Iron-related fitness epistasis is antagonistic to the evolution of silver resistance in *Escherichia coli*. 15th Korea-US Forum on Nanotechnology
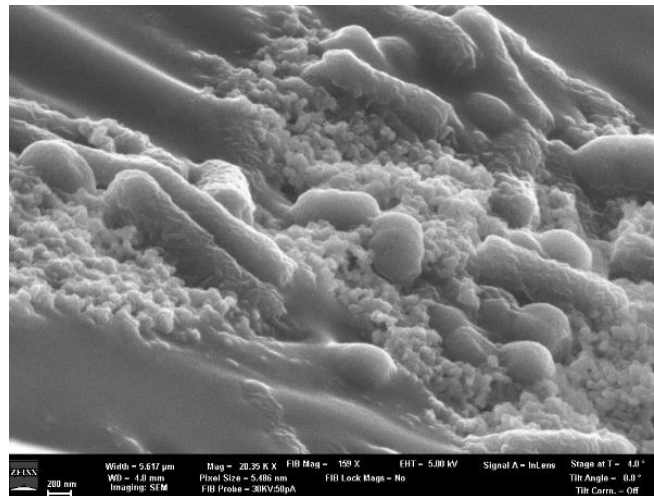
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- Lisa Garrison, Biotechnology Program, Alamance CC.
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Minding my business in Groningen

- Well there I was enjoying a cup of tea during the session break at 3rd Meeting of the International Society for Evolution, Medicine, and & Public Health.

- My citation alert goes off…it goes of when someone cites one of your publications.

nanomaterials   MDPI

Article

3D-Hydrogel Based Polymeric Nanoreactors for Silver Nano-Antimicrobial Composites Generation

Albanelly Soto-Quintero [1], Ángel Romo-Uribe [2], Víctor H. Bermúdez-Morales [3], Isabel Quijada-Garrido [4,*] and Nekane Guarrotxena [4,*]

Heresy!!

- Soto-Quintero et al. 2017 stated:

- "Moreover, both hydrogel nanocomposite systems exhibited a more effective antibacterial activity against *P. aeruginosa* …than against *E. coli*…, as proven with the higher inhibition halo. The explanation of this fact could lie on the ability of *E. coli* to develop heavy metal resistance, particularly for silver."

- To support this claim, they cited our 2015 paper, entitled Rapid evolution of silver nanoparticle resistance in *Escherichia coli* (Graves et al. 2015).

- However this claim is not supported by the results of our paper and it indicates that the authors have a fundamental misunderstanding of the mechanisms that generate antimicrobial resistance.
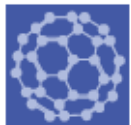
Antimicrobial Nanomaterials: Why Evolution Matters

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Abstract: Due to the widespread occurrence of multidrug resistant microbes there is increasing interest in the use of novel nanostructured materials as antimicrobials. Specifically, metallic nanoparticles such as silver, copper, and gold have been deployed due to the multiple impacts they have on bacterial physiology. From this, many have concluded that such nanomaterials represent steep obstacles against the evolution of resistance. However, we have already shown that this view is fallacious. For this reason, the significance of our initial experiments are beginning to be recognized in the antimicrobial effects of nanomaterials literature. This recognition is not yet fully understood and here we further explain why nanomaterials research requires a more nuanced understanding of core microbial evolution principles.

Keywords: Antimicrobials; metals; acclimation; adaptation; evolution; genomics

Experimental Evolution

- The study of organisms in defined, repeatable conditions in either laboratory or field environments over multiple generations.

- It has utilized since Th. Dobzhansky (1940's; Neo-Darwinian synthesis).

- It predicted the rapid sweep of pesticide and antibiotic resistance long before these phenomena were observed.

- It has been deployed to solve some of the most intractable biologic (aging) and engineering (evolutionary algorithms) problems.

Toxic metals

- Microbes have been exposed to toxic metals since the beginning of life on this planet (Silver and Phoung 2005).

- So the idea that virtually all bacteria have genes to resist toxic metal ions (Ag^+, Cd^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}) is not surprising.

- The largest group of resistance mechanisms involve energy dependent efflux (as we found with Ag^+ resistance in *E. coli*).

- Next in line are enzymatic transformations (oxidation, reduction, methylation, and demethylation) or metal-binding proteins (metallothionein, SmtA, chaperone CopZ, SilE).

- In addition, clones may slow their growth, or cease dividing in the presence of toxic materials (Lewis 2010).

Bacterial resistance to silver

- We have already shown that *E. coli* could rapidly evolve resistance to spherical 10nm silver nanoparticles (AgNPs); as well as to ionic silver: Tajkarimi et al. 2017.

Rapid evolution of silver nanoparticle resistance in *Escherichia coli*

Joseph L. Graves Jr.[1]*[†], Mehrdad Tajkarimi[2], Quincy Cunningham[3], Adero Campbell[4], Herve Nonga[5], Scott H. Harrison[3†] and Jeffrey E. Barrick[6†]

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Selective sweeps were observed in *cusS*, *ompR*, and *rpoB*

Essential micronutrients

- On the other hand, there are the "good" metals required for growth (Fe, S, Mg, Mn, etc.)

- Of these acquiring iron is the greatest challenge for bacteria (Glass 2006.)

- Iron predominantly occurs as ferric iron (Fe^{3+}) under aerobic conditions. Fe^{3+} OH is poorly soluble in aqueous solution (as low as 10^{-18} M at pH 7.0).

- Under anaerobic conditions, the equilibrium shifts to ferrous iron (Fe^{2+}) that is more easily available to microorganisms.

- Iron availability is a key to pathogenesis for a variety of microbes, thus many innate immunity mechanisms utilize iron sequestration (serum albumin, Koropa & Neilands 1984; calprotectin, Nakashire et al. 2015).

Too much of a good thing…

- As iron is so important, microbes evolved means to take it up from the environment; e.g. siderophores such enterobactin.

- Enterobactin in *E. coli* is synthesized by genes such as *ent* and *fep*, and their expression is controlled by the global iron homeostasis regulator, Fur (Shea & McIntosh 1991).

- Excess iron causes oxidative damage, up to the point of cell death.

- As iron nanoparticles are being proposed as a method to control MDR bacteria, we again wanted the ask the question: Can and how does *E. coli* evolve resistance to excess iron?

A — *E. coli* 24h growth assays in iron sulfate
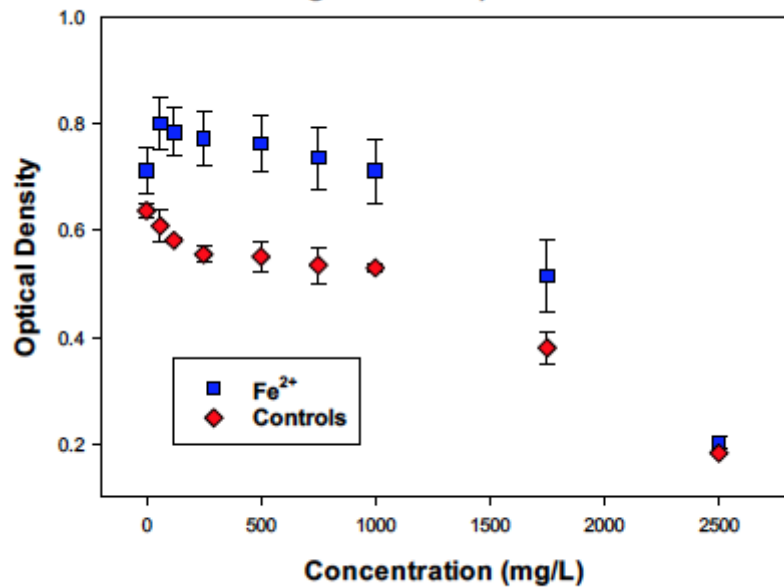
B — *E. coli* 24h growth assays in ionic gallium
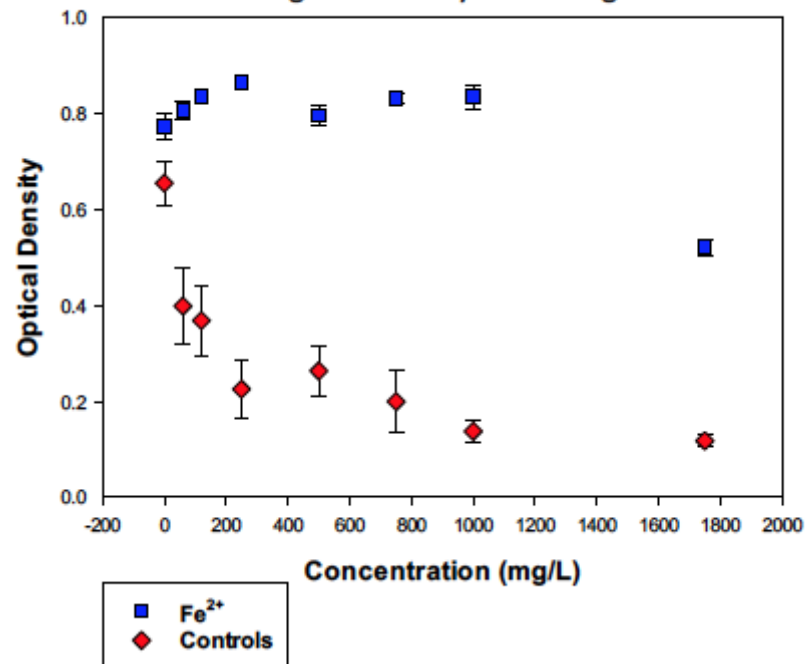
C — *E. coli* 24h growth assays in ampicillin
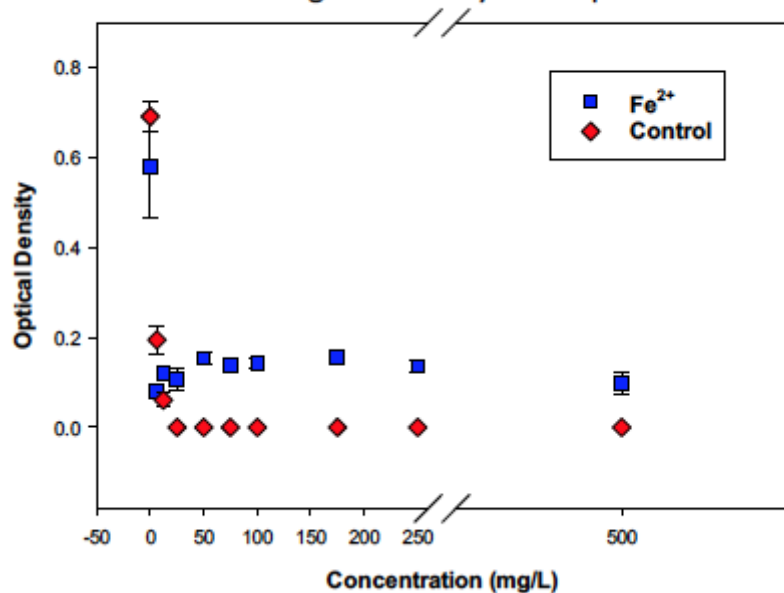
D — *E. coli* 24h growth assays in rifampicin
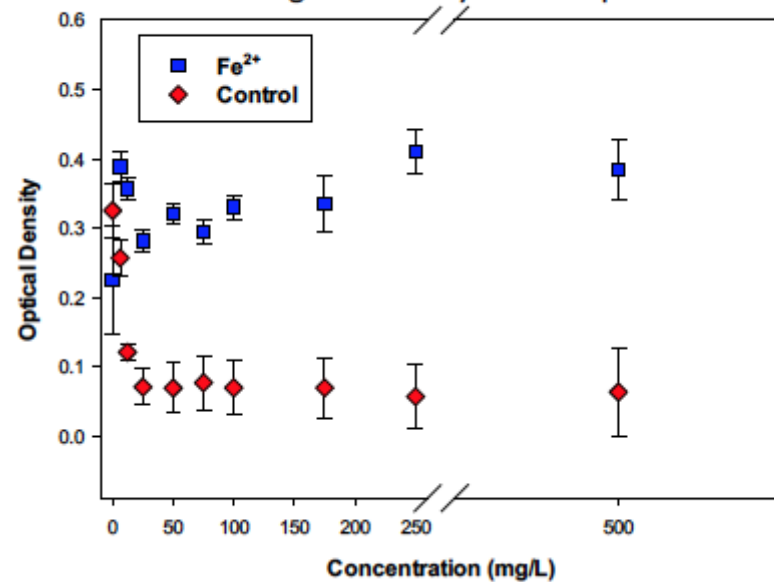
Mechanisms of silver and iron toxicity

Mechanism	Ag	Fe
Reactive Oxygen Species	+	+
Binding to Thiol groups	+	-
Transcription/Translation	+	+
Cell wall/Cell membrane	+	+
Respiration	+	+
Release of cell components	+	+

Why are the adaptations to these antagonistic??

Genomic foundations of iron resistance

- DNA was extracted from each replicate population and genomic libraries prepared for whole genome sequencing on our Illumina MiSeq.

- Depth of coverage was ~ 30-40X for all samples.

- Genomic variants were called via breseq 0.30.1; the methods of this pipeline are described in Deatherage and Barrick 2014.

- Know ancestral variants were filtered out before comparing the population replicates.

- We identified several variants that were not detectable in the ancestral population (see Graves et al. 2015; Tajkarimi et al. 2017) and with frequencies > 0.500 in the experimental populations.

Selective Sweeps – Fe^{2+} selection

Gene	Annotation
murC →	P14S (CCC→TCC)
cueR →	V6L (GTA→CTA)
yeaG →	A441V (GCA→GTA)
fliP →	S39* (TCG→TAG)
ptsP ←	C519* (TGC→TGA)
ilvL → / → ilvX	intergenic (+46/-41)
ilvG →	pseudogene (65/663 nt)
fecA ←	A559T (GCT→ACT)
fecA ←	G243C (GGC→TGC)

The gene *murC* is known to mediate osmotic damage caused by oxidative stress, *cueR* is involved in iron homeostasis, *fecA* (ferric citrate outer membrane transporter) is involved in iron homeostasis.

Gene	Description
murC →	UDP-N-acetylmuramate:L-alanine ligase
cueR →	copper-responsive regulon transcriptional regulator
yeaG →	protein kinase, endogenous substrate unidentified; autokinase
fliP →	flagellar biosynthesis protein
ptsP ←	fused PTS enzyme: PEP-protein phosphotransferase (enzyme I)/…
ilvL → / → ilvX	ilvG operon leader peptide/uncharacterized protein
ilvG →	pseudogene, acetolactate synthase 2 large subunit, valine-insensitive;…
fecA ←	ferric citrate outer membrane transporter

Gene	Mutation
dnaK →	R167G (CGT→GGT)
dnaK →	Q433P (CAG→CCG)
murC →	P14S (CCC→TCC)
fur ←	K9N (AAG→AAT)
ompF ← / ← asnS	intergenic (-122/+481)
iraM ← / ← ycgX	intergenic (-77/+623)
bluR ←	D80E (GAC→GAG)
kgtP ←	coding (586-590/1299 nt)
kgtP ←	E141* (GAA→TAA)
ptsP ←	C519* (TGC→TGA)
yggN ←	I106I (ATC→ATA)
yghS ←	A166V (GCA→GTA)
nudF ← / → tolC	intergenic (-141/-61)
nusA ←	R258C (CGT→TGT)
gltB →	A18V (GCC→GTC)
gltD →	coding (264/1419 nt)
rpoA ←	V282L (GTA→TTA)
rpoA ←	V282L (GTA→CTA)
crp →	C19Y (TGC→TAC)
[yicC]	103 bp deletion
yicO ←	I81R (ATA→AGA)
ilvG →	pseudogene (65/663 nt)
rho →	G63V (GGT→GTT)
rho →	R87S (CGC→AGC)
cyaA →	coding (1615-1616/2547 nt)
fecA ←	A559T (GCT→ACT)

Selective sweeps in Fe^{2+}Ag

murC, *ptsP*, and *fecA* were also observed in Fe^{2+}.
The following genes have been documented to play a role in either iron or metal resistance: **dnaK, fur, bluR, nusA, crp,** and **rho** (Hobman and Crossman 2015; **yicO** is known to interact with **dnaK**).

Gene	Description
dnaK →	chaperone Hsp70, with co-chaperone DnaJ
murC →	UDP-N-acetylmuramate:L-alanine ligase
fur ←	ferric iron uptake regulon transcriptional repressor; autorepressor
ompF ← / ← asnS	outer membrane porin 1a (Ia;b;F)/asparaginyl tRNA synthetase
iraM ← / ← ycgX	RpoS stabilzer during Mg starvation, anti-RssB factor/DUF1398 family protein
bluR ←	repressor of blue light-responsive genes
kgtP ←	alpha-ketoglutarate transporter
kgtP ←	alpha-ketoglutarate transporter
ptsP ←	fused PTS enzyme: PEP-protein phosphotransferase (enzyme I)/GAF domain containing protein
yggN ←	DUF2884 family putative periplasmic protein
yghS ←	putative ATP-binding protein
nudF ← / → tolC	ADP-ribose pyrophosphatase/transport channel
nusA ←	transcription termination/antitermination L factor
gltB →	glutamate synthase, large subunit
gltD →	glutamate synthase, 4Fe-4S protein, small subunit
rpoA ←	RNA polymerase, alpha subunit
crp →	cAMP-activated global transcription factor, mediator of catabolite repression
[yicC]	[yicC]
yicO ←	putative adenine permease
ilvG →	pseudogene, acetolactate synthase 2 large subunit, valine-insensitive
rho →	transcription termination factor
cyaA →	adenylate cyclase
fecA ←	ferric citrate outer membrane transporter

Next step: Gene Expression by Nanostring

- Resequencing only tells you whose at the party, but not necessarily what they are doing.

- Gene expression help you get a better sense of what pathways are involved and how they work to produce the observed phenotype.

- Using a new technology for gene expression (Nanostring) we have designed a pilot study where we targeted 50 genes for their expression profile.

- These 50 were based on those we identified by EERseq, in addition with those known to be association with them.

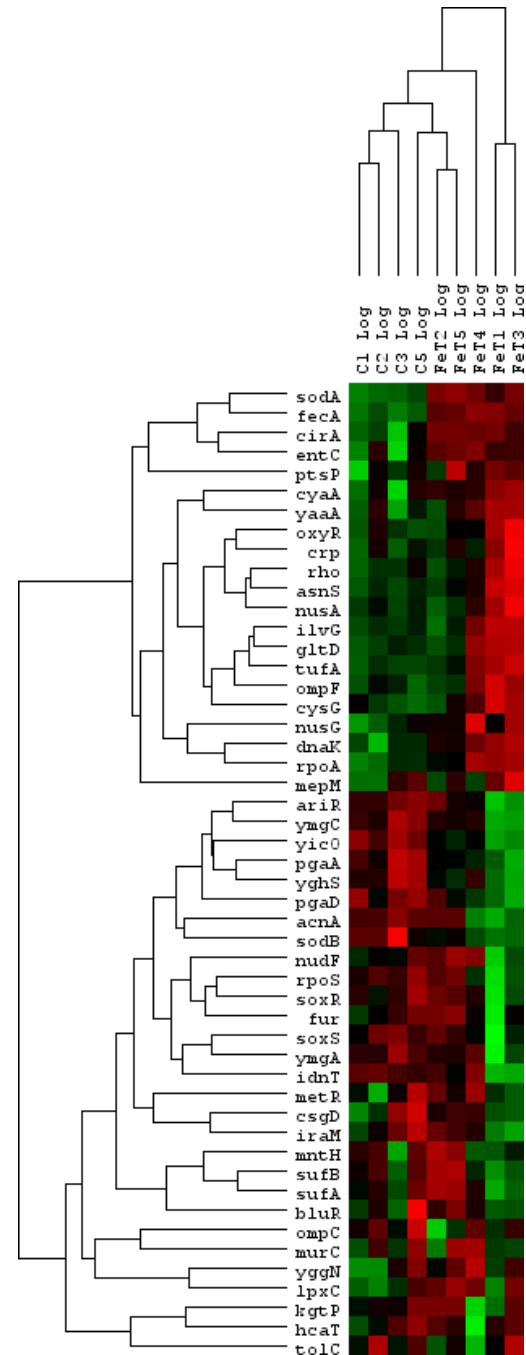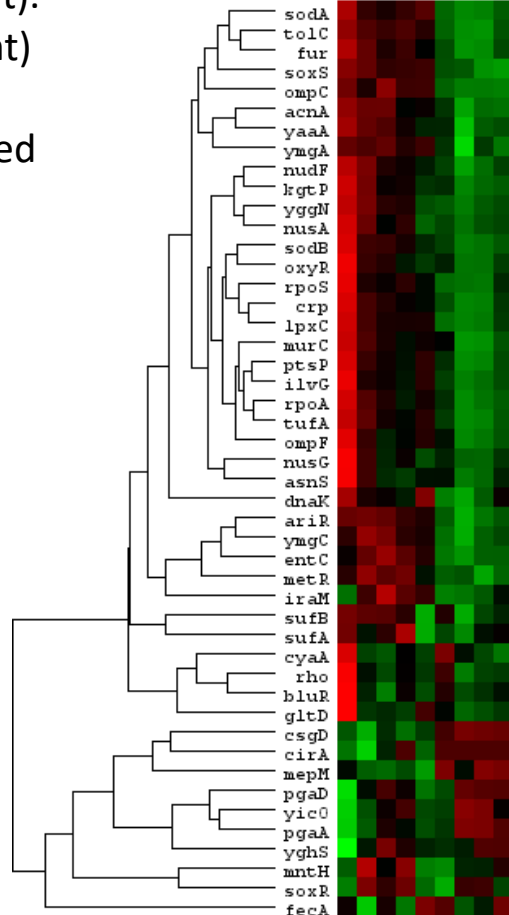**Nanostring technology**

https://youtu.be/85h3vYt3KYg

The nCounter Analysis System utilizes a novel digital barcode technology for direct multiplexed measurement of analytes and offers high levels of precision and sensitivity (< 1 copy per cell). The technology uses molecular "barcodes" and single molecule imaging for the direct hybridization and detection of hundreds of unique transcripts in a single reaction.
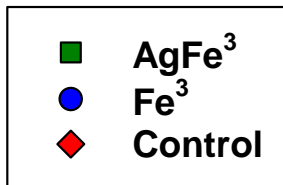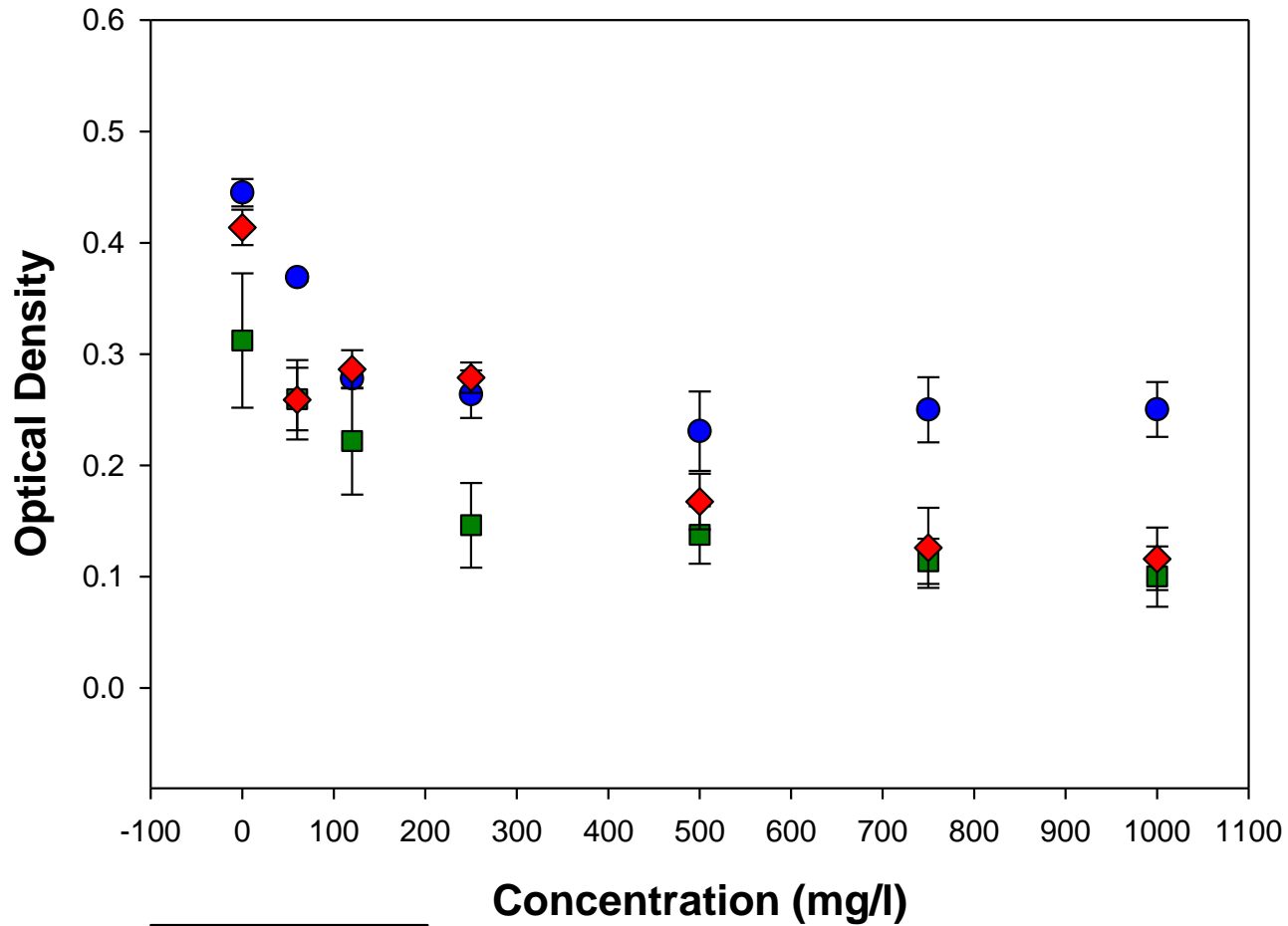
https://www.nanostring.com/scientific-content/technology-overview/ncounter-technology

Heat maps

Gene expression profile shown
In log phase for Fe^{2+}-selected
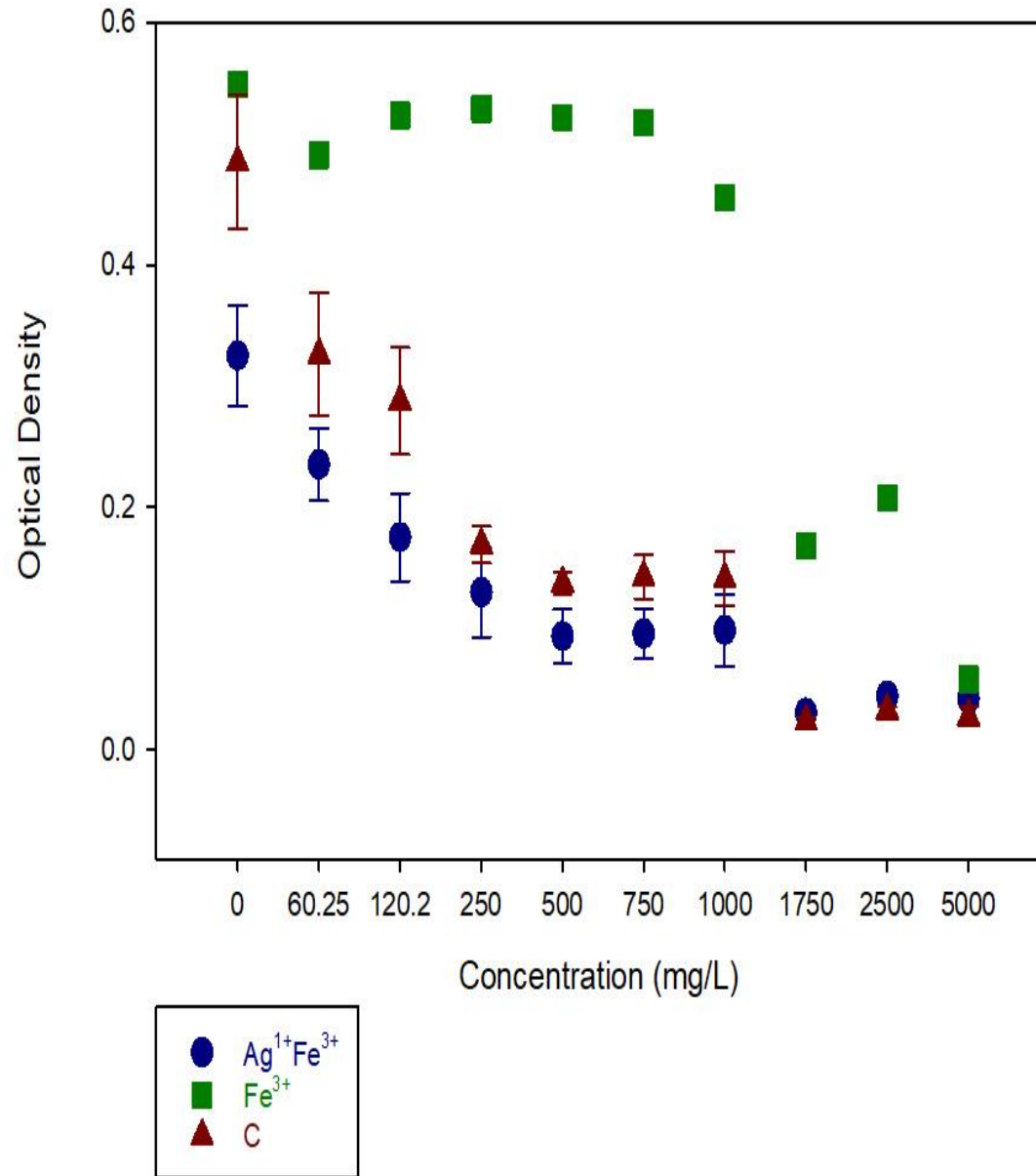v. controls in the presence of toxic
iron concentration (left).
Fe^{2+}Ag v. controls (right)
Red – upregulated
Green – down regulated

Selection for Ag$^+$ resistance with Fe^2 genetic background

Legend:
- ■ AgFe^3
- ● Fe^3
- ◆ Control

Axis labels: Optical Density (y-axis), Concentration (mg/l) (x-axis)

24-hours growth in Ga^{3+}

Optical Density vs Concentration (mg/L)

Legend:
- $Ag^{1+}Fe^{3+}$
- Fe^{3+}
- C

Fe^{3+}	Ag$^+$Fe^{3+}	Gene	Annotation
1.000	0.000	*dnaK* →	R151L (CGT→CTT)
1.000	0.000	*dnaK* →	Q433P (CAG→CCG)
1.000	0.000	*dnaK* →	coding (1712/1917nt) 1 bp del
1.000	0.000	*ompF/asnS*	intergenic (-122/+481 T-A)
1.000	0.000	*nudS/tolC*	intergenic (-141/-61) IS5 (-) +4bp
1.000	0.000	*tolC*	coding (174/1482nt)
1.000	0.000	*tdcR* →/*vhaB*	intergenic (+54/-202) C-A
1.000	0.000	*nusA* ←	R191R (CGT→CGC)
1.000	0.000	*crp*	M1M (ATG→ATA)
1.000	0.000	*crp*	C19Y (TGC-TAC)
0.000	0.060	*cusS*	L388R (CTG-CGG)

Support