986). *B. cereus* was considered harmless until the 1950’s, but is now recognized as the most prevalent cause of food poisoning among the genus *Bacillus*. *B. cereus* causes food poisoning most commonly in reheated fried rice from restaurants. The infectious dose is greater than or equal to one million viable cells. The
spore survives the heating and then germinates in the ideal conditions found in the buffet. *B. cereus* can also cause eye infections such as post-traumatic endophthalmitis, which is most prevalent among drug addicts. Hemolysin is made up of the protein BL, coded by the gene *hblA*, the protein B coded by *hblC* and the proteins L₁ and L₂, coded by *hblD*. The enterotoxins are coded by *bceT*, the cereolysins phospholipase C by *cerA* and sphingomyelinase by *cerB*. Two types of disease are caused by *B. cereus*. The first, “diarrheal syndrome” has an onset of 8-18 hours. The symptoms are abdominal cramps, watery diarrhea, rectal tenesmus, and sometimes nausea and vomiting. The other disease is called “emetic syndrome”, and brings about acute vomiting 1-5 hours after ingestion (Labbe and Garcia, 2001). In addition, *B. cereus*, with a mol\% G+C of 35.7 (Tₘ), produces enzymes such as lecithinase, proteolytic enzymes and enzymes which lyse bacterial cells. The rods of *B. cereus* tend to occur in chains and make colonies with a frosted glass appearance, hence the name, *cereus*, meaning waxen or wax colored (Sneath *et al.*, 1986). *B. cereus* is genetically very similar to *B. anthracis* and *B. thuringiensis*. The three differ in a small amount of chromosomal DNA and in the plasmids they harbor. Also, the type of disease caused differs among the species, with *B. anthracis* causing anthrax and *B. thuringiensis* producing an insecticidal toxin (Helgason *et al.*, 2000). As well as food poisoning, *B. cereus* is a significant problem in both the dairy and paper mill industries. Below is a scanning electron micrograph of *B. cereus* courtesy of SCIMAT, produced from the bottom of a colony provided by Dr. Burton Blais and Mr. Stephen Shaw. The bar at the bottom of the picture is 2 µm across. Figure 31 is the gas chromatograph printout identifying *B. cereus*. 
**Figure 30:** Electron micrograph of *Bacillus cereus*

**Figure 31:** Computer analysis and identification resulting from the gas chromatogram for *Bacillus cereus*
**Bacillus marinus** is found in marine sediments and typically grows between 5-30°C and in up to 7% NaCl. In fact, *marinus* is a Latin term for marine. The mol% G+C content of this species is 37.6 (T<sub>m</sub>). The murein type of *B. marinus* is ND (Sneath et al., 1986). Below is the chromatogram from the gas chromatograph for *B. marinus*.

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**Figure 32:** Gas chromatogram for *Bacillus marinus*

*Bacillus subtilis* is a long, slender rod, with *subtilis* a Latin word meaning slender, that grows at 10°C - 50°C and in conditions of up to 7% NaCl. The pH range is 5.5-8.5. The mol% G+C content is 42.9 (T<sub>m</sub>), and the murein consists of meso-DAP direct. It is important in the soil because it decomposes animal and plant material, such as the pectin and polysaccharides. Some strains infect potatoes, causing potato rot. *B. subtilis* produces and excretes levan and dextran. It can also live in very restrictive conditions, needing only glucose and ammonium salts. Ropy or slimy bread is caused by *B. subtilis* (Sneath et al., 1986). *B. subtilis* has been found in dried skim milk and causes both diarrhea and vomiting. In fact, the supernatant for *B. subtilis* cultures that have been heated for 90 minutes at 100°C induces vomiting in cats when injected intravenously (Labbe and Garcia, 2001). *B. subtilis* has been used as a probiotic in chicken feed. It
causes an increase in the speed of weight gain, while decreasing the number of coliforms, especially *Campylobacter* (Prescott *et al*., 2002). In addition, seedling disease can be controlled by inoculation of the seeds with *B. subtilis* as well as diseases in cut carnations (Walker, 1975). The following is an electron micrograph of both vegetative cells and spores of *B. subtilis*, courtesy of SCIMAT. The bar on the bottom is 2 µm across. Figure 34 is the chromatogram from the gas chromatograph for *B. subtilis*.

![Electron micrograph of Bacillus subtilis](image)

**Figure 33:** Electron micrograph of *Bacillus subtilis*

**Figure 34:** Computer analysis and identification resulting from the gas chromatogram for *Bacillus subtilis*
**Brevibacillus** spp. are used in biocontrol of the mold *Botrytis cinerea*. *Botrytis* is a type of fungus which infects crops as well as contributing to indoor air quality problems. Below is the chromatogram from the gas chromatograph identifying *Brevibacillus agri*.

**Figure 35**: Computer analysis and identification resulting from the gas chromatogram for *Brevibacillus agri*

*Brevibacterium iodinum* is a Gram-positive, nonmotile, obligate aerobe commonly isolated from milk. *Brevis* is a Latin adjective meaning short and *bacterium* is Greek for small rod. *Iodinum* is middle Latin for iodine. The natural habitat of this organism is unknown. *B. iodinum* can be confused with *Arthrobacter* because both exhibit a marked rod-coccus pleomorphic growth cycle. The cells are variable in length, due to the rod-coccus growth cycle, but are approximately 0.6-1.0 μm in diameter.

Although members of *Brevibacterium* are nonmotile, “weak or paralyzed” flagella were detected in *B. iodinum* by electron microscopy. The mol% G+C content is 60-63 (Tm). It is also oxidase positive and positive for hydrogen sulfide on cysteine. *B. iodinum*
produces a capsular-like, slimy coating, but capsule formation is not a usual characteristic of brevibacteria. Most brevibacteria are proteolytic, capable of hydrolyzing gelatin, casein, and milk. *B. iodinum* also produces extracellular purple crystals of iodonin (Sneath *et al.*, 1986). The following is an electron micrograph of *Brevibacterium linens*, provided by the website of the Utah State University Electron Microscopy Lab. Figure 37 shows the chromatogram from the gas chromatograph used to identify this organism.

![Figure 36: Electron micrograph of *Brevibacterium linens*](image)

*Figure 36:* Electron micrograph of *Brevibacterium linens*
The Deinococcaceae family contains only one genus, namely Deinococcus.

Deinos is a Greek adjective meaning strange or unusual and coccus is the Greek noun for a grain or berry. These bacteria are catalase positive, nonmotile, and aerobic. The members are Gram-positive, although the cell wall of Deinococcus contains an outer membrane with lipids and proteins, a feature uncharacteristic of other Gram-positive bacteria. The fatty acid profile is also unlike that of a typical Gram-positive organism, showing a high quantity of even numbered, straight chained, saturated and unsaturated acids. The cells are usually spherical, 0.5-3.5 μm in diameter, but may elongate and show dividing pairs. The mol% G+C of the DNA is 60-70 (Tm). One unique characteristic of almost all strains is that they are radiation and desiccation resistant. For example, many strains of D. radiodurans are resistant to 1.5 Mrad of gamma radiation and up to 500 Jm⁻² of U.V. radiation. This radiation resistant property is also found with the

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**Figure 37:** Computer analysis and identification resulting from the gas chromatogram for *Brevibacterium iodinum*
*D. erythromyxa.* However, *D. erythromyxa*, formally known as *Sarcina erythromyxa*, is more closely related to *Micrococcus* than *Deinococcus* on the basis of peptidoglycan and phospholipid typing (Sneth et al., 1986). Figure 38 is an electron micrograph of *Deinococcus* courtesy of Marc Angee from Les Decouvertes Impossibles. Figure 39 shows the chromatogram from the gas chromatograph used to identify this organism.

**Figure 38:** Electron micrograph of *Deinococcus*

**Figure 39:** Computer analysis and identification resulting from the gas chromatogram for *Deinococcus erythromyxa*
*Flavobacterium* is a genus of Gram-negative coccobacilli to slender rods that are 0.5 µm wide and 1.0-3.0 µm long (Sneath *et al.*, 1986). These facultative aerobes are chemoorganotrophic and are catalase and oxidase positive. The mol % G+C ranges from 31-42% (Tm). *Flavobacterium* is found in soil, plants, food stuffs and almost every water system including distilled water lines. In soils, *Flavobacterium* species are concentrated throughout the rhizosphere, the layer of soil immediately surrounding roots. *Flavobacterium* are fastidious microbes and some strains are pathogenic to humans and animals. Some species can cause infection in premature infants and immunocompromised individuals. The colonies may have a yellow, orange, or red to brown pigment and emit a fruity odor. The colonies are often circular, smooth and shiny. *Flavobacterium* is able to decompose biphenols and selectively stimulate root exudates (Tate, 1995). The following is an electron micrograph of *Flavobacterium psychrophilum*, provided by the Link Aquaculture Programme website. The magnification is 4300x. Figure 41 shows the chromatogram from the gas chromatograph used to identify *F. johnsoniae*.

**Figure 40:** Electron micrograph of *Flavobacterium psychrophilum*
Figure 41: Computer analysis and identification resulting from the gas chromatogram for *Flavobacterium johnsoniae*.

A member of the family Enterobacteriaceae, the *Klebsiella* genus consists of Gram-negative, nonmotile, facultative anaerobe. The cells are straight rods, 0.3-1.0 µm in diameter and 0.6-6.0 µm in length, occurring singly, in pairs, or in short chains. The mol% G+C content is 53-58 (Tm). *Klebsiella* possess a large polysaccharide capsule to aid in resistance to phagocytes, with some having fimbriae (pili). These virulence factors allow Klebsiellae to be important opportunistic pathogens that can cause bacteremia, pneumonia, urinary tract infection and several other types of infection in humans. All *Klebsiella* strains are resistant to ampicillin, and there is a problem with nosocomial...
strains having multiple antibiotic resistances (Sneath *et al*., 1986). In fact, *K. pneumoniae* is second only to *E. coli* as a cause of nosocomially acquired bacteremia. Nosocomial transmission occurs via in-dwelling catheters and endotracheal tubes (Warinner, 2001).

Klebsiellae are widely distributed in nature, found in soil, water and grain, as well as other places. They are also normal inhabitants of the intestinal tract. *K. pneumoniae* is the type species for this genus (Sneath *et al*., 1986). *K. pneumoniae* inhabits the rhizosphere and is a diazotroph that fixes nitrogen. Anaerobic conditions are needed because the nitrogenase, the enzyme that fixes nitrogen, from *K. pneumoniae* is sensitive to oxygen so nitrogen is not fixed in the presence of oxygen. Ammonium production by *K. pneumoniae* is 200-fold greater at low molecular oxygen (2 KPa) versus 10 KPa (Tate, 1995). Shown below is a Gram-stain, magnified 1000x on the oil immersion lens of a light microscope, of sputum from a patient with pneumonia caused by *K. pneumoniae*. The picture is provided by Douglas Hornick, MD at the University of Iowa’s Virtual Hospital.

![Figure 42: Gram stain of Klebsiella pneumoniae](image-url)
Microbacterium saperdae and M. liquefaciens (type strain) were formally named Aureobacterium saperdae and A. liquefaciens, respectively. Microbacterium is a genus of Gram-positive, motile or nonmotile, aerobic coryneforms. The motile organisms have between one and three flagella. The mol% G+C content of the DNA is 69-75.4 (Tm). The cells change from small, slender, irregular rods (~0.4-0.8 μm in diameter by ~1.0-4.0μm in length) in log phase to shorter rods with some cocci being produced during the stationary phase. V-shaped cell arrangements are seen during log growth as well as the longer rods. The genus does not exhibit a marked rod to coccus cycle, but is very similar. The optimal temperature for Microbacterium is 25-30°C, with a minimum of 10°C and a maximum growth temperature of 36-40°C. The members of this genus are also chemoorganotrophs, capable of some fermentation although the vast majority undergoes respiration. Microbacterium is catalase positive, non acid-fast, and are not sporeformers. They are also catalase positive and some require siderophores found in the soil, an example being terregens factors. M. liquefaciens is found in dairy products, most likely as a result of improper cleansing of dairy equipment. M. saperdae is isolated from the body cavity of dead insects (Sneath et al., 1986). Below are the two chromatograms produced by the gas chromatograph in order to identify M. saperdae and M. liquefaciens.
**Figure 43:** Computer analysis and identification resulting from the gas chromatogram for *Microbacterium saperdae*

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**Figure 44:** Computer analysis and identification resulting from the gas chromatogram for *Microbacterium liquefaciens*
The *Morganella* genus, a member of the Enterobacteriaceae family, consists of Gram-negative, motile, facultative anaerobes. The cells are straight rods, 0.6-0.7 µm in diameter and 1.0-1.7 µm in length. Members of *Morganella* were once classified as *Proteus*, but were removed because, along with other factors, analysis revealed the organisms were related not more than 20% to *Proteus*. *Morganella* strains are distinguished by their ability to ferment carbohydrates. *M. morganii* is the type species for this genus.

*Morganella* is probably involved in urinary tract infections, particularly those of nosocomial origin. It is a secondary opportunistic pathogen and has been isolated from blood, pus, and sputa of patients with bacteremia, respiratory tract infections and wound infections. Although the habitat is not known, *Morganella* has been found in the intestines of humans, dogs, other mammals and reptiles (Sneath *et al.*, 1986).

*Paenibacillus* spp. are able to break down a number of natural polymers. One member has the ability to produce an antifungal toxin (peptide). Figure 45 shows the chromatogram produced by the gas chromatograph and used to identify *P. macerans*. 
Figure 45: Computer analysis and identification resulting from the gas chromatogram for *Paenibacillus macerans*

*Rhodococcus* is a genus of 14 Gram-positive, aerobic, nonmotile species. The name *Rhodococcus* comes from the Greek noun *rhodon*, meaning the rose, and *coccus* meaning a grain. The mol% G+C of the DNA varies between 63-72% (Tm). Many strains are able to form hyphae that fragment into rods and cocci, but do not form conidia or endospores. The species of *Rhodococcus* were first assigned to *Mycobacterium* until further chemical and genetic tests warranted the creation of a new genus. *Rhodococcus* is well known for its ability to break down toxic compounds in the soil. It can grow on n-butyramide, formamide, acetamide, propionamide as well as malonamide, benzamide, á-phenylacetamide and 3-aminopropionitrile. Rhodococci are involved in the degradation of lignin-related compounds and humic acid. In addition, they have been isolated from petroleum contaminated soil, and also produce enzymes that are utilized in the transformation of xenobiotics, especially those associated with nocardiae. Some rhodococci can degrade cyanide-substituted compounds, such as the acrylamide
monomer (a neurotoxin), by deaminating it to acrylic acid, ammonia and water.

Camphor is broken down by *R. rhodochrous*. The first step is hydroxylation, followed by dehydrogenation to â-diketone and cleavage of the ring. Next, oxygen is inserted adjacent to the ketone group, causing opening of the second ring. The lactone product can be broken down by other organisms (Sneath *et al.*, 1986).

*R. rhodochrous*, the type species, is isolated from the soil, while *R. luteus*, also known as *R. fascians*, is isolated from the soil and the skin of the intestinal tract of the carp (Sneath *et al.*, 1986). Below is an electron micrograph of *Rhodococcus* provided by the Canada’s Michael Smith Genome Sciences Centre. Figure 47 is the chromatogram produced by the gas chromatograph used to identify *R. luteus*.

Figure 46: Electron micrograph of *Rhodococcus*
Figure 47: Computer analysis and identification resulting from the gas chromatogram for *Rhodococcus luteus*

*Shigella* is of the family *Enterobacteriacea* and shares many genetic characteristics with *E. coli*. *Shigella* is named after K. Shiga, the discoverer of the dysentery microorganism, while *sonnei* is derived from the name Carl Sonne. *Shigella* species are Gram negative, facultative anaerobic straight rods, with the human GI tract as their reservoir. They also tend to be lactose nonfermenters, and are catalase positive and oxidase negative. In fact, *S. sonnei* is one of the few *Shigella* species that ferments lactose. The mol % G+C content of the DNA is 49-53% (Tm) (Sneath et al., 1986). All four species of *Shigella* are non-motile, and are differentiated by group-specific polysaccharide antigens of the LPS.
*Shigella* organisms are highly infectious human pathogens, with a low inoculum dose to produce illness. For instance, as few as 10-100 of the most virulent species can cause disease. Once ingested, *Shigella* colonizes the lower end of the intestinal tract and invades the mucosal surface of the intestinal epithelial cells. This results in a local inflammatory response and dysentery. *S. sonnei* is the principal cause of shigellosis in the United States, and results in watery diarrhea (Labbe and Garcia, 2001). Although *Shigella* infects primarily humans and primates, it has also been known to infect dogs (Sneath *et al.*, 1986). Figure 48 is an electron micrograph of *Shigella* provided by Dennis Kunkel.

**Figure 48:** Electron microgram of *Shigella*
MATERIALS AND METHODS

Isolation and Identification of Soil Isolates

By design, some of the bacteria used in this study were laboratory strains, while the majority of the organisms were isolated from the soil. The bacteria were extracted from the soil by taking 10 grams of soil and mixing it with 95 ml of phosphate buffered saline pH 7 ((PBS) 8.5 g NaCl, 0.3 g KH$_2$PO$_4$, 0.6 g Na$_2$HPO$_4$, 0.1 g peptone bring up to 1L with ultra pure water and adjust to pH 7 using HCl or NaOH). This mixture was then blended in a blender for 3 bursts of 20 seconds on high, with 20 second pauses in between bursts. Serial dilutions were then performed using sterile water. Plates of one-half strength TSA (2.5 g NaCl, 10 g Peptone, 1.25 g potassium phosphate dibasic, and 1.25 g D-glucose; brought up to 1 L with deionized water) were spread with 100 µl of dilutions to produce plates with dilution factors of $1 \times 10^{-3}$ to $1 \times 10^{-6}$. The plates were incubated for 3-5 days at 30°C. To assure purity, isolated colonies were streaked onto a one-half strength TSA plate. These plates were allowed to grow at 30°C for 1-2 days. After the bacteria were determined to be pure, they were then streaked for harvest onto a full-strength TSA plate (5g NaCl, 20g Peptone, 2.5g potassium phosphate dibasic, and 2.5g D-glucose; brought up to 1L with deionized water) followed by incubation for 24 hours at 30°C. Next, 40 mg of the cells were harvested from the third quadrant of the harvest plate. A harvest plate is streaked by spreading a loop full of the isolated colony onto the first quadrant of a new plate. Without flaming, the loop is dragged into an adjacent quadrant. Again, without flaming the loop, the third quadrant is streaked from the second quadrant. Finally, the loop is flamed and a fishtail streak is made from the third quadrant. The cells were saponified with sodium hydroxide in methanol by heating
to 10°C. This disrupted the cells and cleaved the fatty acids from lipids. The fatty acids were then methylated with a methanol/hydrochloric acid mixture and heated to 8°C, producing methyl esterified fatty acids. These methylated fatty acids were then extracted using methyl tert-butyl ether in hexane. Decontamination (removing compounds that would clog the column) was performed with NaOH dissolved in water. The resulting solution was loaded on the Gas Chromatograph (GC), which was fitted with a 25 meter by 0.2 mm phenyl methyl silicone fused silica capillary column. The temperature was ramped from 17°C to 27°C at 5°C per minute during each run. Hydrogen was used as the carrier gas and air was used to support the flame (MIDI handbook). The methylated fatty acids were then separated based on size, carbon to carbon bond saturations, and other chemical properties in the column. After coming out of the column, the fatty acids are completely oxidized by combustion with air, and their concentrations are determined by the flame-ionization detector. With this information, a chromatograph is prepared to show a profile of the different fatty acids based on size (between 9 and 20 carbons in length). The chromatograph also reveals how much of each fatty acid is present by examining the area of each peak. This profile is then compared to a database and the organism or organisms with the most similar profiles are listed, as well as the percent similarity.

Preparing Bacteria for Salvage Enzyme Assays

All bacteria used in this study were washed by resuspending the cell pellet in 10mL of 50mM Tris pH 7 (6.055 g TRIS, 3.8 ml concentrated HCl; bring to 1L using ultra pure water). Next, the cells were centrifuged at 908 x g for 20 minutes.

Relative Centrifugal Force (RCF)=11.17 \( r \) (rpm/1000)² 
\[ r = \text{radius in cm} \]
The Gram negative bacteria were sonicated by resuspending 100 mg of wet cell pellet in 3 ml of 50 mM TRIS pH 7. These tubes of bacteria were placed in an ethanol ice bath, and then sonicated with a Branson Sonifier Cell Disruptor 2000. They were sonicated 3 times on ice, for 3 minutes, with a 3 minute pause between bursts. The burst intensity was level three. The cells were checked for breakage by viewing a wet mount of the cells. The wet mount was prepared by adding one drop of the sonicated cells to a slide. A cover slip was then carefully placed on top of the cell extract, while trying not to introduce air bubbles. This slide was viewed under 1000x magnification. If most of the cells were disrupted and no longer moving, the sonication was successful. If not, the sonication was repeated.

The Gram positive bacteria were broken by lysozyme enzymatic digest by resuspending 100 mg of washed, wet cell pellet in 3 ml of 50 mM TRIS pH 7 and adding lysozyme to a final concentration of 2 mg/ml. This solution was allowed to digest for 1 hour at 25°C. One half volume of Biospec 0.1 mm glass beads was then added to the solution, and the mixture was vortexed for 5 minutes. The cell extract was dispensed into 1.5 ml microcentrifuge tubes and spun for 10 minutes at 10,053 x g. The supernatant was used for all enzymatic assays.

Salvage Enzyme Assays and Analysis by HPLC

HPLC, or high performance (pressure) liquid chromatography, is used to separate molecules based on a wide variety of chemical properties. The properties being exploited for separation are determined by the type of column on the HPLC. In this study, a C18 reverse phase column was utilized. The C18 column separates compounds based on their polarity. A Gilson™ HPLC with two 322 pumps, a 156 UV/VIS detector, and a 234
Autoinjector, courtesy of Dr. C. Maier at Texas Woman’s University, was used and run by Unipoint™ 2.1 software. An isocratic flow of 100mM potassium phosphate pH 6 was the mobile phase. Three programs were written for the HPLC. The first program was used to slowly increase the flow of buffer from 0 ml/min to 1 ml/min and to turn the light source on. This program was named “startup”. The flow rate was slowly ramped up to reduce wear on the pump and column. This program also ensured that the lamp had been warmed for 30 minutes before use and that the column was cleaned for 30 minutes before the first injection. “Startup” was written in the following manner. At time zero, the pump was told to pump buffer at a rate of 0 ml/min and to slowly increase the rate to 1 ml/min over the next two minutes. The detector was switched on at time 0.08, followed by the wavelength being set to 261 nm at 0.1 minute. Time 1.98 was set for the detector to autozero. From minute 2.00 until 30, buffer ran through the column at a rate of 1 ml/min and the lamp warmed up at the wavelength 261 nm. The next stage was the “run” program, which kept the flow rate and lamp wavelength constant. In addition, the next sample was injected onto the column and the data recorded from the run. Runs were setup for 12 minutes to ensure that all compounds were eluted from the column. The first command in the “run” program, occurring at time zero, was to pump buffer at a rate of 1 ml/min. At 0.02 minutes the injector was programmed to inject 20 µl of the appropriate sample along with a loopful of buffer, approximately 20 µl. 0.02 minutes later, the system controller synchronized. At 0.06 minutes the data channel started recording the chromatogram, and 0.02 minutes later the detector autozeroed. Finally, at 11.98 minutes into the run, the data channels stopped recording the chromatogram. The last program was the “shut down” program, an inversion of “startup”. The first command allowed for
the detector to turn the lamp off. Next, the rate of buffer being pumped through the system was decreased at a constant rate from 1 ml/min to 0 ml/min in a two minute period. During sample preparation, the enzyme solutions were filtered using the polyvinylidene fluoride (PVDF) filter. This filter can be used with aqueous or organic solutions and has a low protein binding membrane with throughput of up to 10 ml. The PVDF filter was used because it stops the reaction by removing the enzyme from the reaction mix, and it removes any particles large enough to occlude the column.

1. Cytidine Deaminase assay (Cdd)

Cytidine + H$_2$O $\rightarrow$ Uridine + NH$_3$

This mix consisted of 200 µl of cell extract and 800 µl of 1 mM cytidine incubated at 37°C for 10 minutes. This solution was then filtered with a 1ml syringe through a 0.45 µm polyvinylidene fluoride (PVDF) filter, and 20 µl was injected into a C18 reverse phase column with a flow rate of 1ml/minute of 100mM potassium phosphate pH 6. Peaks were recorded using a wavelength of 261 nm. A peak of uridine is a positive indication of the presence of cytidine deaminase.

   Molecular weight of cytidine is 243.2. To make up 10 ml of 10 mM, add 0.02432g of cytidine to 10 ml of 50mM TRIS pH 7.

2. Cytidine Hydrolase assay

Cytidine + H$_2$O $\rightarrow$ Cytosine + ribose

This mix consisted of 200 µl of cell extract and 800 µl of 1 mM cytidine incubated at 37°C for 10 minutes. This solution was then filtered with a 1ml syringe through a 0.45 µm PVDF filter, and 20 µl was injected into a C18 reverse phase column with a flow rate
of 1ml/minute of 100mM potassium phosphate pH 6. Peaks were recorded using a
wavelength of 261nm. A peak of cytosine indicates the activity of cytidine hydrolase.

3. Cytidine Kinase assay (Udk)

Cytidine + ATP or GTP → CMP + ADP or GDP

This mix consisted of 200 µl of cell extract, 400 µl of 2mM cytidine, and 400 µl of 2 mM
ATP/GTP incubated at 37°C for 10 minutes. This solution was then filtered with a 1ml
syringe through a 0.45 µm PVDF filter, and 20 µl was injected into a C18 reverse phase
column with a flow rate of 1ml/minute of 100 mM potassium phosphate pH 6. Peaks
were recorded using a wavelength of 261nm. A CMP peak is a positive indication for
this enzyme.

Molecular weight of ATP is 551.2. To make up 1ml of 20 mM, add 0.011 grams
of ATP to 1ml of 50mM Tris pH 7.

4. Cytosine Deaminase assay (Cod)

Cytosine + H₂O → Uracil + NH₃

This mix consisted of 200 µl of cell extract and 800 µl of 1 mM cytosine incubated at
37°C for 10 minutes. This solution was then filtered with a 1ml syringe through a 0.45
µm PVDF filter, and 20 µl was injected into a C18 reverse phase column with a flow rate
of 1ml/minute of 100 mM potassium phosphate pH 6. Peaks were recorded using a
wavelength of 261 nm.

Molecular weight of cytosine is 111.1. To make up 10 ml of 10 mM, add 0.01
g of cytosine to 10 ml of 50mM TRIS pH 7.

5. Orotate Decarboxylase assay

Orotate → Uracil + CO₂
This mix consisted of 200 µl of cell extract and 800 µl of 1mM orotate incubated at 37°C for 10 minutes. This solution was then filtered with a 1ml syringe through a 0.45 µm PVDF filter, and 20 µl was injected into a C18 reverse phase column with a flow rate of 1ml/minute of 100 mM potassium phosphate pH 6. Peaks were recorded using a wavelength of 261 nm.

Molecular weight of sodium orotate is 194.2. To make up 10 ml of 10 mM, add 0.019 g of sodium orotate to 10 ml of 50 mM TRIS pH 7.

6. Uridine Hydrolase assay (Udh)

Uridine + H₂O → Uracil + ribose

This mix consisted of 200 µl of cell extract and 800 µl of 1 mM uracil incubated at 37°C for 10 minutes. This solution was then filtered with a 1ml syringe through a 0.45 µm PVDF filter, and 20 µl was injected into a C18 reverse phase column with a flow rate of 1ml/minute of 100 mM potassium phosphate pH 6. Peaks were recorded using a wavelength of 261 nm.

Molecular weight of uridine is 244. To make up 10 ml of 10mM, add 0.024 g of uridine to 10 ml of 50 mM TRIS pH 7.

7. Uridine Kinase assay (Udk)

Uridine + ATP or GTP → UMP + ADP or GDP

This mix consisted of 200 µl of cell extract, 400 µl of 2 mM uridine, and 400 µl of 2 mM ATP/GTP incubated at 37°C for 10 minutes. This solution was then filtered with a 1ml syringe through a 0.45 µm PVDF filter, and 20 µl was injected into a C18 reverse phase column with a flow rate of 1ml/minute of 100 mM potassium phosphate pH 6. Peaks
were recorded using a wavelength of 261 nm. A uridine peak indicates uridine kinase activity.

8. Uridine Phosphorylase assay (Udp)

Uridine + Pi $\rightarrow$ Uracil + ribose-1-P

This mix consisted of 200 µl of cell extract and 800 µl of 1 mM uridine incubated at 37°C for 10 minutes. This solution was then filtered with a 1 ml syringe through a 0.45 µm PVDF filter, and 20 µl was injected into a C18 reverse phase column with a flow rate of 1ml/minute of 100 mM potassium phosphate pH 6. Peaks were recorded using a wavelength of 261 nm.

9. Cytidine Phosphorylase assay

Cytidine + Pi $\rightarrow$ cytosine + ribose-1-P

This mix consisted of 200 µl of cell extract, 400 µl of 2 mM cytidine, and 400 µl of 2 mM phosphate buffer incubated at 37°C for 10 minutes. This solution was then filtered with a 1 ml syringe through a 0.45 µm PVDF filter, and 20 µl was injected into a C18 reverse phase column with a flow rate of 1ml/minute of 100 mM potassium phosphate pH 6. Peaks were recorded using a wavelength of 261 nm.

Molecular weight of cytidine is 243.2. To make up 10 ml of 10 mM, add 0.024 g of cytidine to 10 ml of 50 mM TRIS pH 7.

10. Uracil Phosphoribosyltransferase

Uracil + PRPP $\rightarrow$ UMP + PP$_i$

This mix consisted of 200 µl of cell extract, 400 µl of 2 mM uracil, and 400 µl of 2 mM PRPP incubated at 37°C for 10 minutes. This solution was then filtered with a 1 ml syringe through a 0.45 µm PVDF filter, and 20 µl was injected into a C18 reverse phase column with a flow rate of 1ml/minute of 100 mM potassium phosphate pH 6.
column with a flow rate of 1 ml/minute of 100 mM potassium phosphate pH 6. Peaks were recorded using a wavelength of 261 nm.

Molecular weight of PRPP is 499.6. To make up 1 ml of 20 mM, add 0.1 g of PRPP to 1 ml of 50 mM Tris pH 7.

Molecular weight of uracil is 112.1. To make up 10 ml of 10 mM, add 0.01 g of uracil to 10 ml of 50 mM TRIS pH 7.

11. Microorganism Control

This mix consisted of 200 µl of cell extract and 800 µl of 50 mM Tris pH 7 incubated at 37°C for 10 minutes. This solution was then filtered with a 1 ml syringe through a 0.45 µm PVDF filter, and 20 µl was injected into a C18 reverse phase column with a flow rate of 1 ml/minute of 100 mM potassium phosphate pH 6. The results were monitored using a wavelength of 261 nm.
RESULTS

More than 20 different indigenous soil bacteria were isolated and identified from local Texas soils. Strains were identified by MIDI and their salvage pathways delineated by HPLC. This work represents an expansion and continuation of the work of D.A. Beck (1995) who examined a number of laboratory strains for their pyrimidine salvage. She placed these into nine groups as shown in Table 1. In the present study an additional 10 groups were added (Table 2). Groups were defined as having one or more enzymes changes defining one group from another. A summary illustrating profiles of all 19 groups is found in Table 3. Tables 4 and 5 show the enzymes present or absent and their groupings for 21 organisms. Figure 49 shows the retention times in minutes on the X-axis and peak heights on the y-axis expressed in millivolts of a mixture of standards. All HPLC chromatograms follow this format. Figure 50 shows Group 1 where the salvage pathway for *E. coli* and other organisms is shown in full. It is followed by the 12 chromatograms for each of two organisms (Figures 51-62). These are followed by Figures 63-70 which contain groups 2-9 for which no representative organisms exist in this study. Figure 71 representing group 10, is followed by 12 chromatograms as before (Figures 71-77). This template is followed throughout the remaining groups (Figures 78-178).
<table>
<thead>
<tr>
<th>Group I</th>
<th>Escherichia coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Citrobacter freundii</td>
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<tr>
<td></td>
<td>Enterobacter cloacae</td>
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<tr>
<td></td>
<td>Hafnia alvei</td>
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<td></td>
<td>Klebsiella pneumoniae</td>
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<td></td>
<td>Proteus mirabilis</td>
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<tr>
<td></td>
<td>Salmonella typhimurium</td>
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<tr>
<td></td>
<td>Serratia odorifera</td>
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<tr>
<td></td>
<td>Yersinia enterocolitica</td>
</tr>
<tr>
<td></td>
<td>Vibrio alginolyticus</td>
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<tr>
<td></td>
<td>Vibrio parahaemolyticus</td>
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<tr>
<td></td>
<td>Shewanella putrefaciens</td>
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<tr>
<td></td>
<td>Enterococcus faecalis</td>
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<tr>
<td>Group II</td>
<td>Burkholderia cepacia</td>
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<tr>
<td></td>
<td>Haemophilus influenzae</td>
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<tr>
<td></td>
<td>Mycobacterium smegmatis</td>
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<td></td>
<td>Proteus vulgaris</td>
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<tr>
<td></td>
<td>Pseudomonas mendocina</td>
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<td></td>
<td>Pseudomonas pseudoalcaligenes</td>
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<td></td>
<td>Pseudomonas stutzeri</td>
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<td></td>
<td>Rhizobium loti</td>
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<td>Group III</td>
<td>Pseudomonas indigofera</td>
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<td></td>
<td>Saccharomyces cerevisiae</td>
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<tr>
<td>Group IV</td>
<td>Bacillus megaterium</td>
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<td></td>
<td>Bacteroides fragilis</td>
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<td></td>
<td>Micrococcus luteus</td>
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<tr>
<td>Group V</td>
<td>Staphylococcus aureus</td>
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<tr>
<td>Group VI</td>
<td>Pseudomonas aeruginosa</td>
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<tr>
<td></td>
<td>Pseudomonas aureofaciens</td>
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<tr>
<td></td>
<td>Pseudomonas fluorescens</td>
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<tr>
<td></td>
<td>Alcaligenes faealis</td>
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<tr>
<td></td>
<td>Brevundimonas (Pseudomonas) diminuta</td>
</tr>
<tr>
<td></td>
<td>Comamonas (Pseudomonas) acidovorans</td>
</tr>
<tr>
<td></td>
<td>Comamonas (Pseudomonas) testosterone</td>
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<td></td>
<td>Rhizobium leguninosarum</td>
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<td>Rhizobium meliloti</td>
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<td>Stenotrophomonas (Xanthomonas) maltophilia</td>
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<td>Group VII</td>
<td>Pseudomonas putida</td>
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<td>Group VIII</td>
<td>Acinetobacter baumannii</td>
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<tr>
<td></td>
<td>Moraxella (Branhamella, Neisseria) catarrhalis</td>
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<tr>
<td></td>
<td>Neisseria meningitidis</td>
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<tr>
<td></td>
<td>Neisseria subflava</td>
</tr>
<tr>
<td>Group IX</td>
<td>Clostridium perfringens</td>
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</table>

**Table 1:** Organisms assorted by salvage groups from earlier study (provided by Debrah Beck, 1995)
<table>
<thead>
<tr>
<th>Group I</th>
<th><em>Flavobacterium johnsoniae</em> (positive for orotate decarboxylase activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Aeromonas trota</em> (positive for orotate decarboxylase activity)</td>
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<td>Group II</td>
<td><em>Burkholderia cepacia</em></td>
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<td>Group III</td>
<td><em>Pseudomonas indigofera</em></td>
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<td>Group IV</td>
<td><em>Bacillus megaterium</em></td>
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<td>Group V</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
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<td>Group VI</td>
<td><em>Pseudomonas aeruginosa</em> (positive for orotate decarboxylase activity)*</td>
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<tr>
<td>Group VII</td>
<td><em>Pseudomonas putida</em></td>
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<td>Group VIII</td>
<td><em>Acinetobacter baumannii</em></td>
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<td><em>Clostridium perfringens</em></td>
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<td>Group XIV</td>
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<td>Group XV</td>
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<td>Group XVI</td>
<td><em>Microbacterium liquefaciens</em> (positive for orotate decarboxylase activity)</td>
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<td>Group XVIII</td>
<td><em>Bacillus cereus</em> (positive for orotate decarboxylase activity)</td>
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<tr>
<td>Group XIX</td>
<td><em>Acinetobacter lwoffii</em> (positive for orotate decarboxylase activity)</td>
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</table>

*Results from a previous study, D.A. Beck, PhD Dissertation, 1995

**Table 2:** Organisms assorted by salvage groups from this study
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<thead>
<tr>
<th>Group</th>
<th>CrD (cdd)</th>
<th>CrH (nuh)</th>
<th>CrK (udk)</th>
<th>CD (cod)</th>
<th>OD</th>
<th>UrH (nuh)</th>
<th>UrK (udk)</th>
<th>UrP (udp)</th>
<th>CrP</th>
<th>CMP Gly (nmg)</th>
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+ indicates a positive result for enzyme assayed
- indicates a negative result for enzyme assayed

**Table 3**: Pyrimidine salvage enzymes present in each salvage group
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<th>Group I</th>
<th>CrD (cdd)</th>
<th>CrH (nuh)</th>
<th>CrK (udk)</th>
<th>CD (cod)</th>
<th>OD</th>
<th>UrH (nuh)</th>
<th>UrK (udk)</th>
<th>UrP (udp)</th>
<th>CrP</th>
<th>CMP Gly (nmg)</th>
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+ indicates a positive result for enzyme assayed  
- indicates a negative result for enzyme assayed  
P indicates probable, but further investigation is required

**Table 4**: Results of enzyme assays for each microorganism
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<th>Organism</th>
<th>CrD (cdd)</th>
<th>CrH (nuh)</th>
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<th>CD (cod)</th>
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<th>UrP (udp)</th>
<th>CrP</th>
<th>CMP Gly (nmg)</th>
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+ indicates a positive result for enzyme assayed  
- indicates a negative result for enzyme assayed  
P indicates probable, but further investigation is required  
N. A. indicates this enzyme not assayed  

**Table 5:** Results of enzyme assays for additional microorganisms
Figure 49: HPLC chromatogram of a mixture of the standards cytosine, uracil, cytidine, and uridine.
Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. uridine hydrolase (Udh), 4. cytosine deaminase (Cod), 5. cytidine deaminase (Cdd), 6. uridine kinase (Udk), 7. 5'-nucleotidase, 8. CMP glycosylase, 9. CMP kinase (Cmk)

**Figure 50:** Group I Salvage Scheme
Figure 51: HPLC chromatograms of Flavobacterium johnsoniae cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 52: HPLC chromatograms of *Flavobacterium johnsoniae* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
**Figure 53**: HPLC chromatograms of *Flavobacterium johnsoniae* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 54: HPLC chromatograms of *Flavobacterium johnsoniae* uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
**Figure 55**: HPLC chromatogram of *Flavobacterium johnsoniae* cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
Figure 56: HPLC chromatogram of *Flavobacterium johnsoniae* CMP glycosylase and 5’-nucleotidase assays. (a) CMP glycosylase assay. (b) 5’-nucleotidase assay.
Figure 57: HPLC chromatograms of *Aeromonas trota* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 58: HPLC chromatograms of *Aeromonas trota* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
**Figure 59:** HPLC chromatograms of *Aeromonas trota* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 60: HPLC chromatograms of *Aeromonas trota* uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
Figure 61: HPLC chromatograms of *Aeromonas trota* cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
Figure 62: HPLC chromatogram of *Aeromonas trota* CMP glycosylase and 5’-nucleotidase assays. (a) CMP glycosylase assay. (b) 5’-nucleotidase assay.
Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. cytosine deaminase (Cod), 4. cytidine deaminase (Cdd), 5. uridine kinase (Udk), 6. 5'-nucleotidase, 7. CMP glycosylase, 8. CMP kinase (Cmk)

**Figure 63**: Group II Salvage Scheme
Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine hydrolase (Udh), 3. cytosine deaminase (Cod), 4. cytidine deaminase (Cdd), 5. uridine kinase (Udk), 6. 5’-nucleotidase, 7. CMP glycosylase, 8. CMP kinase (Cmk)

Figure 64: Group III Salvage Scheme
Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. cytidine deaminase (Cdd), 4. uridine kinase (Udk), 5. 5'-nucleotidase, 6. CMP kinase (Cmk)

**Figure 65**: Group IV Salvage Scheme
Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine hydrolase (Udh), 3. cytidine deaminase (Cdd), 4. uridine kinase (Udk), 5. 5'-nucleotidase, 6. CMP kinase (Cmk)

**Figure 66:** Group V Salvage Scheme
Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine hydrolase (Udh), 3. cytosine deaminase (Cod), 4. 5'-nucleotidase, 5. CMP glycosylase, 6. CMP kinase (Cmk)

**Figure 67**: Group VI Salvage Scheme
Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. uridine hydrolase (Udh), 4. cytosine deaminase (Cod), 5. 5'-nucleotidase, 6. CMP glycosylase, 7. CMP kinase (Cmk)

**Figure 68:** Group VII Salvage Scheme
Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. CMP kinase (Cmk)

*Figure 69: Group VIII Salvage Scheme*
Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. uridine hydrolase (Udh), 4. cytidine deaminase (Cdd), 5. uridine kinase (Udk), 6. 5'-nucleotidase, 7. CMP kinase (Cmk)

**Figure 70:** Group IX Salvage Scheme
Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine phosphorylase (Udp), 3. cytidine phosphorylase, 4. uridine hydrolase (Udh), 5. cytidine deaminase (Cdd), 6. uridine kinase (Udk)
Figure 72: HPLC chromatograms of *Microbacterium saperdae* (*Aureobacterium*) cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 73: HPLC chromatograms of *Microbacterium saperdae* (*Aureobacterium*) cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 74: HPLC chromatograms of Microbacterium saperdae (Aureobacterium) orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
**Figure 75:** HPLC chromatograms of *Microbacterium saperdae* (*Aureobacterium*) uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
Figure 76: HPLC chromatograms of *Microbacterium saperdae* (*Aureobacterium*) cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
Figure 77: HPLC chromatogram of *Microbacterium saperdae* (*Aureobacterium*) CMP glycosylase and 5’nucleotidase assays. (a) CMP glycosylase assay. (b). 5’-nucleotidase assay.
Figure 78: Group XI Salvage Scheme

Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine hydrolase (Udh), 3. uridine phosphorylase (Udp), 4. cytidine deaminase (Cdd), 5. cytosine deaminase (Cod), 6. uridine kinase (Udk), 7. CMP glycosylase (Nmg)
Figure 79: HPLC chromatograms of *Klebsiella oxytoca* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 80: HPLC chromatograms of *Klebsiella oxytoca* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 81: HPLC chromatograms of *Klebsiella oxytoca* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 82: HPLC chromatograms of *Klebsiella oxytoca* uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
Figure 83: HPLC chromatograms of *Klebsiella oxytoca* cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
Figure 84: HPLC chromatogram of *Klebsiella oxytoca* CMP glycosylase and 5’nucleotidase assays. (a) CMP glycosylase assay. (b) 5’-nucleotidase assay.
**Figure 85**: Group XII Salvage Scheme

Enzymes: 1. uracil phosphoribosyl transferase (Upp), 2. uridine hydrolase (Udh), 3. uridine kinase (Udk), 4. cytidine deaminase (Cdd)
Figure 86: HPLC chromatograms of *Bacillus marinus* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 87: HPLC chromatograms of *Bacillus marinus* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 88: HPLC chromatograms of *Bacillus marinus* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 89: HPLC chromatograms of *Bacillus marinus* uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
Figure 90: HPLC chromatograms of *Bacillus marinus* cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
Figure 91: HPLC chromatogram of *Bacillus marinus* CMP glycosylase and 5’-nucleotidase assays. (a) CMP glycosylase assay. (b) 5’-nucleotidase assay.
**Figure 92**: Group XIII Salvage Scheme

Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. cytosine deaminase (Cod), 3. cytidine deaminase (Cdd), 4. uridine kinase (Udk)
Figure 93: HPLC chromatograms of *Brevibacillus agri* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 94: HPLC chromatograms of *Brevibacillus agri* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 95: HPLC chromatograms of *Brevibacillus agri* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 96: HPLC chromatograms of *Brevibacillus agri* uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
Figure 97  HPLC chromatograms of *Brevibacillus agri* cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
Figure 98: HPLC chromatogram of *Brevibacillus agri* CMP glycosylase and 5’nucleotidase assays. (a) CMP glycosylase assay. (b). 5’-nucleotidase assay.
**Figure 99**: Group XIV Salvage Scheme

Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine hydrolase (Udh), 3. uridine phosphorylase (Udp), 4. cytidine deaminase (Cdd), 5. cytosine deaminase (Cod), 6. cytidine kinase, 7. 5'-nucleotidase, 8. CMP glycosylase (Nmg), 9. orotate decarboxylase

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**Diagram Details**

- **De novo pathway** indicated by dashed arrows.
- **Salvage pathway** indicated by solid arrows.
Figure 100: HPLC chromatograms of *Morganella morganii* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 101: HPLC chromatograms of *Morganella morganii* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 102: HPLC chromatograms of *Morganella morganii* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 103: HPLC chromatograms of *Morganella morganii* uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
Figure 104: HPLC chromatograms of *Morganella morganii* cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
Figure 105: HPLC chromatogram of *Morganella morganii* CMP glycosylase and 5’nucleotidase assays. (a) CMP glycosylase assay. (b). 5’-nucleotidase assay.
Figure 106: Group XV Salvage Scheme

Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine hydrolase (Udh), 3. uridine phosphorylase (Udp), 4. cytosine deaminase (Cod), 5. cytidine deaminase (Cdd), 6. CMP glycosylase (Nm), 7. uridine kinase (Udk), 8. 5'-nucleotidase, 9. orotate decarboxylase
Figure 107: HPLC chromatograms of *Brevibacterium iodinum* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 108: HPLC chromatograms of *Brevibacterium iodinum* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 109: HPLC chromatograms of *Brevibacterium iodinum* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 110: HPLC chromatograms of *Brevibacterium iodinum* uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
Figure 111: HPLC chromatogram of *Brevibacterium iodinum* cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
Figure 112: HPLC chromatogram of *Brevibacterium iodinum* CMP glycosylase and 5’nucleotidase assays. (a) CMP glycosylase assay. (b) 5’-nucleotidase assay.
Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine hydrolase (Udh), 3. uridine phosphorylase (Udp), 4. cytosine deaminase (Cod), 5. cytidine deaminase (Cdd), 6. CMP glycosylase (Nmg), 7. 5'-nucleotidase, 8. orotate decarboxylase

**Figure 113:** Group XVI Salvage Scheme
Figure 114: HPLC chromatograms of *Microbacterium liquefaciens* (Aureobacterium) cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 115: HPLC chromatograms of *Microbacterium liquefaciens* (*Aureobacterium*) cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 116: HPLC chromatograms of *Microbacterium liquefaciens* (*Aureobacterium*) orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 117: HPLC chromatograms of *Microbacterium liquefaciens* (Aureobacterium) uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
Figure 118: HPLC chromatograms of *Microbacterium liquefaciens* (*Aureobacterium*) cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
Figure 119: HPLC chromatogram of *Microbacterium liquefaciens* (*Aureobacterium*) CMP glycosylase and 5’nucleotidase assays. (a) CMP glycosylase assay. (b). 5’-nucleotidase assay.
Figure 120: Group XVII Salvage Scheme

Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine hydrolase (Udh), 3. uridine phosphorylase (Udp), 4. cytidine deaminase (Cdd), 5. 5’-nucleotidase
Figure 121: HPLC chromatograms of *Aeromonas sobria* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
**Figure 122**: HPLC chromatograms of *Aeromonas sobria* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 123: HPLC chromatograms of *Aeromonas sobria* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 124: HPLC chromatograms of *Aeromonas sobria* uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
Figure 125: HPLC chromatogram of *Aeromonas sobria* cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
Figure 126: HPLC chromatogram of *Aeromonas sobria* CMP glycosylase and 5’-nucleotidase assays. (a) CMP glycosylase assay. (b) 5’-nucleotidase assay.
Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. uridine hydrolase (Udh), 3. cytidine deaminase (Cdd), 4. 5'-nucleotidase, 5. CMP glycosylase (Nmgl), 6. orotate decarboxylase

**Figure 127:** Group XVIII Salvage Scheme
Figure 128: HPLC chromatograms of *Bacillus cereus* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 129: HPLC chromatograms of *Bacillus cereus* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 130: HPLC chromatograms of *Bacillus cereus* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 131: HPLC chromatograms of *Bacillus cereus* uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
Figure 132: HPLC chromatograms of Bacillus cereus cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
Figure 133: HPLC chromatogram of Bacillus cereus CMP glycosylase and 5’ nucleotidase assays. (a) CMP glycosylase assay. (b). 5’-nucleotidase assay.
**Figure 134**: Group XIX Salvage Scheme

Enzymes: 1. uracil phosphoribosyltransferase (Upp), 2. cytosine deaminase (Cod), 3. orotate decarboxylase
Figure 135: HPLC chromatograms of *Acinetobacter lwoffii* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 136: HPLC chromatograms of *Acinetobacter lwoffii* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 137: HPLC chromatograms of *Acinetobacter lwofii* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 138: HPLC chromatograms of *Acinetobacter lwofii* uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
Figure 139: HPLC chromatograms of *Acinetobacter lwofii* cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
Figure 140: HPLC chromatogram of *Acinetobacter lwofii* CMP glycosylase and 5’nucleotidase assays. (a) CMP glycosylase assay. (b). 5’-nucleotidase assay.
Figure 141: HPLC chromatograms of *Klebsiella pneumoniae* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 142: HPLC chromatograms of *Klebsiella pneumoniae* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 143: HPLC chromatograms of *Klebsiella pneumoniae* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 144 HPLC chromatograms of *Klebsiella pneumoniae* uridine kinase and filtered cell extract (control) assays. (a) uridine kinase assay. (b) filtered cell extract assay.
Figure 145: HPLC chromatograms of *Deinococcus erythromyxa* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 146: HPLC chromatograms of *Deinococcus erythromyxa* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 147: HPLC chromatograms of *Deinococcus erythromyxa* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 148: HPLC chromatograms of *Deinococcus erythromyxa* uridine kinase and filtered cell extract (control) assays. (a) uridine kinase assay. (b) filtered cell extract assay.
Figure 149: HPLC chromatograms of *Rhodococcus luteus* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 150: HPLC chromatograms of *Rhodococcus luteus* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 151: HPLC chromatograms of *Rhodococcus luteus* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 152: HPLC chromatograms of *Rhodococcus luteus* uridine kinase and filtered cell extract (control) assays. (a) uridine kinase assay. (b) filtered cell extract assay.
Figure 153 HPLC chromatograms of *Bacillus subtilis* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
**Figure 154**: HPLC chromatograms of *Bacillus subtilis* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
**Figure 155:** HPLC chromatograms of *Bacillus subtilis* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 156: HPLC chromatogram of *Bacillus subtilis* uridine kinase and filtered cell extract (control) assay. (a) uridine kinase. (b) filtered cell extract assay.
Figure 157: HPLC chromatograms of *Paenibacillus macerans* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 158: HPLC chromatograms of *Paenibacillus macerans* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
**Figure 159:** HPLC chromatograms of *Paenibacillus macerans* orotate decarboxylase and cytidine phosphorylase assays. (a) orotate decarboxylase assay. (b) cytidine phosphorylase assay.
Figure 160: HPLC chromatograms of *Paenibacillus macerans* filtered cell extract (control) assays.
Figure 161: HPLC chromatograms of *Aeromonas hydrophila* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 162: HPLC chromatograms of *Aeromonas hydrophila* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 163: HPLC chromatograms of *Aeromonas hydrophila* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 164: HPLC chromatograms of *Aeromonas hydrophila* uridine kinase and filtered cell extract (control) assays. (a) uridine kinase assay. (b) filtered cell extract assay.
Figure 165: HPLC chromatograms of *Shigella sonnei* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 166: HPLC chromatograms of *Shigella sonnei* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 167: HPLC chromatograms of *Shigella sonnei* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 168: HPLC chromatograms of *Shigella sonnei* uridine kinase and filtered cell extract (control) assays. (a) uridine kinase assay. (b) filtered cell extract assay.
Figure 169: HPLC chromatograms of *Arthrobacter globiformis* cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 170: HPLC chromatograms of *Arthrobacter globiformis* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 171: HPLC chromatograms of *Arthrobacter globiformis* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 172: HPLC chromatograms of *Arthrobacter globiformis* uridine kinase and filtered cell extract (control) assays. (a) uridine kinase assay. (b) filtered cell extract assay.
Figure 173: HPLC chromatograms of Agrobacterium radiobacter cytidine deaminase and cytidine hydrolase assays. (a) cytidine deaminase assay. (b) cytidine hydrolase assay.
Figure 174: HPLC chromatograms of *Agrobacterium radiobacter* cytidine kinase and cytosine deaminase assays. (a) cytidine kinase assay. (b) cytosine deaminase assay.
Figure 175: HPLC chromatograms of *Agrobacterium radiobacter* orotate decarboxylase and uridine hydrolase assays. (a) orotate decarboxylase assay. (b) uridine hydrolase assay.
Figure 176: HPLC chromatograms of Agrobacterium radiobacter uridine kinase and uridine phosphorylase assays. (a) uridine kinase assay. (b) uridine phosphorylase assay.
Figure 177: HPLC chromatograms of *Agrobacterium radiobacter* cytidine phosphorylase and filtered cell extract (control) assays. (a) cytidine phosphorylase assay. (b) filtered cell extract assay.
**Figure 178**: HPLC chromatogram of *Agrobacterium radiobacter* CMP glycosylase and 5’nucleotidase assays. (a) CMP glycosylase assay. (b) 5’-nucleotidase assay.
DISCUSSION

Soil bacteria are an integral key to life on the planet today. Therefore, the ways in which the enzymes that soil microorganisms depend on are influenced by environmental conditions is an important topic of study. In a world of expanding human population, decreasing acres of arable soil, and an escalating amount of toxins in the soil, the existence of these microorganisms will be of value more than ever. If humans continue these dire trends, the field of soil microbiology will have to increase in importance.

This body of work concentrated on natural soil organisms that had not previously been studied in the laboratory, as well as laboratory strains whose salvage schemes had not yet been determined. The pyrimidine salvage schemes are a diagramed view of how an organism recycles the breakdown products of RNA and DNA, how pyrimidines are used for other purposes in the cell, as well as any preformed pyrimidines obtained from exogenous sources. If the salvage profile of an organism differs from that of another pathway by one enzyme or more, it was placed in a new group. In the process of this study, it became necessary to add 10 new salvage groups to accommodate the bacteria studied (Table 2), as seen in Figures 72, 78, 85, 92, 99, 106, 113, 120, 127, and 134. This work, as well as previous work from our laboratory (D.A. Beck, Ph. D dissertation, 1995), both indicated that different species of a common genus exhibited diverse salvage pathways. For example, *Aeromonas trota* is a member of salvage Group I (Figure 50), while *Aeromonas sobria* is placed in Group XVII (Figure 120). This is due to the fact that *A. trota* converts cytidine and uridine into CMP and UMP respectively and cytosine to uracil, whereas *A. sobria* does not. A similar finding was reported between species of *Pseudomonas*. *Pseudomonas aeruginosa* lacks uridine phosphorylase, therefore is placed
in Group VI, however, *Pseudomonas putida*, a member of Group VII, produces uridine phosphorylase (Beck, 1995). The *Microbacterium* genus was also split between two groups. *M. saperdae* falls into Group X while *M. liquefaciens* is a member of Group XVI. In addition, an enzyme capable of converting orotate to uracil (orotate decarboxylase), an activity not previously seen, was found in nine of the organisms used in this project (Table 2). It is uncertain whether this activity is due to an enzyme specific for orotate decarboxylation, or the result of a nonspecific decarboxylase. The excretion of uracil into the medium from cells grown on orotate has been previously observed (Vogels and Van der Drift, 1976). It is interesting to note that the soil isolates belonging to the same natural environments overcome similar hardships, such as nutrient scarcity, temperature fluctuation, and varying water availability, with very dissimilar pyrimidine salvage.

The salvage groups were devised under the design that one enzyme difference between the microorganisms warranted their separation into different salvage groups. There are at least seven different enzymes that can be present or absent in pyrimidine salvage, with the potential of 13 depending on specificity. Examining just seven enzymes, each with the possibility of being present or absent, there is a possibility of 128 ($2^7$) salvage groups. It is likely that many of these possible combinations will not be seen in nature.

Typically, the following assays were carried out: cytidine deaminase, cytidine hydrolase, cytidine kinase, cytosine deaminase, orotate decarboxylase, uridine hydrolase, uridine kinase, uridine phosphorylase, cytidine phosphorylase, CMP glycosylase and 5'-nucleotidase. All 11 assays were run on 14 of the microorganisms shown. For seven
microorganisms, uridine phosphorylase, cytidine phosphorylase, CMP glycosylase and 5’-nucleotidase were not assayed, and on *Paenibacillus macerans*, the CMP glycosylase and 5’-nucleotidase were not assayed. Since uracil phosphoribosyltransferase has been shown in the literature (Neuhard and Kelln, 1996) to be present in all bacteria with the exception of *upp* mutants and since all organisms in this study were wild type strains, this assay was not performed. In addition, uridine 5’-monophosphate kinase (*pyrH*), nucleotide diphosphokinase (*ndk*) and cytidine 5’-triphosphate synthetase (*pyrG*) were all assumed to be present and therefore not assayed.

As the HPLC column ages, retention times vary and peak shouldering occurs. For this reason, standards were loaded and assayed with every set of assays performed with the HPLC.

In some cases, further investigation is needed to determine if an enzyme is present or not. For example, if an organism has a 5’-nucleotidase, it can breakdown CMP to cytidine. If cytidine hydrolase is present, cytosine is produced. When one is looking for the presence or absence of CMP glycosylase a cytosine peak is a positive result, however if 5’-nucleotidase and cytidine kinase are present, it is impossible to discern if CMP glycosylase is also present. Thus, to confirm the presence or absence of this enzyme, a cytidine hydrolase deficient mutant would be needed.

Orotate decarboxylase has not been used to define which salvage scheme a microorganism should be placed in. This is because orotate decarboxylase can be considered as part of the pyrimidine biosynthetic pathway or as a link between the biosynthetic and salvage pathways. Whereas orotate decarboxylase can be considered an catabolic enzyme in a catabolic pathway, either by the oxidation or reduction pathways, it
can also be thought of as an anabolic enzyme in an anabolic pathway, where uracil is produced for later conversion to UTP and CTP.

CMP glycosylase is probably in the organism *Flavobacterium johnsoniae*. CMP glycosylase converts CMP to cytosine, however, in this case, cytosine deaminase is present which converts cytosine to uracil. Figure 56 indicates that uracil was present.

Similarly, in *Brevibacterium iodinum*, a CMP glycosylase is probable because the cytosine peak could be produced by cytidine hydrolase acting on the cytidine that was produced by the 5’-nucleotidase (Figure 112).

*Microbacterium saperdae* produces a cytidine phosphorylase, an enzyme not previously reported. Uridine phosphorylases are reversible and it is assumed that any cytidine phosphorylase would be reversible also. This would allow *M. saperdae* to make cytidine from cytosine and ribose-1-P.

When *Microbacterium liquefaciens* is incubated with CMP, a uracil peak is observed. If the uracil is made by the deamination of cytosine, then a CMP glycosylase is present. However, if all of the uracil seen is produced by the hydrolytic cleavage of uridine (produced by the deamination of cytidine produced by 5’-nucleotidase), then there is no CMP glycosylase. Furthermore, if the uracil is produced from the cytidine being hydrolytically cleaved to cytosine before being deaminated to uracil, then no CMP glycosylase is present (Figure 119).

When *Morganella morganii* is incubated with CMP there are several methods by which the resulting uracil peak can arise. First, uracil may be produced by the conversion of the cytidine (produced by 5’-nucleotidase) to uridine and subsequently broken down to uracil. In this situation, CMP glycosylase can not be proven to be present. Alternately,
uracil may be produced by the combined action of CMP glycosylase and cytosine deaminase. *Aeromonas trota* displays a similar pattern to that of *Morganella morganii.*

The salvage enzymes present and absent to make up Group I (devised in Beck dissertation, 1995) are presented in Table 3. An outline of the pathway derived from these enzymes is seen in Figure 50 and the chromatograms for the two soil isolates *Flavobacterium johnsoniae* and *Aeromonas trota* that fall into this Group are seen in Figures 51-62. Group I differs from Group II in that Group II lacks the uridine hydrolase present in Group I. Group III differs from Group I only by the absence of uridine phosphorylase from Group III. Group I and Group XIV are identical except that Group XIV is devoid of uridine kinase.

The set of salvage enzymes that comprise Group X are shown in Table 3 and as a pathway in Figure 71. The representative soil isolate for Group X is *Microbacterium saperdae,* formerly known as *Aureobacterium saperdae.* The chromatograms from this microorganism are seen in Figures 72-77. Group X differs most significantly due to the presence of a putative cytidine phosphorylase, an enzyme proposed for the first time. It is of special interest that *M. liquefaciens* (Group XVI) and *M. saperdae* (Group X) are in different groups. They differ in that *M. saperdae* lacks cytosine deaminase, CMP glycosylase and 5’-nucleotidase, while all three are present in *M. liquefaciens.* Whereas *M. liquefaciens* lacks uridine kinase and cytidine phosphorylase found in *M. saperdae,* it is also to be noted that *M. liquefaciens* decarboxylates orotate. In this pathway, cytidine can be converted to either uridine or cytosine. Cytosine can be converted back to cytidine by a phosphorylase. Uridine can be converted directly to UMP or to uracil and then to UMP.
Group XI comprises the set of salvage enzymes seen in Table 3 and are depicted as a pathway in Figure 78. The representative soil isolate is *Klebsiella oxytoca*. *K. oxytoca*’s chromatograms are seen in Figures 79-84. Group XI differs from Group XV in that Group XV contains organisms with 5’-nucleotidase and Group XI does not. In this salvage scheme, cytidine can be converted in one step to uridine, cytosine or CMP. CMP can be catabolized in one step to cytosine, which can then be converted directly to uracil. In addition, uridine can be converted directly to uracil or UMP.

*Bacillus marinus* fits into Group XII, as shown by Table 2 and Figure 85. Chromatograms of the salvage enzymes for *B. marinus* are shown in Figures 86-91. Group XII is similar to Group XVII in that both contain only the enzyme cytidine deaminase in order to handle cytidine, while possessing no enzymes to metabolize cytosine. However, the two differ significantly in the remainder of their salvage. As the pathway of Group XII outlines, cytidine can be converted only to uridine. This may be phosphorylated to UMP or degraded to uracil.

Group XIII, shown in Table 3 and Figure 92, is represented by the soil isolate *Brevibacillus agri*, whose chromatograms are seen in Figures 93-98. Groups XIII and XVIII handle cytosine and cytidine identically, with the exception of both microorganisms lacking uridine phosphorylase, they handle uridine in "different" manners. Group XIII, Group XIX, Group XII, Group X and Group VIII are groups which do not breakdown CMP to either cytosine or cytidine. The pathway of Group XIII

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Cytosine → Cytidine → Uridine → Uracil → UMP
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243
shows cytidine proceeding to uridine and thence only to UMP. Cytosine is converted to uracil, which may then be converted to UMP.

Group XIV (seen in Table 3 and Figure 99) is identical to Group I with the exception of Group XIV lacking uridine kinase. Group XIV is represented by the human pathogen \textit{Morganella morganii}. The chromatograms of \textit{M. morganii} can be seen in Figures 100-105. CMP can be converted to cytidine or to cytosine, which may be converted to uracil or to cytidine. Cytidine may also proceed to CMP or to uridine, which may then be broken down only to uracil. Any uracil, derived directly from orotate, may also proceed to UMP.

\textit{Brevibacterium iodinum} isolated from the soil fits into the salvage Group XV, as shown in Table 2 and Figure 106. The chromatograms for \textit{B. iodinum} are in Figures 107-112. Group XV is most similar to group XI as discussed previously. In addition, it is interesting to note that Group XV has all seven salvage enzymes.

Group XVI, shown in Table 3 and Figure 113, contains the soil isolate \textit{Microbacterium liquefaciens}, formerly known as \textit{Aureobacterium liquefaciens}. The chromatograms for this organism can be seen in Figures 114-119. Group XVI is closely related to Group XV, with the only difference being that Group XVI lacks cytidine kinase and uridine kinase, while Group XV contains both. As can be inferred from the pathway, CMP produces cytosine or cytidine with the latter being converted to uridine or to cytosine. Cytosine and uridine both are converted to only uracil. Likewise, orotate can be converted directly to uracil to produce additional UMP.

\textit{Aeromonas sobria}, belongs to Group XVII (see Table 2 and Figure 120) and whose chromatograms are seen in Figures 121-126. Group XVII is similar to Group XII,
as discussed previously. The pathway for this group shows that CMP is converted to cytidine, and then to uridine. Uridine is then converted to uracil before proceeding to UMP.

The penultimate group, Group XVIII, is shown in Table 3 and Figure 127. This group is represented by Bacillus cereus, an organism isolated from the soil for this study and is a common human pathogen. As stated earlier, Group XVIII is similar to Group XIII. The salvage scheme of Group XVIII suggests that CMP can be degraded to either cytosine or cytidine. Cytidine may then be converted to uridine, before being phosphorylized to uracil. Here, too, orotate is decarboxylated to uracil and then to UMP.

The last group proposed in this study is the Group XIX, found on Table 3 and Figure 134. This group is represented by the soil isolate Acinetobacter lwaffii and its chromatograms are shown in Figures 135-140. Group XIX contains cytosine deaminase and uracil phosphoribosyltransferase only, making it most similar to Group XVIII, which has almost no salvage. In fact, the only reactions shown are cytosine and orotate being converted to uracil to produce UMP.

Ribosomal RNA sequences are used to classify bacteria, however, a disadvantage is a one dimensional view. Incorporating another characteristic of the organism, such as a traditional enzyme pathway, greatly strengthens this approach. Traditional enzyme pathways have evolutionary constraints that allow for little variation. As opposed to a linear pathway, the pyrimidine salvage pathway is web-like in that there are 19 different methods of producing UTP and CTP instead of only one. In other words, there is greater variability in the path that an organism can choose to yield the same results. It does not
escape notice that it is most probable that additional salvage groups will be discovered in the future.

One may ask the question: Why have orotate decarboxylase? When the cell needs to store pyrimidines, i.e. in the stationary phase, it cannot store them as monophosphates because they are toxic in high concentration. So the key product of the biosynthetic pathway, UMP, which requires the consumption of PRPP, must be broken down to uracil. When conditions are favorable, then uracil can be converted back to UMP by uracil phosphoribosyltransferase (upp), and this consumes a second PRPP. One advantage of orotate decarboxylase is that orotate is converted directly to uracil, thus saving the PRPP. In addition, if the cell needs to convert orotate or other early intermediates of the pyrimidine pathway to carbon or energy sources, orotate decarboxylase saves the costly PRPP by converting orotate to uracil directly. The uracil can then be broken down oxidatively to barbiturate and on to malonyl-CoA. It can also be broken down reductively to â-alanine which can be used as an energy source or converted to pantothenic acid to produce CoA. The pyrimidine biosynthetic pathway is considered to be virtually identical in all organisms, with the exception of a few which lack this pathway completely. The "existence" of the enzyme orotate decarboxylase changes this precept.

This work was designed to be a survey of the pyrimidine salvage schemes of soil microorganisms. The intended purpose was to set up the framework for an exhaustive study to follow. In order to further "extend" this work, a number of experiments are needed. First, each strain should be grown in a different medium with various carbon and
energy sources to mitigate the effects catabolic repression or any other operonic or gene
specific repression has on the enzymes.

It is possible that if grown in a different medium, an organism might not have
expressed an enzyme shown here. Or, the organism might have expressed an enzyme not
seen here. An example of this would be an organism shown in this study containing a
hydrolase, but not expressing the hydrolase if uridine and cytidine are not present in the
growth medium as inducers. In this scenario, the hydrolase would not appear on the
chromatogram, despite the organism possessing the gene for that enzyme.

In addition, a set of pyrimidine salvage mutants would be useful in any future
study. For example, a mutant of *Brevibacterium iodinum* would be of value. It is
difficult to say if *B. iodinum* has a CMP glycosylase. This is due to the fact that a 5’-
nucleotidase is present and breaks CMP down to cytidine. The cytidine is then converted
to cytosine, which is the same product made by CMP glycosylase. Therefore, if a mutant
with a change in the 5’-nucleotidase gene in *B. iodinum* were assayed for CMP
glycosylase and cytosine was produced from CMP, then this microorganism would be
positive for this enzyme. Mutants would also useful in assays where the product of the
assay being performed acts as the substrate for another enzyme present. The second
enzyme not being assayed can remove all traces of the product of the enzyme of interest.
This situation can be corrected by the use of a mutant devoid of the interfering enzyme.

Most of the organisms used in this study were isolated from the soil and have not
been characterized further. Moreover, no mutants have been isolated or studied to date.

As humans, we rely on bacteria throughout our lives in many ways; for the
production of food, recycling of waste, detoxification of the environment, production of
vitamins and antibiotics to out compete other microorganisms just to name a few. They are with us from birth until death. Consequently, as Louis Pasteur eloquently states, “The microbes will have the last word”.

APPENDIX
<table>
<thead>
<tr>
<th>Organism</th>
<th>Origin</th>
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</thead>
<tbody>
<tr>
<td>Acinetobacter lwoffi</td>
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</tr>
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</tr>
<tr>
<td>Aeromonas sobria</td>
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</tr>
<tr>
<td>Agrobacterium radiobacter</td>
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<tr>
<td>Arthrobacter globiformis</td>
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<td>Bacillus cereus</td>
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<td>Bacillus marinus</td>
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<td>Bacillus subtilis</td>
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<td>Brevibacillus agri</td>
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<td>Brevibacterium iodinum</td>
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<td>Klebsiella pneumoniae</td>
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<td>saperdae</td>
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<td>Wards 85W1888</td>
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<td>Paenibacillus macerans</td>
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<td>Rhodococcus luteus</td>
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<tr>
<td>Shigella sonnei</td>
<td>Wards 85W1936</td>
</tr>
</tbody>
</table>

**Table 6:** List of bacterial strains used in this study
LIST OF METHODS

Preparation of Growth Medium for *Arthrobacter*

Minimal medium for culturing *Arthrobacter globiformis* was made by adding the following to a total of one liter:

- 10 ml 5% MgSO₄
- 10 ml of 2.5 M Potassium phosphate buffer, pH 7
- 10 ml Trace Salts
- 10 ml 10% (NH₄)₂SO₄
- 25 ml 20% Glucose
- 935 ml distilled H₂O

* 5% MgSO₄ is made by adding 5 g MgSO₄ to ultra pure water until volume is 100 ml, and autoclave separately.
* 2.5 M Potassium phosphate, pH 7, is made by adding 23.12 g of dibasic Potassium Phosphate to 13.94 g of monobasic Potassium Phosphate, bringing to a total volume of 100 ml using ultra pure water, and autoclaving separately.
* Trace salts are made by bringing the following to a total volume of 0.1 L and autoclaving the resulting solution separately: 1.5 g nitrilotriacetic acid, 3.0 g MgSO₄, 0.5 g MnSO₄, 1.0 g NaCl, 0.1 g FeSO₄, 0.1 g CaCl₂, 0.1 g CoCl₂, 0.1 g ZnSO₄, 0.01 g CuSO₄, 0.01 g AlK(SO₄)₂, 0.01 g H₃BO₃, and 0.01 g Na₂MoO₄.
* 10% (NH₄)₂SO₄ is made by adding 10 g (NH₄)₂SO₄ to ultra pure water until a final volume of 100 ml is reached.

Growth Cycle

1. A sterile 250 ml Klett flask with 100 ml of *Arthrobacter* minimal growth medium was inoculated with 1 ml of a 2 day old culture of *Arthrobacter globiformis*, which had been grown on *Arthrobacter* minimal medium at 30°C.
2. The starting optical density was determined using a Klett-Summerson photoelectric colorimeter.
3. This flask was allowed to shake at 250 rpm in a 30°C incubator for the entire length of the growth curve.

4. Optical density was read every hour using the Klett meter.

**Gel Electrophoresis**

**Preparation of Activity Gel**

1. A PAGE clamp with two glass plates, one short and one long, and two 0.75 mm spacers was assembled.

2. The clamp assembly was then placed into a casting stand.

3. The separating gel was made up as shown below:

   - 2.67 ml Solution A (recipe to follow)
   - 2.5 ml Solution B (recipe to follow)
   - 4.83 ml ultra pure H₂O
   - 0.02 g Ammonium Persulfate
   - 5 µl TEMED
   - 10 ml

4. The separating gel was poured into the cassette until it hit the line 2 cm below the top of the short plate.

5. Enough N-butanol was added to cover the top of the gel.

6. The gel was allowed to polymerize, 15-30 minutes.

7. The N-butanol was next poured off and the top of the gel was rinsed with ultra pure water. The top of the gel was dried with paper towels.

8. A stacking gel was prepared as follows:

   - 4.6 ml ultra pure H₂O
   - 1.34 ml Solution A
   - 2 ml Solution C (recipe to follow)
   - 0.02 g Ammonium Persulfate
   - 5 µl TEMED

252
9. The stacking gel was poured up to the top of the short glass plate.

10. The comb was inserted into top of the gel until top of teeth reached the top of the front plate. The gel was allowed to polymerize for 15-30 minutes.

11. The clamp assembly was removed from the casting stand.

12. The comb was then removed from the clamp assembly.

13. The clamp assembly was inserted into the electrode assembly.

14. Another clamp assembly was inserted into the other side of the electrode assembly.

15. The electrode assembly, with both clamp assemblies, was placed into a buffer tank.

16. The non-denaturing PAGE buffer was prepared, as described below, and poured into the inner chamber created by the two assemblies and then into the outer chamber inside the buffer tank. The top and the bottom of the gel were immersed in buffer.

   Non-denaturing PAGE buffer:
   30 g Tris
   144 g glycine
   H₂O to total volume of 1 liter (pH should be 8.8)

17. The samples were mixed with 5x Loading Dye (recipe to follow) so that the final concentration of dye was 1x.

18. 25 µl of each sample-dye mixture was loaded into the wells created after removal of the comb.

19. The cell lid with power cables was placed on top of the running chamber.

20. The leads were connected to a power supply.
21. The gel was electrophoresed at 150 V for until the dye ran off the bottom of the gel (about 1.5 hours).

22. The leads were then disconnected from the power supply.

23. The cell lid with power cables was removed.

24. The electrode assembly was removed from the buffer tank and clamp assembly removed from electrode assembly.

25. The glass plates were removed from the gel clamp assembly.

26. The hydrogen bonds between the glass plates were broken as the gel was gently removed.

**Solution A: Acrylamide Stock Solution**

30% Acrylamide, 0.8% Bis-acrylamide

Mix 30 g acrylamide and 0.8 g bis-acrylamide and add enough distilled water to make 100 ml. Stir until completely dissolved. Work under hood and keep acrylamide solution covered with Parafilm until acrylamide power is dissolved.

**Solution B: 4x Separating Buffer**

1.5 M Tris-Cl, pH 8.8

Combine 18.2 g Tris with 40 ml H₂O. Add enough HCl until pH 8.8 reached. Then, add enough H₂O to make a total of 100 ml.

**Solution C: 4x Stacking Buffer**

0.5 M Tris, pH 6.8

Combine 6 g Tris with 40 ml H₂O. Add enough HCl until pH 6.8 is reached. Then, add enough H₂O to make a total of 100 ml.

**5x Loading Dye:**

Combine 3.1 ml 1M Tris-Cl, pH 6.8, with 5 ml glycerol, 0.5 ml 1% bromophenol blue, and 1.4 ml H₂O.
**ATCase Activity Stain**

1. The polyacrylamide gel was placed in a large Petri plate and equilibrated for 5 minutes in 50 ml ice cold 50 mM histidine, pH 7.0.

   \[0.388 \text{g histidine/50 ml water}\]

2. 1 ml of 1.0 M aspartate (0.1712 g of aspartate into 1 ml of water) and 2 ml of 0.1 M carbamoylphosphate (0.0306 g of carbamoylphosphate/2.0 ml of water) were then added. The carbamoylphosphate was stored at -20.0°C.

3. The enzymatic reaction was allowed to occur for 20 minutes at 25°C.

4. The gel was rinsed 3X with **ice cold** distilled water to remove reactants. The enzymatic release of orthophosphate trapped in the gel will precipitate by adding ice cold 3 mM lead nitrate in ice cold 50 mM histidine pH 7.0.

   \[\text{To make 3mM lead nitrate add 0.05 g lead nitrate to 50 ml of 50 mM histidine, pH 7.}\]

5. After 10 minutes, the lead nitrate mixture was removed with 3 changes of ice cold water.

6. The gel was stored overnight at 4°C in water.

7. Increasing the resolution of the white water-insoluble lead phosphate precipitate can be obtained by converting it to a dark lead sulfide precipitate. Submerge the gel in 300 ml 1% sodium sulfide (3 g sodium sulfide into a total of 300 ml ultra pure water).

8. Wait approximately 5 minutes.

9. To remove sulfide solution rinse with deionized water for approximately 30 minutes. This removes any background staining.
10. If background levels remain high, the gel can be further destained with 0.7% nitric acid.

**General Assays**

**ATCase**

The following ingredients were mixed for a total reaction volume of 900 µl:

- 800 µl ddH₂O
- 40 µl Tribuffer, pH 9.5 (recipe to follow)
- 50 µl 20x Aspartate, pH 9.5
- 10 µl partially purified ATCase from *A. globiformis*

1. The tubes were placed in a 30°C water bath for 5 minutes.
2. 100 µl carbamoylphosphate was then added to start reaction and the tubes incubated for 20 minutes in a 30°C water bath.
3. The reaction was stopped by adding an equal volume (1 ml) of Stop Mix (2 parts Antipyrine/1 part Monoxime; mix must be pre-made in a dark flask) to each tube and then covering them with a marble.
4. The tubes then incubated in a 65°C water bath for 1 hour in the light.
5. The tubes were removed from the water bath and the absorbance at 466 nm was read.
6. The specific activity was calculated using a CAA standard curve.

**Tribuffer 5x Stocks**: (Make separately)

- 1.37 g/50 ml ddH₂O- Diethanolamine
- 1.48g/50ml ddH₂O- N-Ethylmorpholine
- 4.88g/50ml ddH₂O- MES

Add 10 ml of each stock above, plus ddH₂O, pH with KOH and bring to a final volume of 50 ml for 1x solution. Final concentrations will be 0.051M Diethanolamine, 0.051M N-Ethylmorpholine and 0.1M MES.
DHOase

The following components were mixed for a total reaction volume of 900 µl:

- 690 µl ddH₂O
- 100 µl Tris, 1M, pH 8.6
- 100 µl EDTA, 10 mM
- 10 µl partially purified ATCase from *A. globiformis*

1. The tubes were placed in a 30°C water bath for 5 minutes.
2. 100 µl carbamoylphosphate was then added to start reaction and the tubes incubated for 20 minutes in a 30°C water bath.
3. The reaction was stopped by adding an equal volume (1 ml) of Stop Mix (2 parts Antipyrine/1 part Monoxime; mix must be pre-made in a dark flask) to each tube and then covering them with a marble.
4. The tubes incubated in a 65°C water bath for 1 hour in the light.
5. The tubes were removed from the water bath and the absorbance at 466 nm was read.
6. The specific activity was calculated using a CAA standard curve.

Kinetics Assays

**ATCase Assay to Determine Optimal pH Conditions for the *Arthrobacter globiformis* Enzyme**

The following ingredients were mixed for a total reaction volume of 900 µl:

- 800 µL ddH₂O
- 40 µL Tribuffer with varying pH’s
- 50 µL 20x aspartate pH 9.5
- 10 µL partially purified ATCase from *A. globiformis*
1. 12 tubes were prepared as described above with Tribuffer varying in pH from 5 to 10, in 0.5 pH unit increments. A blank with ddH₂O instead of Tribuffer was included.
2. The tubes were placed in a water bath at 30°C for 5 min.
3. 100 µL of carbamoylphosphate was added to each tube in 10 second increments to start the reaction. Total amount in each tube then equaled 1000 µL.
4. The tubes incubate for 20 minutes per tube in a 30°C water bath.
5. The reaction was stopped by adding 1 ml of Stop Mix to each tube in 10 second increments in the same order as step 3. (1 part Antipyrine/1 part Monoxime; mix must be pre-made in a dark flask.)
6. The tubes were removed and the absorbance was read on a spectrophotometer at 466 nm. A sample was blanked, which contained everything but the Tribuffer.
7. A graph was drawn to determine enzyme’s optimal pH. The pH was graphed on the X-axis and absorbance on the Y-axis. The pH with the highest enzyme activity was the optimal pH.
8. The experiment was repeated 2 additional times.

**ATCase Assay to Determine Optimal Enzyme Concentration for Assays**

The following was mixed for a total reaction volume of 900 µL:

- X µL ddH₂O (volume adjusted to a total of 900 µL)
- 40 µL Tribuffer, at optimal pH
- 50 µL 20x aspartate pH 9.5
- X µL partially purified ATCase from *A. globiformis* with concentrations varying from 1 µL of enzyme to 45 µL; adjust ddH₂O to change
1. 11 tubes were prepared with enzyme amount varying from 1µL - 45µL in 5 µL increments. A blank with no enzyme, only ddH₂O, was included.

2. The tubes were placed in a water bath at 30°C for 5 min.

3. 100 µL of carbamoyl phosphate was added to each tube in 10 second increments to start the reaction. Total amount in each tube then equaled 1000 µL.

4. The tubes then incubate for 20 minutes per tube in a 30°C water bath.

5. The reaction was stopped by adding 1 ml of Stop Mix to each tube in 10 second increments in the same order as step 3. (1 part Antipyrene/1 part Monoxime; mix must be pre-made in a dark flask.)

6. The tubes were removed and the absorbance read on a spectrophotometer at 466 nm. A blank sample containing everything but the enzyme was also read.

7. The enzyme’s optimal concentration was determined by graphing enzyme volume on the X-axis and absorbance on the Y-axis.

8. The experiment was repeated 2 additional times.

ATCase Assay with Varying Aspartate Concentration

The following components were mixed for a total reaction volume of 900 µL:

X µL ddH₂O (volume adjusted to a total of 900µ)
40 µL Tribuffer, at optimal pH
X µL 20x aspartate pH 9.5 (varying concentration 1 mM to 60 mM)
X µL partially purified ATCase from A. globiformis (optimal volume used)

1. 14 tubes were prepared with aspartate concentration varying from 1mM – 60 mM in 5-mM increments. A blank with ddH₂O instead of enzyme was also prepared.

2. The tubes were placed in a water bath at 30°C for 5 min.
3. 100 µL of carbamoylphosphate was added to each tube in 10 second increments to start the reaction. Total amount in each tube then became 1000 µL.

4. The tubes then incubated for 20 minutes per tube in a 30°C water bath.

5. The reaction was stopped by adding 1 ml of Stop Mix to each tube in 10 second increments in the same order as step 3. (1 part Antipyrine/1 part Monoxime; mix must be pre-made in a dark flask.)

6. The tubes were removed and the absorbance was read on a spectrophotometer at 466 nm. A sample containing everything but aspartate was blanked.

7. The aspartate concentration was graphed on the X-axis and change in concentration per minute on the Y-axis.

8. The experiment was repeated 2 additional times.

**Assay to Determine Protein Concentration**

**Bradford Assay (1976)**

1. The Bradford reagent was first gently mixed in the bottle.

2. The standard protein solutions ranging from 50 µg/ml to 1,000 µg/ml were prepared using lysozyme. Dilutions were made from a stock of 1mg/ml (1,000µg/ml) lysozyme.

3. The appropriate amount of lysozyme was added to each appropriately labeled tube along with the appropriate amount of ddH₂O to bring the volume up to 100 µl. In the blank tubes, 100 µl of distilled water was added.
4. 100 µl of the unknown was added to the tube marked unknown. All standards and samples were prepared in duplicate.

5. 100 µl of Bradford Reagent was then added to each tube and mixed.

6. The spectrophotometer was blanked with distilled water prior to reading the absorbances.

7. The absorbances were read between 5-60 minutes at 595 nm using disposable cuvets. The protein-dye complex is stable up to 60 minutes.

8. The absorbance vs. concentration of all trials of the standard protein solutions were plotted on a graph.

9. The protein concentration of the unknown sample was determined by comparing the A595 values against the standard curve.

10. The experiment was repeated 2 additional times.
REFERENCES


hybrid gene formation with the pyrB gene of Escherichia coli, leading to the production of chimeric ATCases. Dissertation. Texas A&M University, College Station.


