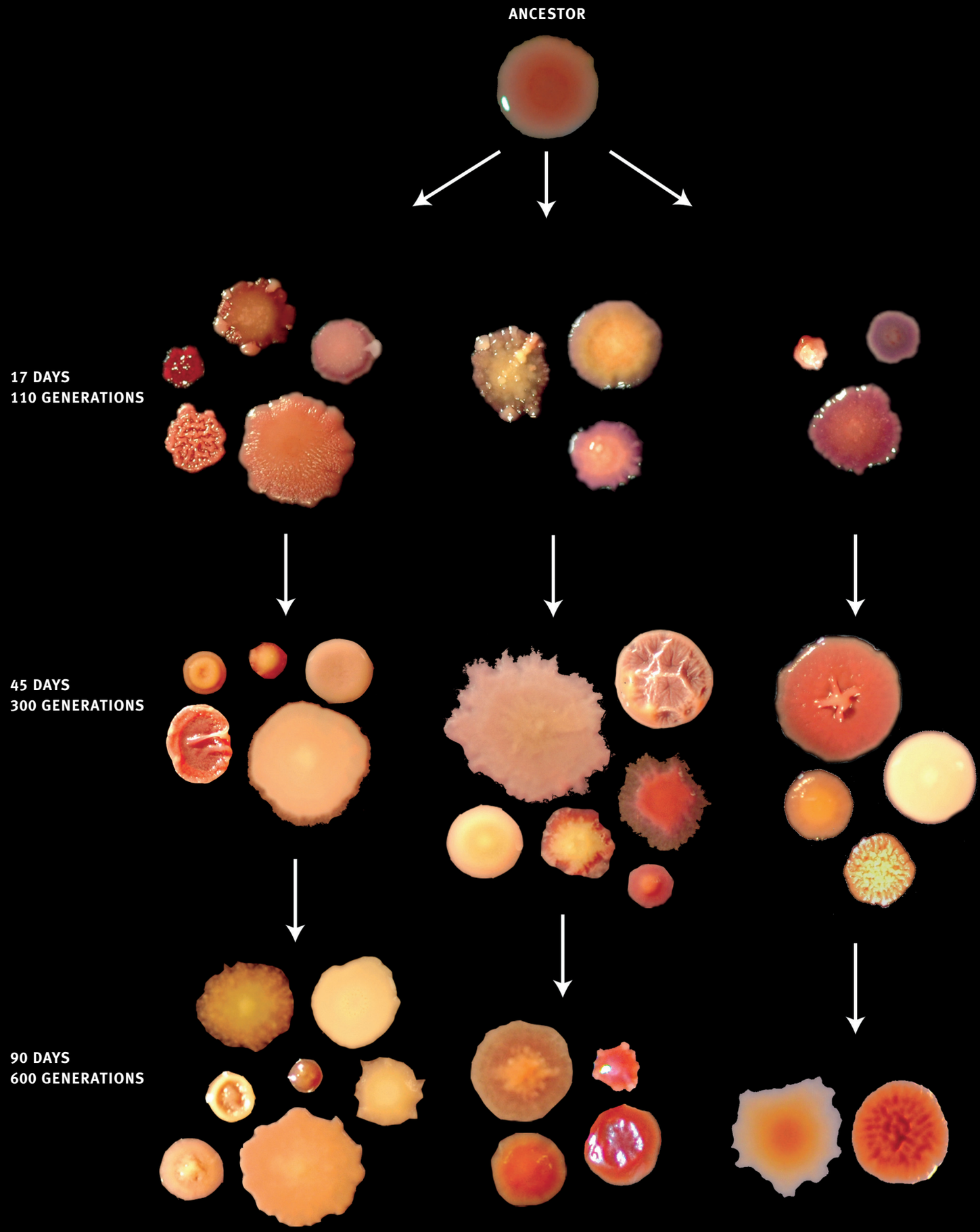

ANCESTOR
17 DAYS
110 GENERATIONS
45 DAYS
300 GENERATIONS
90 DAYS
600 GENERATIONS

EVOLUTION CAUGHT IN THE ACT

BY ELAINE VITONE

UNTANGLING DARWIN

On a recent summer afternoon, in a Bridgeside Point laboratory overlooking the Monongahela River, the University of Pittsburgh's Vaughn Cooper and his team are coaxing bacteria to do in captivity what they've always done so artfully in the wild, as have humans and every other life form.

We evolve.

It's a never-ending story: This environment selects for this trait, that for another trait, and so on, in a highly complex network of falling dominoes. Evolutionary biologists like Cooper have been working to retrace this network—the life story of life itself—for the past 150 years.

Cooper's experiment is a little different from his progenitors', though.

Instead of hypothesizing about evolution after the fact, his team is watching it happen. He figured out how to catch it in the act about a decade ago. And it's actually "ridiculously simple," he says.

In studying evolution in action, Vaughn Cooper has seen patterns emerge. As biofilming bacteria attach to surfaces and set up shop, they mutate by multitudes. This is not a "winner-take-all" operation; rather, the bacteria seem to evolve together to fill complementary metabolic niches. LEFT: Here, a single *Pseudomonas aeruginosa* ancestor yields "a zoo." In a matter of months, the colony becomes an interdependent community.

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The experiment goes like this: Stick some bacteria in a test tube with a plastic bead, give the cells some nutrients to nosh on and a nice warm spot in an incubator, and leave them be for 24 hours. Overnight, these tiny pioneers make a happy home of their 7 millimeter–wide world—just as they would on, say, a hospital handrail, or a catheter, or the lung of a patient in the ICU. Then, the bacteria asexually beget a whole mess of babies, many wee teams of clones and mutants amassing in a motley crew.

Of course, which ones among them survive to make their own broods is up to chance, at least in part. And as chance would have it for this particular bacterial family, the lab introduces a do-or-die ultimatum.

Each day, the researchers replace the nutrients, add a second bead, and let the test tube simmer for 24 hours. Then they swap out the older bead for a brand new one. The old bead's breeds are dead, as far as the experiment is concerned. (Well, retired to the freezer, anyway—more on them later.) In this daily test of fitness, survival favors the bacteria that are best at pressing on to new frontiers.

The bacteria Cooper primarily studies, *Burkholderia cepacia* and *Pseudomonas aeruginosa*, are infamous frontiersmen. They're able to claim new territory with the help of what are known as biofilms.

Put simply, a biofilm is a microbial growth on a surface, usually encased in "slime," Cooper explains. The goo not only gives bacteria sticking power, but it also makes these cells up to 1,000 times tougher to kill. "They are physically protected," says the PhD and Pitt associate professor of microbiology and molecular genetics. "And once the cells start to accumulate in these biofilms, they start to grow more slowly—and it's a lot harder to kill slow-growing cells." For bacteria in no particular metabolic hurry anyway, a drug that inhibits what feeds them is like water off a duck's back. "This is the same reason it's hard to kill slow-growing solid tumors," Cooper notes.

There's a lot to be gained from studying evolution in action, he says. To his amazement, Cooper has seen patterns appear in evolution, leading him to believe what was once unthinkable: that we may be able to *predict* evolution, at least in certain contexts.

Such an ability would have real medical value. Evolutionary biology is at the center of some of the most vexing public health challenges of our time.

In cancer, tissue gives rise to mutations—naturally, inevitably, and continuously—until one day, spurred by some challenge, or selective pressure, certain cells escape the normal checks and balances of our biology.

Caught between do and die, they *do*.

In antibiotic resistance, bacteria face evolution in a hospital patient's body, a cruel selective-pressure cooker of drug after murderous drug and an immune response at the boiling point. So the bacteria fight fire with fire, armies of mutants redoubling and re-emerging, emboldened. If just one cell out of a million survives, it's a chance to win.

And they *do*.

Most bacteria live on surfaces, in biofilms, but historically, that has been a tough setting to study. Simply sticking some scum in a tank full of swirling warm liquid and letting nature take its course is kinda like sending the bacteria off to Vegas, Cooper says. "What happens there stays there. You can't go into that environment and figure out the forces that led to those changes.

"But our system, because it has this daily cycle of renewal, allows you to define those forces as they happen. Because the whole population has to disperse and recolonize."

Not only that, but each time a bead comes out of a test tube, it goes to the freezer to join thousands of other ancestors, their icy afterlife amounting to an exquisitely detailed fossil record.

On a counter in the lab, catercorner from the incubator, is a machine that looks a bit like a gigantic microwave—a genetic sequencer the researchers affectionately named Roz (for chemist Rosalind Franklin). With her help, the team can tell exactly what mutations appear, and when. They can track entire bacterial family trees and study biofilms down to the level of individual organisms.

Cooper says he's living in the best time ever to study evolutionary biology and microbiology.

His studies focus on these evolving organisms not only in test tubes and animals, but also in samples from UPMC patient volunteers. Across these bacterial habitats, he's found common themes:

Bacterial communities quickly become diverse, and they stay diverse. A lot of diversification is driven by relatively small numbers of genes, no matter how many times Cooper's team repeats the experiment. And, when biofilming bacteria reinvent themselves to adapt to new territory, the adaptation they prioritize above all else is sticking, regardless of whether they land on a surface or inside a living, breathing host. "It doesn't matter what they stick to, very much," he says.

History is repeating itself again and again with a predictability he finds "stunning."

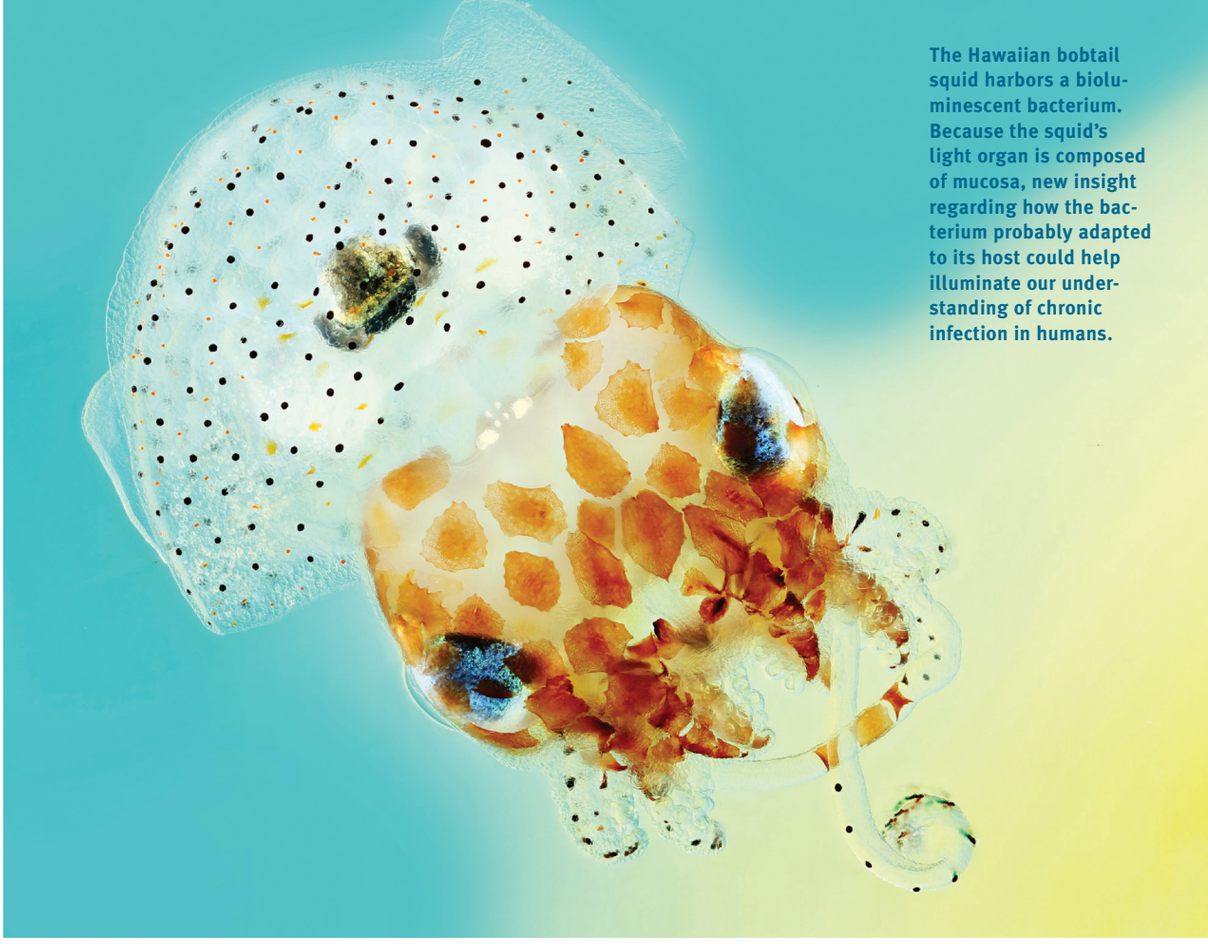
Evolution has always been taught as a retrospective science, says Cooper. "But now, we can almost look at it from the perspective of an engineer."

Gavin Sherlock, a Stanford geneticist who studies evolution in yeast, notes that evolution is largely driven by randomness, thus we can never predict its future with perfect clarity.

But we *can* project probabilities, he says (as scientists now do in determining which flu vaccine to create each year). And, in cases of extreme selective pressure—like that of antibiotics on bacteria—there are only so many mutations that will be viable. Then, the probabilities get much higher.

Cooper acknowledges that there's work to do yet before the consistency, and thus predictability, of evolutionary patterns is clear. But he's hopeful—and he says he's not alone. "We've had a few [international] meetings where we've talked about predicting evolution. And that really is . . . *gosh*. That's a game changer."

Evolution has always been taught as a retrospective science, says Cooper. "But now, we can almost look at it from the perspective of an engineer."



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The Hawaiian bobtail squid harbors a bioluminescent bacterium. Because the squid's light organ is composed of mucosa, new insight regarding how the bacterium probably adapted to its host could help illuminate our understanding of chronic infection in humans.

Cooper, a lean 44-year-old from Massachusetts, completed his 20th Ironman triathlon a few years ago before his knees "mostly" retired him from the sport. "I still love to swim-bike-run, though," he says. He's also an avid naturalist—he taught himself to fish at age 5. These days, he mostly casts in trout streams near his home in the North Hills and in various area ponds and lakes for bass—always with his kids (Soren, 10, and Harlan, 7).

As an undergrad at Amherst College, Cooper had designs on an honors program thesis on river ecology and the dynamics that shape selective pressure therein. Cooper recalls his adviser, an evolutionary biologist named Paul Ewald, telling him, *Yeah, Vaughn, that's great, but you're never going to get that done as an undergraduate—or a PhD candidate, for that matter. How about we try some experiments that are a bit more feasible?* Cooper then enlisted in Ewald's study of a particular virus that was decimating a regional species of moths. That experience reeled in Cooper, so to speak. He has been studying the evolution of pathogens ever since.

In grad school he worked in the lab of virus-evolution luminary Richard Lenski of Michigan State University. It was the presequencing era, and yet the lab discovered ("by a combination of luck and a lot of sweat," Cooper says) some of the first mutations that drove adaptation in *E. coli.*

Along the way, Cooper was waking up to the fact that most microbes don't live in well-mixed cultures. When he got his own lab at the University of New Hampshire, he developed his bead model, which made the covers of *ISME* (from the International Society for Microbial Ecology) in 2011 and the *Journal of Bacteriology* this October.

Collaborations with his New England colleagues continue. Just this April, *eLife* published Cooper and Cheryl Whistler's study of a dazzling little luminescent sea-beast known as the Hawaiian bobtail squid. "This is an amazing creature," Cooper swoons. "It cultivates bacteria to produce light for it. … [Then, the animal] doesn't cast a shadow, so it's harder for predators to detect it."

The squid and its mutualistic symbionts, *Vibrio fischeri*, are darlings of science literature for a host of reasons (forgive the pun). But one mystery is how the bacteria adapt to this symbiosis. So basically, the team simulated it. They took a close cousin of the bacterium and paired it with the squid. (Native strains of *Vibrio fischeri* themselves wouldn't do—they've already evolved to inhabit this host.)

As the cousin adapted to the fix-up, Cooper sequenced the bacterial DNA and found the mutation that made the marriage possible. "I think it's one of the best things we've ever been a part of," Cooper says.

His gushing isn't just his naturalist's tendencies talking. He explains that little is known about bacteria/host interactions at all, let alone on mucosal surfaces like the squid's light organ—or our own noses, lungs, and guts. He calls the paper a sign of things to come. The lab is deeply involved in deciphering how bacteria establish chronic infections.

Working with Jennifer Bomberger, a PhD assistant professor of microbiology and molecular genetics at Pitt, Cooper's lab is beginning to study the evolution of pathogens in the airways of people with cystic fibrosis. It's long been known that once these patients catch certain viruses, chronic biofilming bacterial infection tends to follow. By studying cultured cells from the airways of these patients, Bomberger may have uncovered the reason why. Respiratory syncytial virus appears to cause these epithelial cells to jettison their stores of iron—which the bacteria gladly eat up. Iron is biofilm fuel.

Recently, thanks to a $25,000 seed grant from Pitt's Clinical and Translational Science Institute (CTSI), Bomberger teamed up with Stella Lee—MD assistant professor of otolaryngology at Pitt and director of the Division of Sino-nasal Disorders and Allergy at UPMC—for a pilot study on how well this hypothesis holds up in humans. Its success led to further funding from Gilead, a biopharmaceutical company, to begin recruiting a larger group of patient volunteers this summer.

Pseudomonas is not the kind of bacterium that particularly likes living in the lungs. It prefers much more moisture, gravitating to enclaves like the puddles you pass on the street. In a pinch, it might colonize your nose. "The thought in the field has been that it adapts in the sinuses, and then some event happens that allows it to move to the lung," Bomberger says.

That event may well be viral infection, and the bacteria's adaptation to it. By studying sinus and sputum samples collected from Lee's surgical patients over time, Cooper and Bomberger hope to find out.

In an increasingly more mainstream view of human disease, our vulnerabilities are thought to stem from some trade-off our ancestors' DNA made to stay afloat. Did, say, your forefathers dodge malaria? Maybe you'll wind up with sickle cell disease. Did your Old Country kin survive plague? Perhaps your autoimmune disease is the price your family paid.

But it's still a win, because you're *here*. If you make it long enough to reach reproductive age, that's really all evolution cares about. "Selection doesn't optimize, right?" Cooper says. "It just sort of acts on the lowest-hanging fruit."

The same goes for bacteria, explains Yohei Doi, an MD/PhD, associate professor of medicine, director of Pitt's Center for Innovative Antimicrobial Therapy, and Cooper collaborator. But if you put bacteria in the environment of a hospitalized patient, suddenly it's "evolution on steroids." The bacteria are forced into mutating mucho, and fast, and those rapid-fire changes can't be pleasant. This leads Doi to believe that the so-called "superbugs" we read about in the headlines probably aren't built to last after all. "It's so totally unnatural for them," he says.

In 2008 or so, Doi, who was a Pitt fellow at the time, saw a worldwide spike in super-resistant strains hit home. Working with his mentor, David Paterson, he began collecting samples from patients at multiple time points. (He directs a hat-tip to UPMC's clinical microbiology lab, directed by Pitt associate professor of pathology A. William Pasculle, for so proactively rounding up specimens with each new outbreak.) Today, Pitt/UPMC has one of the largest collections of longitudinal strains of *Acinetobacter baumannii* in the country.

In six months, a dozen strains can evolve in a single hospitalized patient, says Doi. He's working with Cooper to learn what traits distinguish them: fitness levels, rates of growth, damage they do to host cells, and so on. And they are finding that, as the bacteria bend over backward to cope, new vulnerabilities do indeed bubble up. Doi is hoping to eventually capitalize on these shortcomings in the clinic. Say, for example, Mutant A warps its structure so antibiotics can't bind to it. Are there weaknesses in the walls? Say Mutant B produces an enzyme that eats up the antibiotic *du jour* like acid. Can that enzyme be blocked?

In some cases, the solution may be much simpler, says Doi. Sometimes, the bacteria mutate to survive an antibiotic, but then when the coast is clear, they mutate back. Which means that antibiotic can be used again.

In this new Big Data era, getting data is the easy part, says Doi. The rub is distilling it all down to a manageable number of targets to work on in-depth. "That's where there's a chasm. And [Cooper] is one of the people who bridges that."

Since landing at Pitt in 2015, Cooper's lab has published several papers with Doi, as well as others at Pitt. This productivity suggests a bright future for the emerging field of evolutionary medicine.

In February, Cooper and Pitt associate professor of medicine Cornelius Clancy published in *Clinical Infectious Diseases* a study pinpointing the mutations that enabled *P. aeruginosa* to gain resistance to a class of go-to antibiotics known as beta-lactamase inhibitor compounds.

The month before that, *mBio* published the Cooper lab's study of a pediatric leukemia patient case at St. Jude Children's Research Hospital. The infant, whose immune system had been completely wiped out by her chemo, unfortunately caught an *Enterococcus faecium* infection in spite of the hospital's best efforts. The bacterium resisted every drug they threw at it—until, that is, the girl's medical team infused her with certain cells that essentially amounted to an immune-system reboot. She cleared the infection within days and recovered.

Throughout the course of her infection, the medical team had the forethought to collect samples. They later invited Cooper to analyze the samples to learn why the pathogen had persisted for so long. After sequencing the genomes of 22 variants, Cooper found the bacteria had mutated to create "a Faustian bargain for that bacterial population," he says. The bacterial cells had adapted to this depleted immune status. And then, once the immune system was back online, *adios*.

But the bacteria's story didn't play out as you might expect, with mutant begetting warped and mangled mutant over and over again. "It turns out there was one mutation. That's it. That means that the path to pan-resistance is very short for that organism." The reason for this outlier is that infection in immunocompromised people is its own separate, terribly formidable animal. (Cooper is working to further understand its underpinnings.)

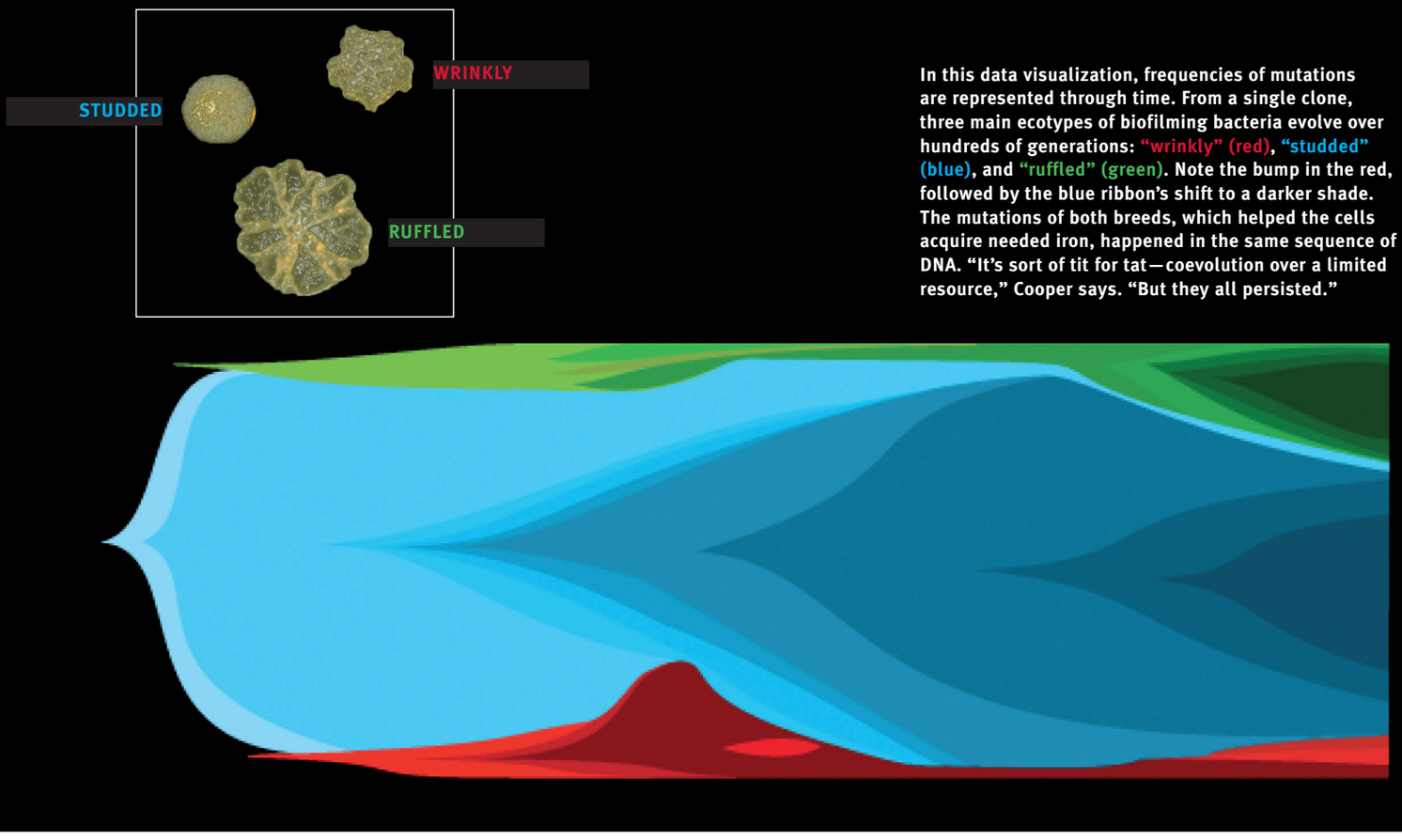
In another way forward with broad implications for human health, Cooper is chasing down a goal shared by just about every other branch of biomedical research: precision medicine.

As microbial evolution keeps playing out predictably right before his eyes, he has high hopes (albeit with a healthy dose of skepticism). Perhaps in some cases, we will know at the onset which kinds of antibiotics are worth prescribing to a patient, and which ones are a dead end. And we will know this based on genetic sequencing—an approach that, thanks to Roz and her ilk, is quite fast compared to the days or even weeks a lab culture can take. Just give Roz a sputum sample, and within hours you're in business.

In fact, it has been done before. In March, a colleague of Cooper's in Oxford predicted a resistant strain of tuberculosis (TB). "We know enough about what allows TB to resist various drugs that the sequence will tell you the answer," Cooper says.

They are finding that, as the bacteria bend over backward to cope, new vulnerabilities do indeed bubble up. Doi is hoping to eventually capitalize on these shortcomings in the clinic.

VAUGHN COOPER LAB



In this data visualization, frequencies of mutations are represented through time. From a single clone, three main ecotypes of biofilming bacteria evolve over hundreds of generations: "wrinkly" (red), "studded" (blue), and "ruffled" (green). Note the bump in the red, followed by the blue ribbon's shift to a darker shade. The mutations of both breeds, which helped the cells acquire needed iron, happened in the same sequence of DNA. "It's sort of tit for tat—coevolution over a limited resource," Cooper says. "But they all persisted."

In a bacterial homestead, a world where every single day is a new test of do or die, you might expect a wild west—an every bug for itself kind of rough and tumble.

But surprisingly, as Cooper's lab was the first to report, that's not the case.

When biofilming bacteria are ready to yield young'uns, they send out single-celled swimmers to stake new claims and plant new seeds. As they land, they glom onto something—like a bead from Cooper's experiments—and start pumping out polysaccharides, extracellular DNA, and a proteiney glue to hold it all together. Cooper hypothesizes that, in time, the goo surrounding this first wave of migrants takes shape, forming a distinct structure he calls a "wrinkly."

Two other kinds of characters show up and put down stakes in a similar fashion. The ones called "ruffled," for their lovely rosettes, fill in the gaps, sticking partly onto the plastic and partly onto their wrinkly brethren. The spurred specimens, which Cooper calls "studded," line the top.

Much like how Pittsburgh's topography has preserved its cultural communities over time (Polish, African American, Jewish, pick your hillside), in biofilms, bacterial societyfolk form physical niches and cultivate group identities. Nestled in their neighborhoods, they adapt and evolve, and adapt and evolve again. But instead of being cutthroat in their ways—with winners, losers, and cheats—these three groups (and ever-growing numbers of subgroups therein) evolve *together*.

"The [studded biofilm] that's on the outside is tending to feed the guys it's sticking to," says Cooper. "That's a food web, right? That's the producer-consumer relationship. . . . Very, very simple experiment producing these really complex phenomena that we see in nature. And it only takes a few months."

This is the story of life, in miniature: a single cell evolving into what Darwin famously called the "tangled bank" of complex, varied, and interdependent species. And it's all told in a replicable and measurable way. A few years ago, NASA took note and awarded the lab a spot in the NASA Astrobiology Institute, a research collective with the goal of uncovering the origins of multicellular life.

In 2014, Cooper used his super simple bead experiment to launch what he says is the most important thing he does. He turned the experiment into a high school science curriculum, a National Science Foundation–funded effort he calls EvolvingSTEM. Using the harmless bacterium *Pseudomonas fluorescens*, ninth-graders in New Hampshire—and, as of last school year, the Pittsburgh area's Peters Township—have been conducting their own bead-transfer experiments and watching wrinkly, studded, and ruffled mutants arise.

"I mean, there's nothing like *doing* science, right?" says Cooper.

"Many of [the teens] are doubtful about whether they *can* do it, but the protocol is so simple that most people wind up succeeding, and it builds a lot of confidence that they can do bench science."

A scientist true to form, Cooper has used surveys to compare EvolvingSTEM students against controls. The former had better learning outcomes. And they said they were more motivated to consider STEM fields, too.

There are other reasons this is his favorite pet project: These experiments completely undermine any suspicion of evolution, says Cooper. And a thin-to-nonexistent understanding of evolution really limits the understanding of life sciences as a whole—which has consequences.

It's easy to forget how clonal we *Homo sapiens* really are. Just 200,000 years ago, for example, there was just one mitochondrion among our species. Every single one of us descends from the same maternal ancestor who spun out a second one, likely under some pressure to survive.

And so we *do*.

When we lose sight of our origins, it is to our detriment, Cooper says. "We tend to focus on the little differences among individuals rather than a broad commonality." ■