

## ***Calidifontibacillus erzuremensis*, *Pantoea agglomerans*, and *Pseudomonas glycinae* identified as antibiotic-producers from freshwater**

Caymen Hoffman<sup>1,2</sup>, Kristina Blanke<sup>1§</sup>

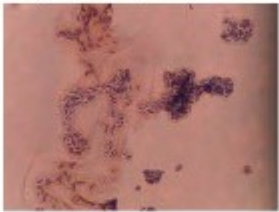
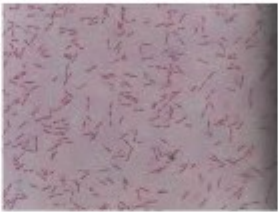




<sup>1</sup>Beloit College, Beloit, Wisconsin, United States

<sup>2</sup>Pakula Biomedical Fellowship

<sup>§</sup>To whom correspondence should be addressed: blankeresearch@gmail.com

### **Abstract**

Three water isolates were previously identified as promising antibiotic producers from freshwater sources in Wisconsin, United States. Each isolate produced effective antibiotics against three or more bacterial relatives of antibiotic resistant pathogens. The isolates were identified as *Calidifontibacillus erzuremensis*, *Pantoea agglomerans*, and *Pseudomonas glycinae* through 16S rRNA sequencing and further characterized with biochemical tests to verify the genus and species of each isolate.

Isolate and Media	A. TC1 50% TSA	B. RR2 PDA	C. WR2 LBA
Genus species	<i>Calidifontibacillus erzuremensis</i>	<i>Pantoea agglomerans</i>	<i>Pseudomonas glycinae</i>
Colony morphology Size Surface Form Margin Color	11 mm dry filamentous lobate white	3 mm glossy circular entire cream	3 mm smooth, glistening circular entire cream
Gram stain; shape	positive; bacillus 	negative; bacillus 	negative; coccobacillus 
MSA	yellow colonies Gram-positive unclear fermentation	no growth	no growth
MacConkey	no growth	yellow growth Gram-negative non-lactose fermenting	mucoïd cream growth Gram-negative non-lactose fermenting
EMB	clear growth non-lactose fermenters noncoliform	clear growth non-lactose fermenters noncoliform	pink growth slow lactose fermenting possible coliform
Motility	motile 	non-motile 	motile 
Antibiotic activity	<i>Acinetobacter baylyi</i> <i>Erwinia carotovora</i> <i>Pseudomonas putida</i> <i>Mycobacterium smegmatis</i>	<i>Bacillus subtilis</i> <i>Erwinia carotovora</i> <i>Pseudomonas putida</i>	<i>Enterobacter aerogenes</i> <i>Erwinia carotovora</i> <i>Escherichia coli</i> <i>Staphylococcus epidermidis</i>

**Figure 1. Characteristics of three freshwater isolates with antibiotic activity against three or four tester bacteria related to antibiotic resistant pathogens:**

Each isolate is labeled based on their sampling location and the isolate number: TC = Turtle Creek, RR = Rock River, WR = Wisconsin River. Culture media included 50% tryptic soy agar (50% TSA), potato dextrose agar (PDA), and lysogeny broth agar (LBA). The selective/differential media was mannitol salt agar (MSA), MacConkey agar, and eosin-methylene blue (EMB) agar.

**Column A.** Isolate TC1 was cultured on 50% TSA and has characteristics aligning with *Calidifontibacillus erzuremensis*. TC1 produced antibiotics that inhibited the growth of four Gram-negative or acid fast tester bacteria.

**Column B.** Isolate RR2 was cultured on PDA and has characteristics aligning with *Pantoea agglomerans*. RR2 produced antibiotics that inhibited the growth of three Gram-positive or Gram-negative tester bacteria.

**Column C.** Isolate WR2 was cultured on LBA and has characteristics aligning with *Pseudomonas glycinae*. WR2 produced antibiotics that inhibited the growth of four Gram-positive or Gram-negative tester bacteria.

## Description

Freshwater from creeks and rivers in Wisconsin contained bacteria that produced antibiotics against Gram-negative, Gram-positive, and acid fast relatives of antibiotic resistant pathogens (Hoffman et al., 2024). Several water isolates inhibited the growth of three or four tester bacteria through a spread/patch screen and warranted further investigation. This study identified three isolates with 16S rRNA sequencing and verified the results with their biochemical characteristics. The lethal outcomes for each isolate against multiple tester bacteria emphasize the need to investigate novel environments to discover new natural classes of antibiotics during the global healthcare crisis of rising antibiotic resistant pathogens.

Isolate TC1 was cultured on 50% TSA and identified as *Calidifontibacillus erzuremensis* (Figure 1.A). TC1 was grown in aerobic conditions, stained Gram-positive with a bacillus shape, and was motile; which aligns with the known characteristics of *C. erzuremensis* (Adiguzel et al., 2020). TC1 growth on 50% TSA led to white colonies with a filamentous form and lobate margin, further correlating with the expected morphology of *C. erzuremensis* on TSA (Adiguzel et al., 2020). TC1 grew as yellow colonies on MSA, indicating this isolate is Gram-positive and can tolerate higher levels of salt in media, as is the case for *C. erzuremensis* (Adiguzel et al., 2020). TC1 also grew as clear colonies on EMB, which demonstrates a lack of lactose and sucrose fermentation and follows the expected characteristics of *C. erzuremensis* (Adiguzel et al., 2020). Additional data gathered for TC1 showed antibiotic activity against four tester bacteria: Gram-negative *A. baylyi*, *E. carotovora*, *P. putida*; acid fast *M. smegmatis*. *C. erzuremensis* is a novel species that has not been thoroughly studied and the antibiotic activity results are encouraging that the secondary metabolites from this species have pharmaceutical potential.

Isolate RR2 was cultured on PDA and identified as *Pantoea agglomerans* (Figure 1.B). RR2 grew in aerobic conditions, stained Gram-negative with a bacillus shape, was isolated from a water source, and appeared glossy with a circular form and entire margin; which aligns with expected characteristics of *P. agglomerans* (Gavini et al., 1989). RR2 was confirmed as a Gram-negative species from yellow colony growth on MacConkey agar and clear growth on EMB. The MacConkey and EMB agar results indicate RR2 is a non-lactose fermenter. *P. agglomerans* is known to be motile (Gavini et al., 1989); however, isolate RR2 was non-motile. Additional data gathered for RR2 showed antibiotic activity against three tester bacteria: Gram-positive *B. subtilis*; Gram-negative *E. carotovora*, *P. putida*. Previous studies found one strain of *P. agglomerans* produced two antibiotics (pantocin A and B) that inhibited growth of *E. carotovora* (Wright et al., 2001). The antibiotics produced by RR2 may include pantocin A and B, which could be effective against *B. subtilis* and *P. putida*. However, it is unclear which secondary metabolites RR2 produces as antibiotics since antiSMASH 7.0 identify four main secondary metabolites for *P. agglomerans* (ASM1904838v1) with 84-100% similarity that included metal sequestering (frederiksenibactin and desferrioxamine) or pigmentation (aryl polyenes and carotenoid) molecules (Blin et al., 2023), though there were no gene clusters for pantocin.

Isolate WR2 was cultured on LBA and identified as *Pseudomonas glycinae* (Figure 1.C). WR2 grew in aerobic conditions, stained Gram-negative with a coccobacillus shape, was motile, and catalase positive; which follows the expected characteristics of *P. glycinae* (Jia et al., 2020). The colony morphology of WR2 also aligned with *P. glycinae* characteristics of being 3 mm in diameter with a circular shape, entire margin, and cream color on LBA (Jia et al., 2020). WR2 was confirmed as a Gram-negative species from mucoid cream growth on MacConkey agar and the results indicated that WR2 was not fermenting lactose; however, the EMB agar results showed pink growth and indicated that WR2 was a slow lactose fermenting bacteria and a possible coliform. In this study, WR2 showed antibiotic activity against four tester bacteria: Gram-positive *S. epidermidis*; Gram-negative *E. aerogenes*, *E. carotovora*, *E. coli*. *P. glycinae* is another novel species that needs a thorough investigation and the additional data for antibiotic activity indicate the secondary metabolites for this species also have pharmaceutical potential.

In conclusion, *Calidifontibacillus erzuremensis*, *Pantoea agglomerans*, and *Pseudomonas glycinae* were discovered in Wisconsin freshwater and produced antibiotics that inhibited the growth of Gram-positive, Gram-negative, and acid fast bacteria.

## Methods

### Sample Selection

Water samples were collected from four water systems in Wisconsin, United States (Hoffman et al., 2024), and screened against tester bacteria provided by the Tiny Earth organization (Hoffman & Blanke, 2024; Hernandez et al., 2018). Colonies chosen for screening were categorized by size, surface, form, margin, and color. Additional protocol information regarding the collection, screening, and identification of antibiotic-producing bacteria from the environment is described in the Hoffman and Blanke (2024) article. Three isolates were selected for further analysis since they produced antibiotics against 3-4 tester bacteria across two of the three bacterial categories: Gram positive, Gram negative, acid fast. Each sample was from a different water system (Turtle Creek, Rock River, Wisconsin River) and cultured on various media (50% TSA, PDA, LBA, respectively) as described by Hoffman et al. (2024).

### Genus species Identification

Each isolate was prepared for 16S rRNA sequencing (Hernandez et al., 2018) performed by Eurofins Genomics (Louisville, KY). Sequencing text was run through BLASTn (courtesy of the National Library of Medicine) and the genus species for each isolate was chosen based on the highest percent identify and alignment (Table 1).

**Table 1.** Isolate 16S rRNA sequences identified the genus and species. The corresponding percent identity and number of aligned base pairs (bp) are listed as compiled by BLASTn.

Isolate	Genus species	Reference	Percent Identity	Alignment
TC1	<i>Calidifontibacillus erzuremensis</i>	<a href="#">NR_180225.1</a>	99.52%	837 bp
RR2	<i>Pantoea agglomerans</i>	<a href="#">NR_041978.1</a>	96.82%	1125 bp
WR2	<i>Pseudomonas glycinae</i>	<a href="#">NR_179889.1</a>	100.00%	924 bp

### 16S rRNA sequence

#### TC1 sequence:

```
AACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATAC
CGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACA
GATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCG
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTG
AGTGATGAAGTTTTCCGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAA
ATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGG
CGGTTTCTTAAGTCTGATGTGAAAGCCCCGGCTCAACCGGGGAGGGTCATTGGAACTG
GGGAACTTGAGTGCAGAAGAGGAGAGTGGAAATCCACGTGTAGCGGTGAAATGCGTAGAG
ATGTGGAGGAACACCAGTGCCGAAGGCGACTCTCTGGTCTGTAAGTACGCTGAGGAGCG
AAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTG
CTAAGTGTTAGGGGGTTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCT
GGCGAGTACGGTCGCAAGACTGAAACTCAAAGGAACGGACGGAGGCCCGCACAAAGCGGTGGAG
```

#### RR2 sequence:

```
CTAACACATGCAGAGTCAACGGTAGCACAGAGAGCTTGCTCTTGGGTGACGAGTGGC
GGACGGGTGAGTAATGTCTGGGGATCTGCCTGACAGAGGGGATAACTACTGGAAACGGT
AGCTAATACCGCATAACCTCGCAAGAGCAAAGAGGGGGACCTTCGGGCTCTCGCTGTCA
GATGAACCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCC
TAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAGTACGACACGGTCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTG
TATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGATGAGGTTAA
TAACCTCGTCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCC
GCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGC
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GGTCTGTCAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGAAACTGG  
CAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA  
TCTGGAGGAATACCGGTGGCGAAGGCGGCCCTGGACAAAGACTGACGCTCAGGTGCGA  
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGAC  
TTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGG  
GGAGTACGGCCGCAAGGTTAAAACCTCAAATGAATTGACGGGGCCCGCACAAGCGGTGGA  
GCATGTGGTTTAAATTCGATGCAACGCGAAGAACCCTTACCTACTCTTGACATCCACAGAAT  
TTGGCAGAGATGCCTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTC  
AGCTCGTGTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCTTTGTTG  
CCACACGTAATGGTGGGAACTCAAAGGAGACTGCCGGTGATAACCGGAGGAAGGGGGGA  
TGACGTCAGTNATCATG

WR2 sequence:

CCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTTCGAAAGGAACGCTAATACCGCAT  
ACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTTCGCTATCAGATGAGCCTAGGTC  
GGATTAGCTAGTTGGTGAGGTAATGGCTACCAAGGCGACGATCCGTAACGGTCTGAGA  
GGATGATCAGTCACACTGGAACCTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG  
GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCT  
TCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTT  
GACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGTAATACAGAG  
GGTGAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTCGTTAAGTTG  
GATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACCTGGCGAGCTAGAGTATG  
GTAGAGGGTGGTGAATTTCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACC  
AGTGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCA  
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGC  
CTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCA  
AGGTTAAAACCTCAAATGAATTGACGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAAT  
TCGAAGCAACGCGAAGAACCCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGA  
TTGGTGCCTTCGGGAACATTGAGACA

#### Gram Staining and Selective/Differential Media

All isolates were categorized by their shape and cell wall structure through Gram staining. Additional selective/differential media tests were performed to confirm Gram staining results and identify isolate fermentation capabilities. MacConkey and EMB agars confirmed Gram-negative isolates, and MSA confirmed select Gram-positive isolates. MacConkey agar identified lactose fermentation, EMB agar identified lactose and sucrose fermentation, and MSA identified mannitol fermentation. EMB agar also indicated coliform status for any isolates with growth. Each isolate was cultured on either 50% TSA or LBA as a control for the selective/differential media tests. Media were incubated at 30°C with light exposure for 24-48 hours after inoculation.

#### Motility

Isolates were inoculated in general media with 2,3,5-triphenyl tetrazolium to visualize bacterial movement and incubated at 25°C for seven days. Isolates with spreading growth were motile, while non-motile isolates grew along the insertion line. Results were either motile or non-motile.

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