High rates of plasmid cotransformation in *E. coli* overturn the clonality myth and reveal colony development

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The concept of DNA transfer between bacteria was put forth by Griffith in 1928. During the dawn of molecular cloning of DNA in the 1980s, Hanahan described how the transformation of DNA plasmids into bacteria would allow for cloning of DNA fragments. Through this foundational work, it is widely taught that a typical transformation produces clonal bacterial colonies. Using low concentrations of several plasmids that encode different fluorescent proteins, under the same selective antibiotic, we show that *E. coli* bacteria readily accept multiple plasmids, resulting in widespread aclonality and reveal a complex pattern of colony development. Cotransformation of plasmids occurs by either CaCl₂ or by electroporation methods. A bacterium rod transformed with three plasmids—each expressing a high level of a unique fluorescent protein—and replated on agar, appears to reassign a random number of the three fluorescent plasmids to its daughter cell during cell division. The potential to simultaneously follow multiple lineages of clonally related bacteria in a bacteria colony would allow for mosaic analysis of gene function. We show that clonally related bacterium rods self-organize in a fractal growth pattern and can remain linked during colony development revealing a potential target against microbiota growth.

Manipulation of *E. coli* colony development by DNA transformation has not been studied. *E.coli* colonies are generally viewed as a collection of bacterium with non-cell autonomous signaling rather than a developing organism. Mutant bacterial rods are studied for their ability to form a colony or biofilm. Typical competition studies are performed by the juxtaposition of large numbers of wild type and mutant bacterial rods. The ability of making mutants mosaically distributed in a developing colony has not been exploited. Here we have taken a de novo analysis of DNA transformation and uncovered that single bacterial rods can readily take up multiple plasmids and reveal for the first time how *E. coli* colonies develop. We show the potential to exploit this process for the study of mutations mosaically distributed within the development of a wild type colony.

Transformation of *E. coli* with DNA plasmids is a step for amplifying DNA fragments of interest, during molecular cloning. A typical procedure for generating DNA plasmids entails the cutting of a parent “vector” backbone carrying resistance and an origin of replication with a restriction enzyme along with alkaline phosphatase treatment to prevent vector re-closure. This vector is subsequently ligated with a fragment of interest. Other methods to incorporate an insert of interest also exist, such as ligations of PCR products with A overhangs directly into vectors that contain T overhangs (Promega) and DNA pairing (Gibson, TOPO). Ligated DNA is then transformed into “competent” bacteria, cells that have been pretreated to readily take up DNA.

Typically, DNA transformation is a two-step process: 1) pretreatment of bacteria with CaCl₂ (or variations thereof) or with water and 2) subsequent heat shock or electroporation, respectively. Pretreatment of bacteria makes them highly competent⁵. Transformation efficiency is measured in transformants or colony forming unit (cfu) per μg DNA used, this being calculated at 10⁸ cfu/μg for CaCl₂ pretreatment and subsequent heat-shock. The competency efficiency is about 10¹¹ cfu/μg for the water pretreatment/electroporation method. Competency status of bacteria is usually established with 1 to 30 nanograms (ng) of supercoiled DNA, which is comparable to the quantity used for DNA cloning procedures. One nanogram of a typical supercoiled 4 kb plasmid contains about 2.3 × 10⁸ molecules and would yield a maximum of 10⁶ colonies. Hanahan demonstrated that transforming

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more than 35 ng of DNA yielded similar numbers of colonies as 1 ng, suggesting that the number of plasmid molecules is at threshold relative to the number of competent bacteria. Finally, the hallmark of DNA cloning is that the methodology yields bacterial colonies that are said to be clonal, because each originate from a single bacterium. By contrast, selection for cotransformants of bacteria with two different plasmids carrying two selectable markers is possible, but does not lead to development of mosaic colonies.

Do standard methods in DNA cloning lead to clonal colonies? In the past, our laboratory has generated large plasmids of up to 20 kb by standard DNA cloning methods to create targeting vectors for homologous recombination in embryonic stem cells. Targeting vectors (TV) DNA consisted of a vector backbone and two arms of genomic DNA (~4 kb each) with a PacI site between the arms. We incorporated reporter DNA cassettes at the PacI site by digestion of a second plasmid and gel isolation of a ~5 kb IRES-tauLacZ (ITL) reporter fragment from its 3 kb vector backbone. Ligation of ITL, PacI insert into a PacI digested TV yielded dark blue colonies after addition of X-gal due to the LacZ gene in the cassette. Such colonies were subsequently grown in liquid culture followed by DNA isolation, sequencing and restriction analysis.

We observed that E. coli cultures growing a correct clone reached saturation very slowly (up to 24 h) and produced low quantities of plasmid DNA. However, many uniformly dark blue colonies that seeded cultures became confluent much faster (12 h) and upon restriction analysis and DNA sequencing were revealed to be a religated PacI vector without ITL, which did not produce dark blue colonies upon retransformation. We found this result especially surprising as the 5 kb (ITL) and 3 kb backbone vector fragments were easily separable by gel electrophoresis. Thus, any contamination of the ITL insert with its 3 kb backbone vector would be very low. This persistent anomaly forced us to grow multiple clones and throw away the rapidly growing cultures, which would firmly establish the cotransformation phenomena. Recent advances have shown GFP protein can be split into two portions capable of re-annealing: beta strands 1–10 and 11 only. In such a circumstance, robust fluorescent can only be observed when both portions of the GFP are expressed in the same cell. Thus, we created expression vectors that express each portion separately using sfGFP9,10 (see Data File S1) and determined whether green fluorescence could be observed. Such fluorescence would parallel patterns of E.coli development observed by the three plasmid cotransformation experiments (Fig. 2L1,L2), since the only fluorescence that could be observed will occur from stable expression of both plasmids in the same bacteria. In fact, this is precisely what we observed. No-fluorescence was seen with either portion of sfGFP alone (Fig. 3A,B), but when both were coexpressed, fluorescence was readily detected (Fig. 3C) and in all bacterial colonies had fluorescence in a mosaic pattern (Fig. 3D). No colony could be observed that was uniformly fluorescent, which also suggests that plasmid equilibrium is a robust competitive process during the earliest rounds of bacterial divisions. By

A direct test for clonality. To distinguish between these two possibilities we constructed a set of nearly identical plasmids differing only in their expression of several fluorescent proteins (mCerulean, Venus—with both derivatives of Aequorea victoriae—and mCherry, aka Cherry, from Discosoma sp.) that can easily be distinguished by confocal microscopy. To generate plasmids, we subcloned into pGEM-T easy (ColE1 origin) PCR products containing a constitutively active lactamase promoter (LamR) upstream of the start codon of a fluorescent proteins (XFP). Each of these plasmids also contain a lactamase resistance gene (lam) in its backbone (see Data File S1).

We initially performed a straightforward CaCl2 transformation experiment by either combining all three plasmids post-heat shock (Separate Transformation) or prior to heat shock (Mixed Transformation). We expected to see the same results from both experiments: circular colonies, each expressing only one fluorescent protein. But, this pilot experiment led to two unexpected observations. From the Separate Transformation, we found that most colonies revealed Cerulean or Venus or Cherry fluorescence alone (Fig. 1A-E), however, some colonies contained multiple fluorescent bacteria and maintained the typical circular shape (Fig. 2A-D). In some circumstances non-circular colonies were observed that were clearly derived from distinct populations of transformants (Fig. 2E-H). When we dispersed tortuous fluorescent routes taken by one fluorescent population within another one (Fig. 2I-A, SIC-C-F) when these resuspended colonies were diluted serially and aliquots of those dilutions were plated in order to obtain plates with single colonies that grew up each from a single cell, distinct fluorescent colonies emerged. An additional colony type was easily observed in the Mixed Transformation, appearing to contain different combinations of fluorescent proteins at different fluorescent intensities in patterns that did not resemble sectors or concentric rings (Figs. 1F-J, 2I–L,1A–d,1La–d). This pattern is different than previously described. When we dispersed and replated these multiple fluorescent colonies at limiting dilutions, the fluorescent mixtures were maintained in nearly all-resulting colonies suggesting that three plasmids were common to each bacterium (Fig. S1B, SIG-J). Based on this result, it was difficult to argue against the idea that a triple transformation event had occurred.

Direct evidence for cotransformation. Validation of cotransformation by genetic complementation would firmly establish the cotransformation phenomena. Recent advances have shown GFP protein can be split into two portions capable of re-annealing: beta strands 1–10 and 11 only. In such a circumstance, robust fluorescence can only be observed when both portions of the GFP are expressed in the same cell. Thus, we created expression vectors that express each portion separately using sfGFP9,10 (see Data File S1) and determined whether green fluorescence could be observed. Such fluorescence would parallel patterns of E.coli development observed by the three plasmid cotransformation experiments (Fig. 2L1,L2), since the only fluorescence that could be observed will occur from stable expression of both plasmids in the same bacteria. In fact, this is precisely what we observed. No-fluorescence was seen with either portion of sfGFP alone (Fig. 3A,B), but when both were coexpressed, fluorescence was readily detected (Fig. 3C) and in all bacterial colonies had fluorescence in a mosaic pattern (Fig. 3D). No colony could be observed that was uniformly fluorescent, which also suggests that plasmid equilibrium is a robust competitive process during the earliest rounds of bacterial divisions. By
contrast, robust fluorescence (Fig. 3C,D) was not observable in colonies there were a mixture of sfGFP1-10 and GFP11 bacteria, showing that bridges between colonies cannot reconstitute full fluorescent sfGFP molecule. At least 11 plasmids can be simultaneously cotransformed. In order to determine cotransformation events, we set up a PCR screen for each fluorescent plasmid. Venus and Cerulean coding sequences are nearly identical, so we altered the codon usage for Venus at its most divergent point to Cerulean to generate specific oligomers and we confirmed by PCR the presence of all three plasmids. Because we readily observed cotransformation of three different plasmids in a bacterial colony, we surmised that it would be possible for even more plasmids to cotransform.

We constructed a set of unique Venus expression plasmids by the addition of nucleotide sequence specific tags after the stop codon to extend our analysis (see Data File S1). In addition, we switched to the brighter blue fluorescent protein mTeal, aka Teal, from Clavularia sp. Here, we made a mixture of 10 Venus plasmids (V1-V10), 1 ng each, along with 1 ng of our Cherry plasmid and cotransformed the 11 ng mixture. However, prior to plating, we added 1 ng of Teal expressing plasmid to control for stickiness of a plasmid that might confound our PCR test (Fig. 4A). It is important to note that Teal, Venus, and Cherry are from three different organisms and share minimal nucleotide homologies.

Venus fluorescent colonies were easily identified with Cherry fluorescent mosaicism by microscopic inspection; no Teal fluorescence was observed in any colony. PCR analysis of 10 mosaic colonies revealed the presence of many combinations of Venus plasmids along with the Cherry plasmid, averaging 5 unique plasmids per bacterial colony (Data File S1). No Teal plasmid could be identified as anticipated (Fig. 4B). We were surprised that one colony (#4) contained all 11 plasmids and another (#8) contained 7 plasmids. Both of these colonies were replated at limiting dilutions to obtain sparse single colonies. These subclones were reanalyzed by PCR: Colony #4 subclones, #4–1 to #4–5, revealed differing subsets of the 11 Venus plasmids and none contained more than 5 plasmids. However, colony #8 subclones, #8–1 to #8–6, revealed that 5 out of 6 colonies contained at least 6 plasmids with #8–4 having all of the original 7 plasmids, showing the surprising stability of these multiple Venus plasmids (Fig. 4C). Thus, at least 11 distinct plasmids could be cotransformed into a single bacterium using 11 ng of plasmid DNA. It should be noted that 11 ng of plasmid DNA is a significant lower number than most laboratories use in transformation experiments. Based on the ease of this observation, it is likely that many more plasmids can be cotransformed.

A smaller plasmid outcompetes a larger one. The above observations suggested that our hypothesis of cotransformation of TV + ITL and the recircularized donor vector must be true. To directly confirm our original observations we took two plasmids, one expressing Cherry (3,805 bp) and a targeting vector containing the ITL cassette (~17.5 kb). We mixed 1 ng of each together and introduced them into bacteria using the CaCl₂ transformation method. We obtained 151 colonies: 146 Cherry fluorescent positive by microscopy and 5 that were devoid of Cherry fluorescence. As expected all 5 of the non-Cherry fluorescent colonies amplified early in the Sybr Green based qPCR detection process solely with primers specific for ITL. However, 4 out of 146 Cherry...
fluorescing colonies also amplified with primers specific for ITL, albeit late in the Sybr Green based qPCR detection process, suggesting they were in much lower abundance (Data File S1). Not surprisingly, replating and repeating the PCR on subclones from the 4 colonies failed to detect any residual ITL. Thus, we now confirm our hypothesis and show directly that the fast replicating 4 kb Cherry plasmid outperformed the larger plasmids. We show that 2 ng total plasmid DNA (1.25 ng molar equivalents of replicons: 1 ng of the Cherry plasmid, ~ 4 kb plasmid DNA, plus 0.25 ng molar equivalent for the 1 ng of ~ 17.5 kb plasmid) can result in observable cotransformation rates of 2.6% (4 out of 151 of the total bacterial colonies). This rate of cotransformation appears to be more common than had been described in the literature.

**Figure 2.** High density plating produces mixed segregating colonies whereas coexpressing colonies are mosaic. (A–C) Low magnification image of many segregated round colonies. (D) is overlay. (E–G), High magnification image; one colony made up of five separate transformants using saturating laser excitation. (H) is overlay. (I–K) Low magnification image of mixed transformations; four of five colonies coexpress using saturating laser excitation. (L) is overlay (arrows 1 and 2 are two coexpressing colonies). L1a-c and L2a-c are high magnification image of colonies 1 and 2, respectively in L observed with non-saturating laser excitation. L1d and L2d are overlays clearly revealing reciprocal mosaicism of fluorescence.
**CaCl₂ mediated cotransformation of plasmids is a common occurrence.**

PCR is a sensitive way to detect cotransformation, however, two confounding variables complicate the use of PCR as a quantitative test of absolute cotransformation rates. First, it is not possible to perform PCR on an entire colony, thereby every cell in a colony cannot be reliably analyzed. Second, even if all bacteria could be assessed, PCR cannot differentiate between a mosaic bacterium carrying multiple plasmids or two neighboring bacteria each carrying different plasmids. We had initially confirmed our findings of cotransformations with multiple plasmids by a strategy of limited dilution and replating and subsequent reanalysis by confocal microscopy. In general, PCR identification of multiple plasmids from our transformations with low DNA concentrations, resulted from cotransformation events rather than contamination from a neighboring colony.

Unfortunately, our Lam→XFP plasmids did not fluoresce with enough intensity to provide us with single bacterium resolution and therefore we could not assess the fates of any single bacterium hiding within a bacterial colony. However, while shuttling fluorescent DNA inserts during pGEM-T cloning we observed a single strong Venus expressing bacterial colony, about 6–8 × more fluorescent intensity (Hi→Venus) during colony formation than our original Lam→Venus version. This new plasmid, to be described in detail elsewhere, relied on the lac operon promoter to create constitutively expressed fluorescent proteins throughout colony formation. Using this vector template, we substituted Venus with other XFPs to generate a series of Hi→XFP plasmids (lam): Hi→
Cerulean, Hi→Teal, Hi→Venus (codon altered) and Hi→Cherry. We analyzed single bacterium fluorescence for all the four XFPs and only Hi→Cerulean fluorescence was not reproducibly observed in single bacteria. Hi→XFP mixtures (Teal/Venus/Cherry) provided the foundation for our endeavor to understand cotransformation rates where single bacterium resolution can be readily imaged (Fig. 5B, S5A-D, Movie S3).

The first version of our cotransforming assay was performed by mixing 0.1 ng of Hi→Venus and 0.1 ng of Hi→Cherry plasmids followed by the addition of 1 ng of Hi→Teal plasmid post heat shock. PCR analysis on 95 colonies and subsequent replating by limited dilutions of two candidate cotransformed bacterial colonies revealed 1 out of 95 colonies was truly a double positive and 1 out of 95 was a mosaic colony of two separate transformants. No Hi→Teal could be amplified or visualized in the colonies. Thus, 0.2 ng total DNA yielded a ~ 1% observed double positive (ODP) rate of cotransformation (Data File S1). However, we do not score for two Hi→Venus or two Hi→Cherry plasmids cotransforming (unobservable double positive, UDP) with the same plasmid. Taking into account these unobserved double positives (Data File S2), the estimated double positive (EDP) rate for 0.2 ng is higher and closer to 2% (Table 1). Our finding that only about 98% of colonies are clonal...

Figure 4. E. coli can take up at least 11 plasmids. (A) 10 versions of the Lam→Venus plasmids were created, each separable by PCR. 1 ng of each were mixed with 1 ng of Lam→Cherry and cotransformed. Following cotransformation, 1 ng of Lam→Teal was added prior to plating (all 12 plasmids carry same resistance). (B) PCR analysis of 10 minimally Cherry fluorescent mosaic colonies and two colonies that appeared Cherry fluorescent only. Lam→Cherry was detectable by PCR, whereas Lam→Teal was not detectable. Colony #4 was positive by PCR for all 11 PCR primer sets and #8 was positive for 7 PCR primer sets. (C) Colonies #4 and #8 were subcloned and a few colonies were tested by PCR. An average of 5 plasmids were stably maintained.
colonies when using 0.2 ng of plasmid DNA is crucial and dispels the current belief that CaCl₂ transformation into bacteria yields clonal colonies (Table S1).

Three plasmid transformations reveal high rates of aclonal colonies. A slight 0.1 ng increase in plasmid DNA to 0.3 ng (0.1 ng of each: Hi→Teal, Hi→Venus, and Hi→Cherry) yielded 3 out of 95 colonies with multiple plasmids or a 3% ODP rate (97% clonal). Again, we cannot score for double positive colonies of two Hi→Teal, Hi→Venus, or Hi→Cherry plasmids, so these rates are likely conservative. When calculating the unobserved events for three plasmid using 0.3 ng, the clonality rate dips to 94% with an EDP rate of cotransformations of 6% and no observed triple positives (OTP). Based on our ability to directly visualize single bacterium fluorescence and discern the difference between two colonies growing together and a true double positive bacterium, we finalized a high-throughput scoring method where 96 random colonies were picked into a microtiter dish, grown overnight and replated as 1 µl droplets on a fresh plate and grown for a second night (Fig. S2 and S3). This replating strategy accurately distinguished neighboring (mosaic) from double or triple positive colonies (Data File S1). We next tested tenfold more plasmid DNA, 3 ng (1 ng for each of Hi→Teal, Hi→Venus, and Hi→Cherry), which yielded 9 double positives and one triple positive and increases the ODP cotransformation rate to 10% and the observed triple transformation rate (OTP) to 1%. Once again, the non-observable triple cotransformation events such as 3 plasmids of Hi→Teal, Hi→Venus or Hi→Cherry or paired combinations of one plus a different plasmid are not counted. The estimated rate of clonality drops to 82%, which is surprising and notable for the practicality of molecular cloning methods, as most laboratory transformation protocols are performed with more than 3 ng of DNA.

Finally, we tested transformation rates in the range used in standard cloning and retransformation, 30 ng of plasmid DNA (10 ng of each Hi→Teal, Hi→Venus, and Hi→Cherry plasmids). Our rates of clonality further decreased to 72% based on 20% ODP and 8% OTP colonies, but the estimated clonality was only 44%. Thus, 30 ng of plasmid DNA transformations using CaCl₂ mediated transformation and plated on carbenicillin (Carb) selection plates yields low rates of clonal colonies, scored by microscopy. We repeated these observations over the course of months and at various concentrations and readily established a colony clonality rate relative to the amount of plasmid used (Table S1 and Data File S1). These observed clonality rates for the transformations were further reduced when we accounted for the unobservable events (EDP and ETP-Data File S2 and Table 1). Finally, performing transformations with 0.1 ng of 0.033 ng for each Hi→XFP using purchased, highly competent (10⁹ cfu/µg) bacteria, we found 3.3% ODP and 0% OTP colonies, similar to our 3% ODP and 0% OTP using 0.3 ng and lower than our 4% ODP and 1% OTP using 1 ng in our laboratory produced competent (10⁷ cfu/µg) bacteria. Thus, using cells 100 times more competent does not increase cotransformation rates by 100 fold (Data File S1) leading to the conclusion that cotransformation rates are dependent on the DNA concentration and not the competency status of the bacterial aliquot.

Cotransformation frequencies also occur using kanamycin selection. Our analysis suggested that using standard cloning methods with calf intestinal phosphatase (CIP) treatment would reduce cotransformation rates that would otherwise be rampant with recircularized vector plasmids. We also observed mixed fluorescent colonies after transforming when performing simultaneous triple insert ligations (Cerulean, Venus
and Cherry) into a CIP treated vector; These mixed clones did not contain vectors with multiple inserts. In all experiments so-far described, the plasmids that were cotransformed contained the same selection marker (lam).

The high rates of cotransformation suggested that the occurrence of mixed fluorescent colonies is solely dependent on DNA concentration and not plasmid selection by a resistance marker. To test this hypothesis in CaCl₂ cotransformation experiments, we mixed plasmids with two different resistance markers, kanamycin (Kan) and carbenicillin (Carb)-kan and lam, respectively (Data File S1). Here we selected only for the Kan plasmid and asked if we could observe fluorescence from the Carb containing plasmid that might have been cotransformed. Initially, we cotransformed 1 ng of a ~5 kb plasmid containing a weak cherry (Lo→Cherry) fluorescent reporter (kan) with 2 ng of a Hi→Teal (lam) or 2 ng of a Hi→Venus ~4 kb plasmid (lam), 3 ng total. We randomly selected 96 colonies that grew on Kan only plates. Colonies were picked and subsequently grown in Carbenicillin media, which confirmed that Carb containing plasmids did indeed co-transform with the Kan containing plasmids. (Fig. S4A-P). We observed 19% of the colonies were cotransformed (17 out of 96 Kan resistant colonies were highly fluorescent for Teal, without this plasmid being selected, and in a separate experiment 20 out of 96 Kan resistant colonies were highly fluorescent for Venus, without this plasmid being selected, and both sets were weakly mosaic for Lo→Cherry). The clonality rate of 81% for 3 ng of total plasmid is similar to the 91% observed Carb resistance alone for 3 ng of plasmid DNA. Transformation rates were based on growth in Carb media followed by direct visualization.

We were in a position to ask if the ODP rates were a property of the total DNA transformed or simply on the Carb resistant plasmids. Using only Kan selection, cotransformation of 0.2 ng of Hi→XFP mix (lam) along with

| Transformation parameters used | Heat shock 1 min HS | Heat shock 1 min HS |
|-------------------------------|---------------------|---------------------|
| Conditions                    | No Wash             | Chilled Cuvettes    |
| DH5alpha                      | PF-Lab              | NEB                 |
| Plasmids                      | Mix                 | Mix                 |
| Method                        | CaCl₂               | Electroformation    |
| Total DNA                     | Carbenicillin       | Kanamycin           |
|                               | *Kan→Carb*          | Carbenicillin       |

Table 1. Summary of cotransformation rates with multiple plasmids under different parameters that include the unobservable events. *The Kan→Carb column reflects concentrations for Carb only. In addition to this concentration, 1 ng of Kan plasmid was added during cotransformation experiments.
1 ng of the Kan plasmid yielded 5% double positive colonies whereas 10 ng of the Hi→XFP mix (λam) along with 1 ng of Kan plasmid yielded 40% double positives, 5% triple positives and 1% quadruple positives (Table 1; Fig. S4Q-T and S5E-F). Because we cannot ascertain multiple Kan resistant colonies: double, triple, or quadruple positive colonies, estimated clonality for the total 1.2 ng and 11 ng cotransformations must be lower than 95% and 54%, respectively. Still using only Kan selection, we cotransformed 11 ng of DNA, but with 1 ng of the Hi→XFP mix (λam) and 10 ng of the kan plasmid, which gave a 88% clonal rate, which is between the 0.2 ng and the 2 ng rates for the Hi→XFP and distant from 10 ng Hi→XFP rates (Table 1). Thus, cotransformation rates in this context were not dependent on the total amount of Kan resistant DNA used. It was surprising that selection on Kan plates did not affect rates of cotransformation of a Carb resistant plasmid. Finally, we created Hi→XFP-Kan ~ 5 kb plasmids, by cloning a 1 kb kan resistant gene within the λam gene and tested if clonality rates under Kan selection differed from Carb selection. The main difference here is that these transformations are required to grow in nonselective media for at least 30 min prior to plating. To our surprise, combined ODP and OTP rates of cotransformation appeared quite low and plateaued at 13% for both 10 ng and 30 ng Hi→XFP-Kan mixes (Table 1). It is possible that these cotransformation rates were lower than the Carb plasmids due to their 25% increase in plasmid size.

**Cotransformation frequencies by electroporation are temperature sensitive.** Thus far we have focused on the standard Hanahan CaCl$_2$ transformation technology and hypothesized that cotransformation may be tightly associated with this poorly understood and unknown mechanism. By contrast, electrocompetent bacteria take up plasmids through a water environment during the process of electroporation (EP)\textsuperscript{11-13} and may be immune to cotransformation. Using our Hi→XFP Carb resistant plasmids, we show ODP rates were fairly low for 1 ng to 30 ng DNA concentrations, ranging between 3–6% aclonality rates. In addition, OTP rates were almost non-existent, 1 out of 1042 colonies analyzed. We were surprised by these results and decided to electroporate bacteria with higher concentrations: 300 ng mixture of Hi→XFP; achieved 36% ODP and 10% OTP rates, driving the clonality down to 54% of the colonies. Thus, the electroporation process is not resistant to high cotransformation rates.

Electrocompetent bacteria are normally prepared by several washes in ice-cold water, followed by maintaining bacteria on ice in water prior to EP in cuvettes kept on ice. These chilled cuvettes are at an elevated temperature relative to the chilled bacteria in a liquid environment. Thus, adding the bacteria/DNA mixture prior to EP has the potential to alter the bacteria making them less competent or able to take up DNA by EP. We asked if maintaining the bacteria in a colder state by keeping cold bacteria in freezer-chilled cuvettes would alter cotransformation rates. Indeed, transformation rates rose dramatically with the identification of triple transformed colonies in the 1 ng Hi→XFP mix (2 out of 168 colonies) and in the 10 ng Hi→XFP mix (5 out of 204 colonies) as compared to a single observation among 1042 colonies from the chilled cuvettes. Thus, if bacteria are maintained in a cold environment during the electroporation, the clonality rates of Hi→XFP plasmids of 97% for 1 ng and 83% for the 10 ng are similar to those observed during CaCl$_2$ treatment (Table 1, S1 and Data File S1). Therefore, cold, but not ice-cold, cuvettes can limit cotransformation rates.

**CaCl$_2$ transformation is dependent on plasmid association with the bacterium.** Using our Kan selection experiments, we show that cotransformation rates are not dictated by the total amount of plasmid DNA in a transformation reaction. Because the mechanism of CaCl$_2$ transformation is unknown, we employed our Hi→XFP plasmids to test if the plasmid DNA-CaCl$_2$ precipitates were associated with the bacterial membrane during the process. If yes, could we wash off the DNA from the membrane of competent cells prior to heat shock with CaCl$_2$, water or Tris–EDTA (TE)? Thus, we designed an experiment to reveal any residual Hi→XFP transformation on Kan plates did not affect rates of cotransformation of a Carb resistant plasmid. Finally, we created Hi→XFP-Mix were lowered to 10% whereas the 30 ng Hi→XFP-Mix colonies were at 27% despite 2×ice-cold CaCl$_2$ washes, and 1% for the Hi→XFP-Sep colonies. Thus, the DNA appears to be tightly associated with the bacteria and cannot be washed off by additional CaCl$_2$. We followed up on this experiment by using two washes with room temperature water (instead of CaCl$_2$), which we expected would solubilize the potential plasmid- CaCl$_2$ composites associated with an individual bacterium. However, transformation still occurred, but cotransformation rates for 30 ng Hi→XFP-Mix were lowered to 10% whereas the 30 ng Hi→XFP-Sep were reduced to 0%. In contrast, no transformations occurred with 2×room temperature TE washes. This latter result seemed inconsistent with the 2×RT water washes, which not only allowed for transformations, but also non-clonal colonies. So either the TE was solubilizing DNA-CaCl$_2$ aggregates or maybe the RT water washes themselves might not be “washing” the DNA of the bacteria, but rather providing a heat shock to the bacteria prior to the placement at 42 °C for 1 min; the 42 °C (2nd heat shock) might cause an increase in lethality due to the extended time that bacteria would be in the presence of elevated temperatures. Thus, we reduced the time at heat shock to just under a minute. CaCl$_2$ transformations with 2×CaCl$_2$ washes and reduced incubation at 42 °C led to increased cotransformation rates up to 40% for the 30 ng Hi→XFP-Mix, and 3% for the 30 ng Hi→XFP-Sep colonies. In stark contrast to the room temperature water washes, the ice-cold water washes yielded no transformations and may have indeed washed off the plasmid from the bacterial membrane. We concluded that the plasmid DNA is loosely associated with CaCl$_2$ treated bacterial membranes prior to heat shock, but unlikely to precipitate the cotransformation event as we observe cotransformation by electroporation without the presence of CaCl$_2$ (Table S2 and Data File S1).
Bacterial colony development suggests complex developmental processes. Finally, we sought to understand how our mixed colonies obtained their patterning. A large segment of the archetypal E. coli literature implies that bacteria colony formation occurs in subsequent expansions of concentric circles. However, our mixed colonies exhibited fractal patterns of development similar to early stages of replicating bacteria. To test if the fractal colony growth pattern is independent of constitutive high fluorescence expression, we decided to express our fluorescent proteins using an arabinose inducible plasmid system with the ColE1 origin of replication and to repeat the cotransformations, inducing XFP expression after colonies had formed. Not only were triple transformants and fractal patterns observed, but we also found that fluorescent intensity varied amongst a set of concentric rings within the bacterial colony, suggesting that differential distance from the colony center dictates gene expression patterns.

In an effort to observe how these Hi→XFP multifluorescent colonies emerge, we set up an extended time course to analyze how a single triple fluorescent bacterium forms a colony. Initially, this colony-founder bacterium divides in a fractal pattern and, after a few hours, different lineages of fluorescent bacteria emerge, presumably due to differential plasmid loads of the Hi→XFP plasmids. Colonies are not initially round, but after about 10 h of the fractal growth pattern, the colonies achieve a more roundish shape; under high power magnification, the edges of the colonies are not in the shape of a perfect circle but rather look uneven like the gyri and sulci observed in cerebral cortex. Thus, the fractal growth pattern is still present when a colony is fully formed.

While following the growth pattern of our E.coli bacteria, we arrived at two important findings. First, many of the colony-founder single bacteria failed to divide and were eventually swallowed up by a colony forming in...
Figure 7. Time course of triple fluorescent colony development. 0 min to 420 min. A triple fluorescent bacterium (A) expressing Hi→Teal, Hi→Venus, and Hi→Cherry was imaged every 20 min for 420 min. All 20 min timepoints are in Fig. S7 and Movie S1A. Mosaicism is quickly revealed after a few cell divisions.
close proximity. This can explain how mosaic bacterial colonies containing other bacteria units form. Second, we often observed the initial colony-founder bacterium producing replicates, but failing itself to divide, creating a point of nucleation for the colony. Finally, our lineage tracing experiments revealed fluorescent bacterium that had divided and stayed attached to each other after replication (Fig. 6M-P) creating a chain of linked progeny. These linked chains could be observed in 3D (Movie S3 and S4). We concluded that our multi-fluorescent assay can be used for identifying genes involved in maintaining progeny linkage during replication, relevant to chemotaxis and lipid secretion behaviors of bacteria.

Mosaicism to study development. In order to study mutant bacteria it is important for those mutations to allow for colony development. It is likely that large numbers of mutants could survive when surrounded by wild-type bacteria secreting or contributing non-autonomous signals. Our proposed mosaic colony screens will determine how colony development are affected when neighboring bacteria are mutated. In particular, mutations that disrupt robust fractal patterning would be most readily identified. There are several types of screens using cotransformation to place multiple plasmids in the same bacterium. We envision two types of mosaic analyses to study these event (Fig. 8A,B): 1) A two population screen: where one plasmid reflects wild-type bacteria segregating from bacteria under or over-expressing a mutant protein. 2) A three-population screen: where each plasmid alone produces a phenotype and a third phenotype is observed when both plasmids are stably expressed in the same bacteria. It should be noted that all experiments described here are with the current plasmids, which constitutively express our proteins of interest. Other versions of these plasmids could yield distinct populations.
as well. We foresee that adding the ability of protein induction to these schemes further allows bacterial colonies to prosper, until the mutation is imposed on their development.

Conclusion
Our observations confirmed that a single bacterium is the foundation of our mixed colony phenotypes. Cotransformation of several plasmids was readily observed with as little as 0.1 ng of each plasmid, a concentration that is remarkably lower than what is commonly used for cloning or retransformation cloned DNA. Thus, many existing plasmid preparations likely contain multiple types of plasmids, some containing mutations. When retransformed at too high a concentration, resulting bacterial colonies may have preferentially selected mutant plasmids capable of outcompeting the original one. Our lab routinely sequences new subclones from insert ligations with primers that can reveal multiple sequences, i.e. multiple insertion events from distinct clones, which are accidently grown at the same time by the process of cotransformation or contamination described above. This analysis provides us with the basis for discarding clones that have multiple sequence traces at any position in the insert. In addition, we recognize that bacterial colonies that are not completely circular can arise from two neighboring colonies. To limit DNA from multiple plasmids in a preparation, we select colonies that are circular in appearance. Finally, we foresee potential of our fluorescent protein expression system to replace the blue/white subcloning techniques with a fluorescent/non-fluorescent cloning methodology. Typical blue/white selection is performed by adding X-gal or IPTG/X-gal to bacterial plates followed by the plating of DNA transformation. X-gal diluted in N, N Dimethylformamide, can reduce the growth rate of bacteria and thus reduce plasmid yield. In contrast, nothing needs to be added to our Hi → Teal, Hi → Venus, Hi → Cherry and Hi → sfGFP vector transformations in order to produce bright fluorescent colonies with an observable color under white/room light after an overnight growth (Fig. S9). When an insert is cloned into a polylinker disrupting its open reading frame, the fluorescence is abolished yielding white colonies. We believe that the Hi → Cherry fluorescent colonies yielded the most contrast with the white insert containing colonies under white/room light (Fig. S9).

We provide a profound set of data to reveal properties of bacterial transformation that challenge how we perceive molecular DNA cloning and unmask fractal growth patterns of E. coli colonies from a single bacterium founder to a whole colony. Importantly, we provide a platform to further pursue either avenue of investigation. In particular, these assays could further the understanding of genes involved in growth and replication patterns of E. coli and potentially other bacteria, and may yield clues to the development of microbial biofilms, relevant to human pathogenesis.

Materials and methods
DNA transformations. All DNA transformations were carried out with 100 mM CaCl₂ competent DH5α E.coli. In general: DNA incubations were performed with cells on ice for 30 min, followed by a 1 min heat shock at 42 °C, then a 1 min incubation step back on ice. For carbenicillin (lam plasmids) plates (1.5% agar), 600 μl of media was added before plating. For kanamycin (kan plasmids) plates (1.5% agar), 600 μl of media was added, followed by 30 min incubation at 37°C prior to plating. For pGLO induction with 2% L-arabinose was performed after overnight growth for 6 h (http://www.bio-rad.com/en-us/product/pglo-bacterial-transformation-kitInduction) All images (except movies) were taken with a 2.5x, 5x, 10 × or 20X/0.5 Plan-Neofluar Zeiss lens using an LSM510 confocal microscope.

Cotransformation of 10 Venus plasmids. Ten Venus plasmids (V1-V10) plus one Cherry plasmid (D355-2), each at 1 ng/µl (~ 11 ng total) plus 50 µl CaCl₂, ice 25 min, heat shock 1 min 15 s, ice 1 min.

1 ng of Teal plasmid was then added (D378-1), then addition of 600 μl of media and plating.

12 Cherry colonies were picked (10 predominantly Venus fluorescence and 2 without). Only a portion of each colony assayed by PCR was picked.

Movies. A mixed colony was picked into a well of a 96-well plate and grown overnight in 100 µl of 2XYT media. 1 µl was taken out and diluted into 1 ml of 2XYT media. 1 µl was again taken out of this dilution and and diluted into 1 ml of 2XYT media. 300 µl of it was dispensed on a agarose plate. The next day, a tip was dipped in a colony, then swirled in 1 ml of 2XYT media. 10 µl -20 µl of this media was placed in the center of a small agarose plate. Small plates were made of a 4.5% agarose main bottom layer topped with a superficial layer of 3% agarose. The plates were placed on a heated microscope stage at 33-35°C and cover-slipped leaving an air interface. Images were taken with 20X/0.5 Plan-Neofluar Zeiss lens on a LSM 510 Zeiss Confocal Microscope at 4 × optical zoom. The developing colony was manually refocused every 20 min by moving the stage up in the Z position by about 20 µm.

Data availability
All data generated or analysed during this study are included in this published article [and its supplementary information files].

Received: 9 September 2021; Accepted: 9 June 2022
Published online: 07 July 2022

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**Acknowledgements**

We thank Ivan Rodriguez, Thomas Bozza, Diana Bratu, Carmen Melendez-Vasquez and Anna-Katerina Hadjan-tonakis for helpful insights throughout the data collection phase. Also, Ivan Diaz, Assistant Professor, Division of Biostatistics Department of Healthcare Policy & Research, Weill Cornell Medical School for helping with the analysis. Many thanks to the members of the lab, especially Eugene Lempert, Ute Schwinghammer, Irena Parvanova, Charlotte D’Hulst for providing thoughtful comments. This work was supported by Research Centers in Minority Institutions Program grant from the National Institute on Minority Health and Health Disparities (MD007599), NIH SC1 GM088114 (P.F), and CTSC UL1 TR000457-06 (I.D.). We would like to dedicate our manuscript to Roger Tsien, who made it possible to peek inside cells with an array of fluorescent technologies.

**Author contributions**

PF and D.T. wrote the manuscript text. PF prepared Figures 1-6, 8. D.T prepared Figure 7. J.B. and P.F. performed experiments in Figures 1-2, 4-6, 8. D.T. performed experiments in Figure 7. L.T performed experiments in Figure 3. J.B., D.T., L.H and P.F. each took part in the design of the experiments. All authors reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-14598-9.

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