Supporting Online Material for

Bacteria Subsisting on Antibiotics

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Bacteria Subsisting on Antibiotics

Supporting Online Material

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Materials and Methods

Growth media

All liquid media used for isolating bacteria capable of subsisting on antibiotics was made by dissolving 1 g/L of the relevant antibiotics (Table S1) into Single Carbon Source (SCS) media containing 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 15 mg EDTA, 4.5 mg $ZnSO_4 \cdot 7H_2O$, 4.5 mg $CaCl_2 \cdot 2H_2O$, 3 mg $FeSO_4 \cdot 7H_2O$, 1 mg $MnCl_2 \cdot 4H_2O$, 1 mg H_3BO_3, 0.4 mg $Na_2MoO_4 \cdot 2H_2O$, 0.3 mg $CuSO_4 \cdot 5H_2O$, 0.3 mg $CoCl_2 \cdot 6H_20$ and 0.1 mg KI per liter water. The pH was adjusted to 5.5 using HCl, and the media was sterilized through a 0.22 µm filter. Solid medium was prepared by adding 15 g agar per liter of liquid SCS media followed by autoclaving before adding antibiotics.

All liquid media used for resistance profiling was made by dissolving 20 mg/L or 1 g/L of the relevant antibiotics into autoclaved Luria Broth containing 5 g Yeast Extract, 10 g NaCl and 10 g of Tryptone in 1

Liter of water. The pH was adjusted to 5.5 using HCl, and the media was sterilized through a 0.22 μm filter.

Culturing of environmental bacteria capable of subsisting on antibiotics

Initial soil microbial inocula (soil description in table S2) were prepared in minimal medium containing no carbon, and inoculated into SCS-antibiotic media (corresponding to approximately 125 mg of dissolved soil in 5mL of media). To significantly reduce the transfer of residual alternative carbon sources present in original inocula, samples were passaged (2.5 uL) into fresh SCS-antibiotic media (5 mL) two additional times after 7 days of growth, resulting in a $5x10^4$ dilution at each passage (resulting in a final carryover of approximately 30 ng of soil in 5mL of media at the third passage). Clonal isolates from the liquid cultures were obtained by plating cultures out on SCS-antibiotic agar medium and resulting single colonies were picked and restreaked on corresponding plates. Three colonies each were then inoculated into fresh SCS-antibiotic liquid media (5 mL) to confirm clonal phenotype. Final culture growth was recorded after 1 month incubation without shaking at 22°C and cultures with at least 10^8 cells/mL were assayed as positive growth.

Since inoculation in media lacking a carbon source (no carbon control) did not show growth in any cases, carbon source contamination of the source media or carbon fixation from the air were considered insignificant to this experiment. The only other alternative carbon substrate for growth could be impurities in the antibiotic stocks. All antibiotics used were purchased from Sigma-Aldrich at the highest purities available – lot purities of each compound used are listed in Table S1. Based on an average carbon mass of $0.15x10^{-12}$ g per bacterial cell, we estimate that at least 15 μg of carbon must be incorporated into bacterial biomass to reach sufficient culture densities in 1 mL of culture to be rated as

successful growth. Assuming 50% carbon content of impurities, and under the most stringent assumptions of (1) 100% incorporation of carbon impurities into biomass, and (2) no loss of carbon as metabolic byproducts (such as CO_2), antibiotics with greater than 97% purity would have insufficient impurities to support sole carbon source growth. Of the antibiotic lots used in this experiment (Table S1), twelve compound stocks are at least 99% pure, two compounds (ciprofloxacin and D-cycloserine) have between 98 and 98.5% purity, one compound (carbenicillin) is 92.9% pure, and no purity information is available for three compounds (kanamycin, gentamicin, and vancomycin).

Phylogenetic profiling

The 16S ribosomal DNA (rDNA) of each of the clonal isolates identified in this study was amplified using universal bacterial 16S primers:

>Bact_63f_62C

5' – CAG GCC TAA CAC ATG CAA GTC – 3'

>Bact_1389r_63C

5' – ACG GGC GGT GTG TAC AAG – 3'

Successful 16S rDNA amplicons were sequenced for phylogenetic profiling. High-quality, non-chimeric sequences were classified using Greengenes (*1, 2*), with consensus annotations from RDP (*3*) and NCBI taxonomies (*4*). Phylogenetic trees were constructed using the neighbor-joining algorithm in ARB (*5*) using the Greengenes aligned 16S rDNA database. Placement in the tree was confirmed by comparing automated Greengenes taxonomy to the annotated taxonomies of nearest neighbours of each sequence in the aligned database.

Resistance profiling of 75 representative isolates capable of subsisting on antibiotics

75 clonal isolates (Table S3) were selected to include multiple isolates capable of subsisting on each of the 18 antibiotics and originating from each of the 11 soils (Table S2). Bacterial cultures were inoculated into Luria Broth from frozen glycerol stocks and were incubated at 22°C for 3 days. 500 nL of this culture was used to inoculate each of the clonal isolates into 200 uL of Luria Broth containing one of the eighteen different antibiotics (See Table S1) at 20 mg/L and 1 g/L. Cultures were incubated without shaking at 22°C for 4 days. Resistance of an isolate was determined by turbidity at 600 nm using a Versamax microplate reader from Molecular Devices.

Analysis of antibiotic removal of penicillin and carbenicillin subsisting bacteria

Representative isolates capable of growth on penicillin and carbenicillin as sole carbon source were selected for analysis of antibiotic removal from the growth media by High Performance Liquid Chromatography (HPLC). 2 uL of these cultures were re-inoculated into fresh SCS- antibiotic medium (5 mL) and allowed to grow for 28 days. Samples of the cultures and un-inoculated media controls were taken at regular intervals throughout the 28 day period and the catabolism of penicillin and carbenicillin was monitored at 214 nm by HPLC of filtered media from samples using a Hewlett Packard 1090 Liquid Chromatograph and a Vydac C-18 column. HPLC was performed at a flow rate of 0.3 mL/min with an acetonitrile gradient going from 5% to 65% in 30 minutes in the presence of 0.1 % trifluoroacetic acid.

The HPLC chromatogram of the penicillin catabolizing culture medium (Fig 1B) started out with a single peak corresponding to the penicillin peak of the un-inoculated control. This peak disappeared at day 4 with the appearance of multiple smaller peaks at lower elution times; by day 20 these peaks had also disappeared in agreement with the complete catabolism of penicillin by the culture in 20 days. In

comparison, the single penicillin peak in the un-inoculated control remained the dominant peak over the same time course. The HPLC chromatogram of the medium from the carbenicillin catabolizing culture (Fig 1B) started out with a bimodal peak corresponding to the un-inoculated carbenicillin control, which remained stable for 2 days. At day 4, corresponding to the appearance of visible turbidity in the inoculated culture, the bimodal peak had almost disappeared and secondary peaks at lower elution times were observed. These secondary peaks almost completely disappeared by the 28th day, suggesting that carbenicillin was almost completely catabolized within 28 days. The bimodal carbenicillin peak remained relatively unchanged in the un-inoculated control over the same time course.

Samples from the penicillin subsisting culture from day 0 and day 4 were prepared for LC/MS using a Waters Sep-Pak Cartridge prior to mass spectrometry analysis using a LTQ-FT from Thermo Electron. Mass spectra were analyzed using XCalibur 2.0.5 and the empirically determined m/z values of all major peaks were compared to predicted m/z values of putative penicillin degradation products calculated using ChemDraw Ultra 9.0 (Fig S3).

Supporting Figures:

Figure S1:

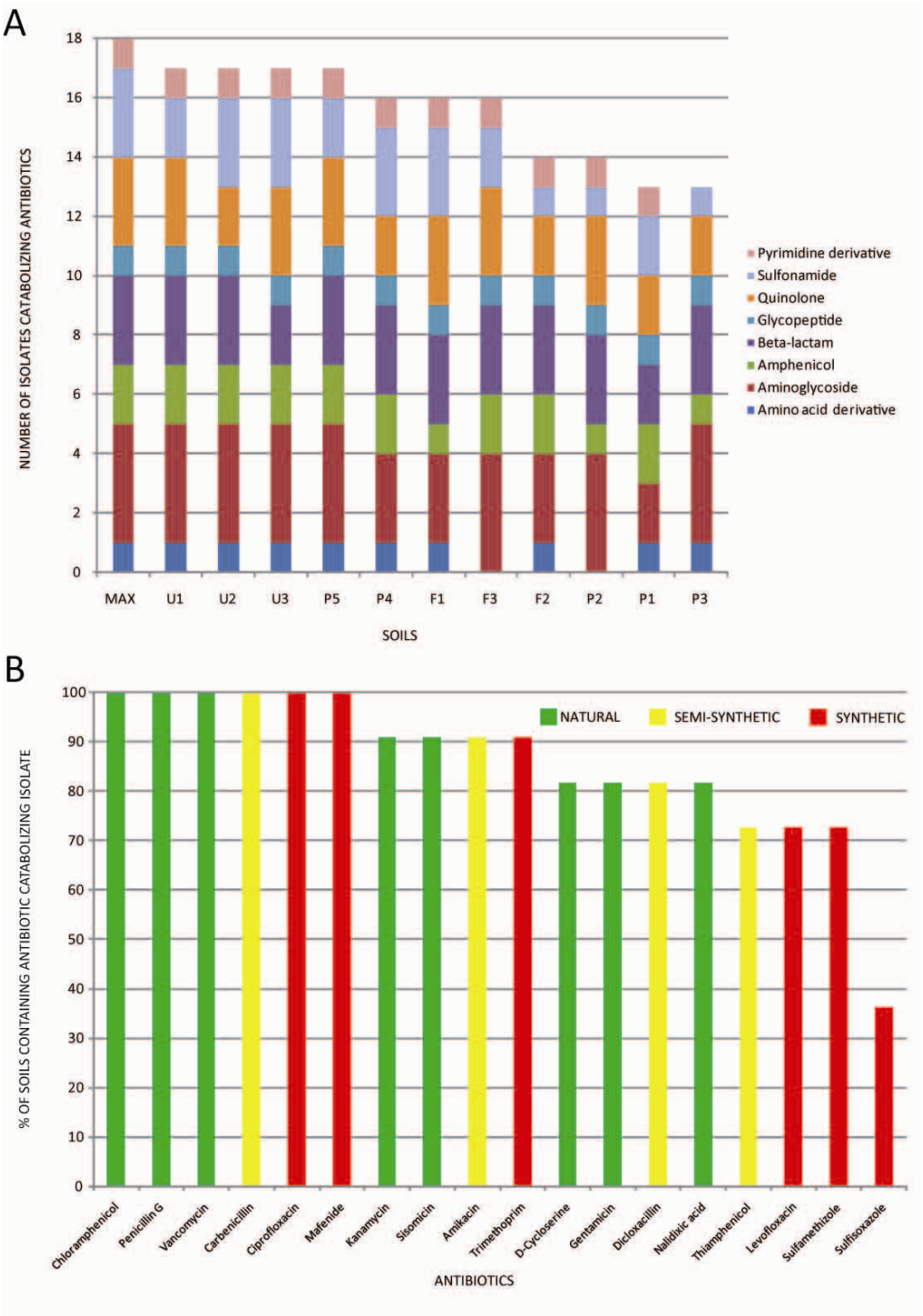

Figure S1. Distribution of antibiotic catabolizing bacterial isolates with respect to antibiotics and soil. (A) Number of antibiotic catabolizing bacteria isolated from 11 soils color-coded by antibiotic class catabolized. (B) Percentage of soils containing antibiotic catabolizing bacteria, color-coded by chemical origin of antibiotic.

Figure S2:

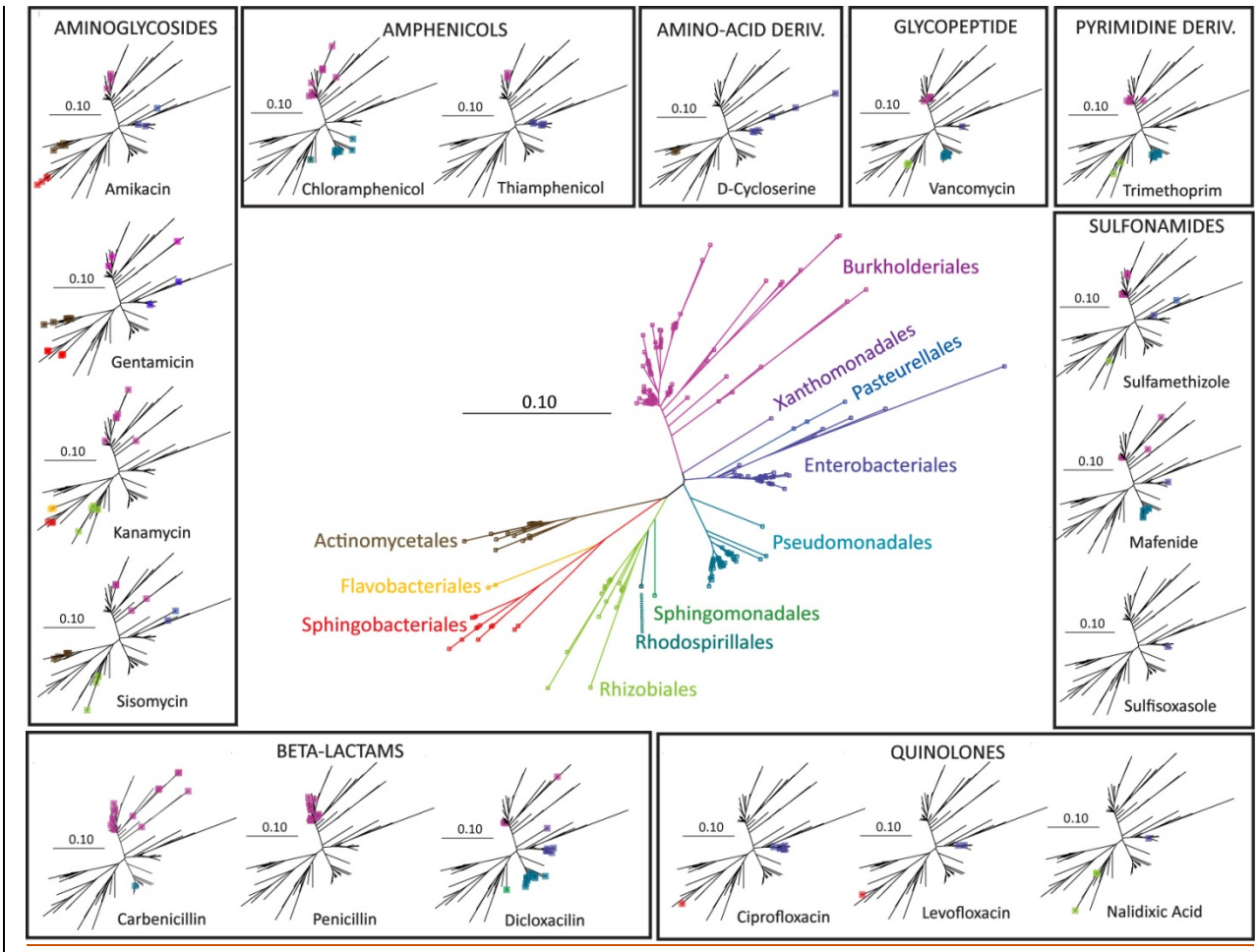

Figure S2. Phylogenetic distribution of bacterial isolates subsisting on antibiotics. Full set of bacteria subsisting on antibiotics is displayed in the centre, with branches color-coded by bacterial orders, and clonal isolates represented as squares. Subsets comprising clonal isolates catabolizing each antibiotic are represented as trees around the periphery, grouped by antibiotic class. 16S ribosomal DNA (rDNA)

was sequenced from antibiotic catabolizing clonal isolates using universal bacterial rDNA primers. High-quality, non-chimeric sequences were classified using Greengenes (*2*), with consensus annotations from RDP (*3*) and NCBI taxonomies (*4*). Phylogenetic trees were constructed using the neighbor-joining algorithm in ARB (*5*) using the Greengenes aligned 16S rDNA database. Placement in the tree was confirmed by comparing automated Greengenes taxonomy to the annotated taxonomies of nearest neighbors of each sequence in the aligned database. The phylogenetic distributions of species isolated from different antibiotics as sole carbon source exhibit some interesting trends. For instance, the fluoroquinolone antibiotics, ciprofloxacin and levofloxacin, have similar phylogenetic distributions, as do the aminoglycoside antibiotics, gentamycin and amikacin, but the two sets are notably different from each other. Interestingly, the orders of bacteria subsisting on amikacin appear more similar to gentamycin than kanamycin despite amikacin being a semi synthetic kanamycin derivative.

Figure S3:

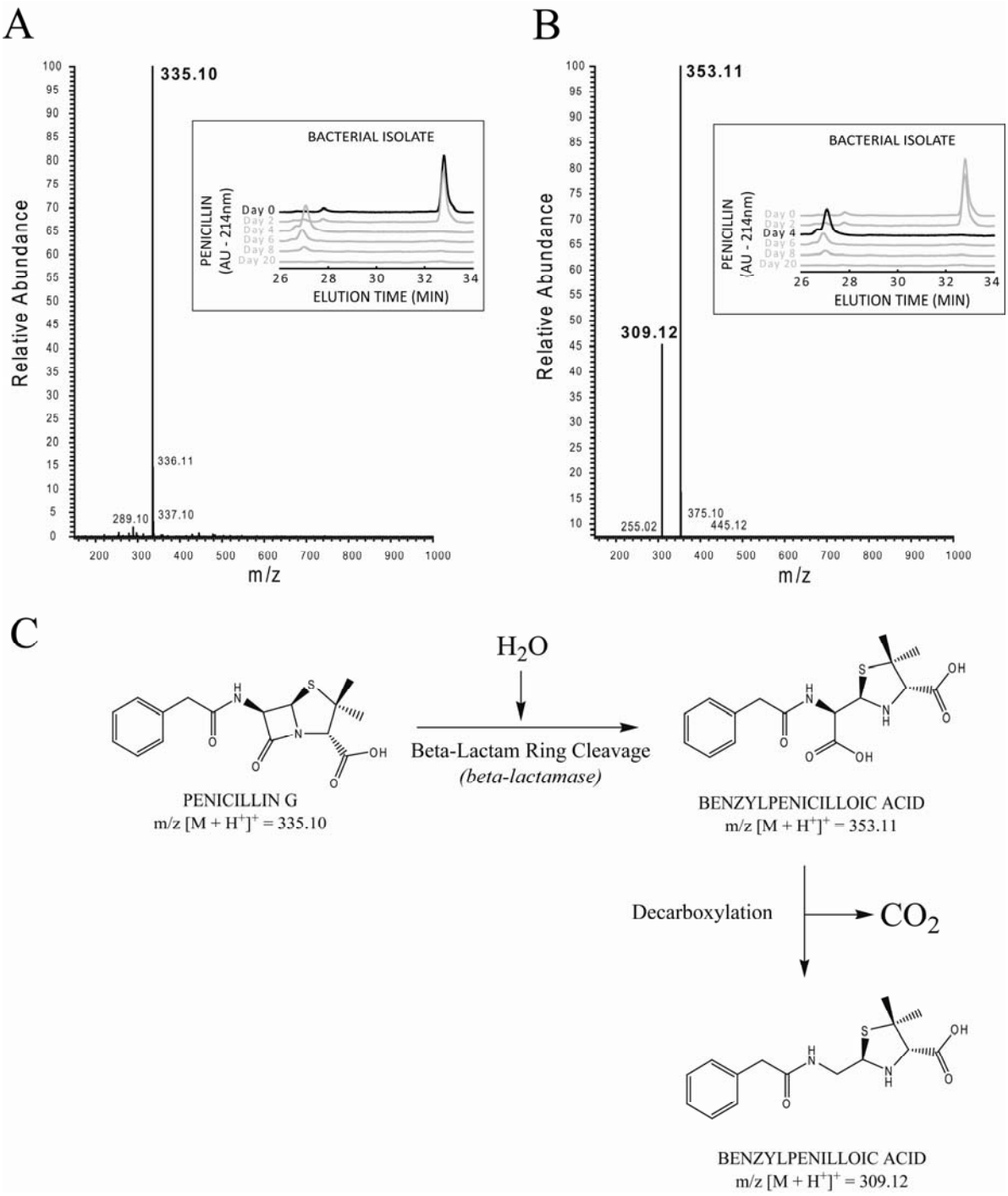

Figure S3: Mass spectrometry analysis of growth media from penicillin subsisting bacterial culture. (A)

Mass spectra of day 0 growth media from penicillin culture with a major peak at m/z of 335.10

corresponding exactly to the protonated penicillin G molecule. (B) Mass spectra of day 4 growth media from penicillin culture with two major peaks at m/z values 353.11 and 309.12 corresponding to protonated benzylpenicilloic acid and benzylpenilloic acid, respectively. (C) Proposed first steps of penicillin G degradation pathway.

Supporting Tables

Table S1. Lot purities of antibiotics used, as reported on Certificates of Analysis from Sigma-Aldrich.

(NR=Not Reported)

Antibiotics	Lot Purity %
Ciprofloxacin	98.5
Levofloxacin	100.0
Sisomicin	99
Gentamicin	NR
Kanamycin	NR
Amikacin	100
Penicillin G	99.7
Carbenicillin	92.9
Dicloxacillin	99.8
Chloramphenicol	>99
Nalidixic acid	100
Thiamphenicol	>99
Sulfisoxazole	99.7
Trimethoprim	100
Mafenide	100
Sulfamethizole	99.9
D-Cycloserine	98
Vancomycin	NR

Table S2: Soil information for the 11 different soils from which bacteria capable of subsisting on antibiotics were isolated.

Figure 1A identifiers	**Soil type**	**Soil name**	**Soil collection location**
F1	Farmland	S1G	Corn Field with Antibiotic Treated Manure, Great Brook Farm, Carlisle, MA
F2	Farmland	S1N	Alfalfa Field with Manure Treatment, Northcroft Farm , Pelican Rapids, MN
F3	Farmland	S2N	Alfalfa Field without Manure Treatment, Northcroft Farm, Pelican Rapids, MN
P1	Pristine	S2R	Raccoon Ledger, Rockport, MA
P2	Pristine	S3N	Prairie next to Northcroft Farm, Pelican Rapids, MN
P3	Pristine	S1R	Brier's Swamp, Rockport, MA
P4	Pristine	S1A	Pristine Forest Soil, Alan Seeger Natural Area, PA
P5	Pristine	S2T	Untreated Forested Area, Toftrees State Gameland Area, PA
U1	Urban	S1T	Waste Water Treated Area, Toftrees State Gameland Area, PA
U2	Urban	S3F	Boston Fens, MA
U3	Urban	S1P	Boston Public Garden, MA

Table S3: Strain information for the 75 clonal isolates used for resistance profiles.

Figure 3A identifier	Strain name	Subsisting on	From soil
1	Levo-S2T-M1LLLSSL-2	Levofloxacin	S2T
2	Kana-S2T-M1LLLSSL-3	Kanamycin	S2T
3	Amik-S2T-M1LLLSSL-1	Amikacin	S2T
4	Carb-S2T-M1LLLSSL-2	Carbenicillin	S2T
5	Chlo-S2T-M1LLLSSL-2	Chloramphenicol	S2T
6	Nali-S2T-M1LLLSSL-1	Nalidixic acid	S2T
7	Thia-S2T-M1LLLSSL-2	Thiamphenicol	S2T
8	Trim-S2T-M1LLLSSL-1	Trimethoprim	S2T
9	Mafe-S2T-M1LLLSSL-3	Mafenide	S2T
10	Cycl-S2T-M1LLLSSL-3	D-Cycloserine	S2T
11	Vanc-S2T-M1LLLSSL-3	Vancomycin	S2T
12	Siso-S2N-M1LLLSSL-1	Sisomycin	S2N
13	Gent-S2N-M1LLLSSL-2	Gentamycin	S2N
14	Kana-S2N-M1LLLSSL-2	Kanamycin	S2N
15	Peni-S2N-M1LLLSSL-2	Penicillin G	S2N
16	Dicl-S2N-M1LLLSSL-1	Dicloxacillin	S2N
17	Trim-S2N-M1LLLSSL-1	Trimethoprim	S2N
18	Vanc-S2N-M1LLLSSL-1	Vancomycin	S2N
19	Dicl-S3N-M1LLLSSL-2	Dicloxacillin	S3N
20	Thia-S3N-M1LLLSSL-3	Thiamphenicol	S3N
21	Trim-S3N-M1LLLSSL-2	Trimethoprim	S3N
22	Mafe-S3N-M1LLLSSL-2	Mafenide	S3N
23	Vanc-S3N-M1LLLSSL-2	Vancomycin	S3N
24	Cipr-S1P-M1LLLSSL-3	Ciprofloxacin	S1P
25	Peni-S1P-M1LLLSSL-2	Penicillin G	S1P
26	Chlo-S1P-M1LLLSSL-1	Chloramphenicol	S1P
27	Thia-S1P-M1LLLSSL-1	Thiamphenicol	S1P
28	Trim-S1P-M1LLLSSL-3	Trimethoprim	S1P
29	Slfm-S1P-M1LLLSSL-2	Sulfamethizole	S1P
30	Cycl-S1P-M1LLLSSL-1	D-Cycloserine	S1P
31	Vanc-S1P-M1LLLSSL-3	Vancomycin	S1P
32	Cipr-S1T-M1LLLSSL-2	Ciprofloxacin	S1T
33	Levo-S1T-M1LLLSSL-1	Levofloxacin	S1T
34	Siso-S1T-M1LLLSSL-1	Sisomycin	S1T
35	Carb-S1T-M1LLLSSL-1	Carbenicillin	S1T
36	Dicl-S1T-M1LLLSSL-1	Dicloxacillin	S1T
37	Chlo-S1T-M1LLLSSL-1	Chloramphenicol	S1T
38	Thia-S1T-M1LLLSSL-3	Thiamphenicol	S1T

Figure 3A identifier	Strain name	Subsisting on	From soil
39	Trim-S1T-M1LLLSSL-2	Trimethoprim	S1T
40	Mafe-S1T-M1LLLSSL-1	Mafenide	S1T
41	Cycl-S1T-M1LLLSSL-2	D-Cycloserine	S1T
42	Vanc-S1T-M1LLLSSL-1	Vancomycin	S1T
43	Levo-S3F-M1LLLSSL-3	Levofloxacin	S3F
44	Slfs-S3F-M1LLLSSL-3	Sulfisoxazole	S3F
45	Trim-S3F-M1LLLSSL-1	Trimethoprim	S3F
46	Mafe-S3F-M1LLLSSL-3	Mafenide	S3F
47	Slfm-S3F-M1LLLSSL-3	Sulfamethizole	S3F
48	Vanc-S3F-M1LLLSSL-2	Vancomycin	S3F
49	Amik-S1R-M1LLLSSL-3	Amikacin	S1R
50	Peni-S1R-M1LLLSSL-2	Penicillin G	S1R
51	Mafe-S1R-M1LLLSSL-2	Mafenide	S1R
52	Vanc-S1R-M1LLLSSL-2	Vancomycin	S1R
53	Trim-S1N-M1LLLSSL-1	Trimethoprim	S1N
54	Vanc-S1N-M1LLLSSL-1	Vancomycin	S1N
55	Kana-S1A-M1LLLSSL-2	Kanamycin	S1A
56	Carb-S1A-M1LLLSSL-2	Carbenicillin	S1A
57	Slfs-S1A-M1LLLSSL-1	Sulfisoxazole	S1A
58	Vanc-S1A-M1LLLSSL-2	Vancomycin	S1A
59	Kana-S2R-M1LLLSSL-2	Kanamycin	S2R
60	Amik-S2R-M1LLLSSL-3	Amikacin	S2R
61	Peni-S2R-M1LLLSSL-2	Penicillin G	S2R
62	Dicl-S2R-M1LLLSSL-1	Dicloxacillin	S2R
63	Mafe-S2R-M1LLLSSL-2	Mafenide	S2R
64	Slfm-S2R-M1LLLSSL-1	Sulfamethizole	S2R
65	Cipr-S1G-M1LLLSSL-1	Ciprofloxacin	S1G
66	Levo-S1G-M1LLLSSL-1	Levofloxacin	S1G
67	Gent-S1G-M1LLLSSL-3	Gentamycin	S1G
68	Kana-S1G-M1LLLSSL-1	Kanamycin	S1G
69	Peni-S1G-M1LLLSSL-1	Penicillin G	S1G
70	Carb-S1G-M1LLLSSL-3	Carbenicillin	S1G
71	Chlo-S1G-M1LLLSSL-3	Chloramphenicol	S1G
72	Nali-S1G-M1LLLSSL-2	Nalidixic acid	S1G
73	Thia-S1G-M1LLLSSL-1	Thiamphenicol	S1G
74	Slfs-S1G-M1LLLSSL-3	Sulfisoxazole	S1G
75	Mafe-S1G-M1LLLSSL-2	Mafenide	S1G

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