Droplet Tn-Seq combines microfluidics with Tn-Seq for identifying complex single-cell phenotypes

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While Tn-Seq is a powerful tool to determine genome-wide bacterial fitness in high-throughput, culturing transposon-mutant libraries in pools can mask community or other complex single-cell phenotypes. Droplet Tn-Seq (dTn-Seq) solves this problem by microfluidics facilitated encapsulation of individual transposon mutants into growth medium-in-oil droplets, thereby enabling isolated growth, free from the influence of the population. Here we describe and validate microfluidic chip design, production, encapsulation, and dTn-Seq sample preparation. We determine that 1–3% of mutants in *Streptococcus pneumoniae* have a different fitness when grown in isolation and show how dTn-Seq can help identify leads for gene function, including those involved in hyper-competence, processing of alpha-1-acid glycoprotein, sensitivity against the human leukocyte elastase and microcolony formation. Additionally, we show dTn-Seq compatibility with microscopy, FACS and investigations of bacterial cell-to-cell and bacteria-host cell interactions. dTn-Seq reduces costs and retains the advantages of Tn-Seq, while expanding the method’s original applicability.
Transposon-insertion sequencing (Tn-Seq), and related techniques including IN-Seq, TraDIS, and HTS, have become the gold standard to determine the quantitative contribution of a gene to fitness under a specific growth condition in high-throughput and genome wide. It has been successfully applied to bacteria, yeast, and eukaryotes, and has enabled discoveries, including gene function, noncoding RNAs, host factors affecting disease susceptibility, bacterial transmission determinants, and vaccine targets. One of the biggest strengths of Tn-Seq is the ability to screen hundreds of thousands of mutants in a single experiment. However, growing mutants en masse, i.e. in a pool, could mask altered fitness of certain mutants. For instance, secreted enzymes that break down complex glycans into smaller units for energy utilization can be viewed as community factors since mutants that do not produce these enzymes can “cheat” and reap the carbon-source benefits. In addition, mechanisms including frequency-dependent selection, bet-hedging, and division of labor can retain mutants with a relatively low individual fitness in a population, which are missed by Tn-Seq. Moreover, being able to evaluate phenotypes through microscopy or FACs could create alternative opportunities to identify leads for gene function.

In order to obtain a comprehensive understanding of a complex bacterial population, it is thus important to consider the growth fitness of each individual cell in isolation as well as in the context of the population. One way to address population patterns that are present within a complex pool of cells, specifically a complex pool of mutants, is to create an ordered mutant library. In such a library, every bacterial mutant is separated into a different well of a microtiter plate. Although ordered cell libraries are a tremendous resource, generating them is prohibitively expensive and time consuming. As a solution, we developed droplet Tn-Seq (dTn-Seq), by combining droplet microfluidics with Tn-Seq. In dTn-Seq, a microfluidic device enables encapsulation of millions of single transposon mutants into micron-sized droplets, in which bacteria are cultured. Each transposon mutant thus starts off in a complex pool of mutants, is then separated and cultured in isolation, and finally cells are pooled back together. Before encapsulation and after pooling, genomic DNA is isolated for sample preparation, and the change in frequency of each mutant over the course of the experiment is determined through massively parallel sequencing to calculate the individual growth rates of each mutant. Therefore, through strategic isolation and pooling, dTn-Seq enables the establishment of single-cell fitness in a genome-wide and high-throughput fashion (Fig. 1). Moreover, besides the ability to resolve complex single-cell behavior, droplets have additional advantages and applications including a substantial reduction in culture media volume (and possible expensive compounds such as host glycoproteins or proteases such as elastase), it enables analysis of bacterial microcolony formation, interbacterial interactions, and bacterial–host cell interactions through FACs and microscopy.

Results and discussion
Microfluidics separate single mutants into droplets. For “standard” Tn-Seq, complex, mutant populations are created, in which each bacterial cell contains a transposon somewhere inserted in the genome, thereby disrupting the function of the affected genetic component. To assess the fitness of each mutant in isolation from the rest of the pool and thereby possibly uncover population masking effects, a microfluidic device was designed and manufactured in-house that enables encapsulation of individual cells into growth medium-in-oil droplets, in which bacteria can be cultured (Fig. 2a; Supplementary Fig. 1, Supplementary Data 1). The size of the device channels, along with the oil and aqueous phase flow rates, determine droplet size and production frequency. For the described device this yields droplets with a ~65–67 µm diameter, ~144–157 pl volume, at a production rate of ~5 × 10^6 droplets/min. Monodisperse droplets are composed of an outer fluorinated oil-surfactant layer and can be filled with liquid growth medium (Fig. 2b, c), which enables 5–8 generations of bacterial growth for Gram-negative and -positive bacteria alike with comparable growth dynamics to bacteria grown in liquid batch culture (e.g., an 8-ml culture; Fig. 3a). Moreover, by adding agarose to the liquid medium at a 1% concentration stable monodisperse agarose droplets are formed that provide a matrix, which supports microcolony formation (Fig. 2d, e; Fig. 3b).

Fitness assessment of individual mutants in isolation. The ability to apply microfluidics to separate a pool of transposon mutants into droplets and culture them successfully creates the opportunity to assess the fitness of each mutant in isolation from
the rest of the population. Similar to Tn-Seq, this can be achieved by determining the frequency of each mutant at the start and at the end of the experiment through Illumina sequencing by using barcodes to determine the frequency of each mutant at the start and at the end of the experiment. Similar to Tn-Seq, this can be achieved by determining the frequency of each mutant at the start and at the end of the experiment. Optimized WGA conditions did not introduce bias into the resulting fitness data compared with the standard Tn-Seq approach ($R^2 = 0.89$; Fig. 4a) and the reproducibility between biological replicates was high ($R^2 = 0.88$; Fig. 4b). Alternatively, sample preparation in random barcode Tn-Seq (RB-Tn-Seq) is simpler than Tn-Seq. For those organisms for which a RB-transposon exists only a PCR on the genomic DNA is needed before sequencing$^{17}$, making it likely that a WGA step is unnecessary if dTn-Seq is combined with RB-Tn-Seq. Lastly, dTn-Seq results could be negatively influenced if a significant proportion of droplets during encapsulation end up loaded with multiple cells. Assuming a Poisson distribution, at a cell concentration of $\sim 2 \times 10^6$ cells/ml the fluidic device should generate a droplet population that consists of $\sim 74\%$ empty droplets, $\sim 22\%$ with single cells, and $\sim 3\%$ with two or more cells$^{18}$. Encapsulation frequencies were empirically determined by mixing two *Streptococcus pneumoniae* strains, one expressing GFP and the other RFP, in a 1:1 ratio and at two different final cell concentrations of $1.75 \times 10^6$ cells/ml and $2.5 \times 10^6$ cells/ml. Mixed populations were encapsulated into agarose droplets, cultured, and imaged by brightfield and fluorescent microscopy to determine the cell occupation frequency. From these analyses, we determined that the device generates $78.8\% - 83.7\%$ empty droplets, $15.3\% - 19.5\%$ droplets with single cells, and $1.0\% - 1.8\%$ droplets with two or more cells (Supplementary Fig. 2). These distributions are thereby within the expected range, which means that $\sim 90\%$ of filled droplets contain a single cell and $\sim 10\%$ of filled droplets will contain double-encapsulated cells. If we hypothesize that $\sim 10\%$ of insertions behave differently in a droplet compared with batch culture than that means that $\sim 1\%$ of those insertion mutants will be double encapsulated. While these sporadic double encapsulations can thereby affect a single cell’s phenotype and add noise to the fitness data, the effects should be limited. Moreover, fitness for a genetic component is, similar to Tn-Seq, calculated from multiple transposon insertions (i.e., from multiple individual mutants with a transposon inserted within the same genetic component) and for each condition multiple libraries (usually six) are sampled$^{4,9}$. This should mostly limit the effects of double encapsulations and result in high-confidence fitness data.

dTn-Seq uncovers population structure-dependent fitness. To determine the functionality of dTn-Seq, six transposon-insertion libraries (\~{}10,000 mutants each) of *S. pneumoniae* were grown in batch culture as pooled populations (“standard” Tn-Seq) and encapsulated in droplets as single cells (dTn-Seq) under four different conditions: (1) growth medium with glucose as the major carbon source; (2) growth medium with the complex host glycan alpha-1-acid glycoprotein (AGP) as the major carbon source; (3) growth medium with glucose and the human leucoyte produced protease elastase, which is responsible for neutrophil-mediated killing of *S. pneumoniae*; and (4) growth medium with glucose and a 1% agarose-droplet density to assess how a solid substrate that provides structural support affects single-cell growth and promotes microcolony formation. For each gene in each condition, relative fitness was calculated as described previously, which represents the growth rate in that specific environment and enables direct cross-environment
to no

This means that a gene of interest thus shows little

replaced with AGP or supplemented with elastase (Supplemen-

ted medium with glucose and droplets, in which glucose was

grown in compact microcolonies for both Gram-positive and -negative bacteria. White arrows indicate developed microcolonies. Source data are

available in the Source Data file.

Fitness is thereby only considered when com-

posed of at least three data points (transposon insertions), and

significant environment effects are assessed by taking into

account the amount of variance in the overall experiment and by

comparing fitness through a Student’s t test with Bonferroni

correction for multiple testing. The goal is to identify insertion

mutants that have a

significance, which is disrupted by separating and culturing each mutant

(Supplementary Data 2)

Overall, depending on the environment, 1–3% of

screened mutants from a variety of categories, including meta-

bolism, transport, regulation, and cell wall integrity have a sig-

ificantly different fitness between batch and droplet growth

(Supplementary Data 2–4). This indicates that population struc-

ture, which is disrupted by separating and culturing each mutant

in its own environment (i.e., a droplet), can significantly affect

clonal fitness. To validate the fitness results acquired from dTn-

Seq, a total of eight genes were picked from the four different

conditions and explored in more detail.

LytB affects competence and cell death in a confined space. Comparing fitness data obtained from batch culture Tn-Seq with
dTn-Seq in the simplest environment with glucose as the carbon

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source shows that a \textit{lytB} (SPT\_1238) knockout in \textit{S. pneumoniae} strain Taiwan-19F has no effect on fitness in batch culture (batch\textsubscript{WSPT\_1238} = 0.98), however, when grown in droplets the mutant has a severe growth defect (droplet\textsubscript{WSPT\_1238} = 0.60) (Fig. 5; Supplementary Data 2). To determine whether the phenotype arises from the absence of a complex mutant population in droplets, \textit{ΔlytB} was grown by itself in a larger 8-ml culture volume, which masked the low-fitness phenotype that was observed in droplets (Fig. 5d). It is possible that the growth difference of \textit{ΔlytB} in a 8-ml culture compared with droplets is due to intrinsic environmental differences that could exist within the droplet. For instance, in the droplet environment, \textit{S. pneumoniae} may experience differences in pH, CO\textsubscript{2}, O\textsubscript{2}, or H\textsubscript{2}O\textsubscript{2} concentration, however, varying these parameters in batch culture did not affect \textit{ΔlytB} growth (Supplementary Fig. 3). This means that the growth defect seems to be due to the small droplet environment, which suggests that in this case it is not the population structure that affects \textit{ΔlytB} fitness, instead it is the
compartment size that triggers a specific phenotype in this mutant.

LyTB is part of the lytic cycle of <i>S. pneumoniae</i> and is involved in cell-chain shortening<sup>[19]</sup>. Indeed, bacterial cell chains of the ΔlyTB mutant are increased when grown in batch. When the mutant is grown in droplets, chain lengths are shortened and similar to wild-type (wt) (Fig. 5b, c). Induction of local hypercompetence, which is in part controlled by the sensing of competence-stimulating peptide (CSP) through the ComDE two-component regulatory system, has been associated with longer cell chains<sup>[20]</sup>. Even though ΔlyTB has shorter chains in droplets, we hypothesized that the shorter chains could be a side effect of further enhancement of competence within the confined droplet environment. Gene expression of the competence-associated genes <i>comD</i>, <i>comE</i>, <i>comX</i>, <i>comM</i>, <i>cbpD</i>, and <i>lytA</i> was determined for wt and ΔlyTB grown in batch and in droplets. Most striking is that <i>comD</i>, <i>comE</i>, <i>comX</i>, and <i>comM</i> all have a lower expression in batch culture in the ΔlyTB background compared with wt. However, in droplets all of these genes get downregulated in the wt, while in ΔlyTB these genes get upregulated between ~two- and eightfold (Fig. 5e, f). In addition, in ΔlyTB the autolysins <i>cbpD</i> and <i>lytA</i> become ~fourfold and ~32-fold upregulated, respectively, while in the wt background only <i>cbpD</i> gets upregulated (Fig. 5e, f). It is likely that due to the confined environment of the droplet, (quorum) signaling molecules, such as CSP, can build up to a high concentration, thereby triggering the competence system and induce a local hypercompetent state. While in the wt, this can be controlled and results in several competence genes becoming downregulated with no measurable effects on fitness, in the ΔlyTB background all of the analyzed competence genes get upregulated (Fig. 5f). Especially, the increased induction of the autolysins in ΔlyTB, which induce lysis and thus cell death, may explain why growth of the mutant in the confined droplet environment is significantly limited and cell chains are shortened. Thus, LyTB seems at least partially involved in controlling a hypercompetent phenotype from becoming too dominant through the reduction of autolysis and fratricide and thus limiting cell death. This underscores that a phenotype uncovered with approaches such as dTn-Seq are not always necessarily straightforward to interpret. Because, even though we set out to identify the effect of complex communities on individual mutant fitness, the phenotype of LyTB seems to be triggered by the confined environmental compartment size. Interestingly, other studies show that a buildup of signaling molecules can occur in host tissue or densely packed biofilms<sup>[21]</sup> and affect bacterial growth states and survival<sup>[22]</sup>, which would indicate that dTn-Seq, where a similar buildup can occur, could be used to mimic and explore such environments.

Community factor genes utilizing AGP as a carbon source. We next tested <i>S. pneumoniae</i> libraries grown in the presence of the host factor alpha-1-acid glycoprotein (AGP). AGP has an immunomodulatory role<sup>[23]</sup> and is also an important carbon source for many bacteria, including <i>S. pneumoniae</i><sup>[24]</sup>. However, bacteria such as <i>S. pneumoniae</i> are unable to take up these large structures and depend on monosaccharides being liberated extracellularly by a variety of specialized secreted or cell-surface-attached bacterial enzymes<sup>[25]</sup>. Four genes (<i>nagB</i>/SP_1415, <i>phosphosugar-binding transcriptional regulator</i>/SP_1674, <i>nanE</i>/SP_1685, and <i>nagA</i>/SP_2056) were identified through dTn-Seq to have a small growth defect in droplet culture in the presence of AGP (Fig. 6a; Supplementary Data 3). Moreover, three out of the four genes had no fitness effect in batch culture with AGP compared with the baseline environment (batch culture with glucose), while <i>nagA</i>/SP_2056 had a small defect in batch culture with AGP, but its negative fitness effect was larger in droplets with AGP (Supplementary Data 3). dTn-Seq thus indicates that each gene is dispensable when grown by itself in medium with glucose, but it is important for growth when grown in isolation with AGP as the sole carbon source. These phenotypes were validated with growth curves performed with individual knockouts for each gene in growth medium with glucose or AGP as the main carbon source (Fig. 6b, c). Importantly, and as suggested by dTn-Seq, when a deletion mutant of <i>nagB</i>, SP_1674, <i>nanE</i>, or <i>nagA</i> is grown in the presence of the wild-type, i.e. in a mixed culture, the growth defect in AGP is largely masked. Even though a small defect for <i>nagB</i> and <i>nagA</i> remains (the latter not being significant), the fitness of each of these mutants is significantly improved and largely compensated by the wt (Fig. 6c). This indicates that the wild-type strain is providing community support when grown in medium with AGP as the main carbon source and can compensate the reduced fitness of the dTn-Seq-identified mutants. While none of the four genes have previously been associated with AGP in <i>S. pneumoniae</i>, or shown to be influenced by the community, each gene is associated with either regulating, releasing and/or processing AGP-linked monosaccharides. Specifically, <i>nagB</i> and <i>nagA</i> have been shown in other species to be involved in processing GlcN and GlcNAc<sup>[26]</sup>; SP_1674 is a predicted transcriptional activator of a regulon containing <i>nanA</i> and <i>nanB</i>, which have been shown to release sialic acid from complex glycan structures<sup>[27]</sup>, and <i>nanE</i> is a putative lipoprotein anchored to the membrane and important for sialic acid utilization<sup>[28]</sup>. These data reveal that dTn-Seq is indeed able to identify gene products and processes that can be shared amongst bacteria and which enable the opportunity for community resource “cheating”, which are missed with traditional Tn-Seq.
A mechanosensitive channel protects against elastase. Other environments in which survival can possibly be influenced due to shareable resources are those in which bacteria interact with components of the host immune system. An example of this is neutrophil-mediated nonoxidative antimicrobial killing, which can involve serine proteases such as elastase, cathepsin G, and proteinase 3, which are critical for killing of a number of bacteria including \textit{S. pneumoniae}, \textit{Klebsiella pneumoniae}, and \textit{Staphylococcus aureus}.\textsuperscript{28–30} Here, bacterial growth in the absence or presence of elastase in droplet culture is compared. The use of elastase as the selective pressure introduced more variation in the presence of elastase, while when both strains are co-cultured, \textit{Amscl}’s survival is indistinguishable from wt, when the mutant is cultured in the presence of the wt. “Percent survival” was calculated relatively to an untreated control (each experiment was performed at least three times and significance was determined through a two-way ANOVA). All error bars are standard error of the mean; ns = not significant, * < 0.05, ** < 0.005, *** < 0.0005, **** < 0.00001. Source data are available in the Source Data file.

### Fig. 6 Mutants sensitive to host-specific factors can be compensated by co-culture

**a** The volcano plot shows genome-wide fitness changes from a dTn-Seq experiment comparing growth in droplets with either glucose or alpha-1-acid glycoprotein as the main carbon source. Validated genes \textit{nagA}, \textit{SP}_1674, \textit{nanE}, and \textit{nagA} are circled in red. **b** While Δ\textit{nanE} (orange) grows similarly to wt (blue) in glucose, there is a growth defect for the mutant (green) compared with wt (red) when AGP is the sole carbon source. **c** Individual and mixed culturing combined with bacterial cell enumeration on agar plates containing antibiotics enabling differentiation between the wt and mt were used to determine relative fitness of the mt. While mutants grow just as well as the wt when glucose is the main carbon source (orange bars), they grow significantly slower than the wt when grown independently (green bars). However, their fitness is significantly improved when grown in the presence of the wt (blue bars), indicating that wt is providing “community support” (each growth experiment was performed at least four times, significance was determined through a one-way ANOVA with Bonferroni correction for multiple testing). **d** Volcano plot comparing growth in droplets in the absence and presence of elastase. **e** Deletion mutant Δ\textit{mscl} (green) has reduced survival in two different concentrations of elastase compared with wt (orange) when cultured as single strains. However, Δ\textit{mscl} survival in elastase is improved, and indistinguishable from wt, when the mutant is cultured in the presence of the wt. "Percent survival" was calculated relatively to an untreated control (each experiment was performed at least three times and significance was determined through a two-way ANOVA). All error bars are standard error of the mean; ns = not significant, * < 0.05, ** < 0.005, *** < 0.0005, **** < 0.00001. Source data are available in the Source Data file.

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has been shown in bacterial species such as S. aureus, Salmonella enterica, and Pseudomonas aeruginosa31 (and is a phenomenon that has also been suggested to occur in S. pneumoniae32,33). Alternatively, MscL might be affecting the environment in such a manner that it leads to a reduction in activity of elastase, thereby also protecting bacteria without the functional gene. We observed that in a mixed culture the survival of the wt does become more variable, as indicated by higher variance in the data, however one could argue in favor of either hypothesis. Most importantly, this result shows that dTn-Seq is able to identify genes that interact with the host immune system, which could lead to detailed insights into how components of the immune system are involved in killing bacterial cells and how bacteria are able to avoid this.

Capsule genes dispensable for microcolony development. We found that by adding agarose to growth medium, solid monodisperse droplets are formed that provide a stable matrix for culture growth. In each condition, the relative growth expansion was determined for each strain by counting the number of cells in the population at the beginning of the experiment and at the end after 5 h of culture. Growth expansion of the mutants is relative to the expansion of the wt strain within each growth environment (each experiment was performed at least four times). Growth is improved when the mutants are embedded in agarose (Kruskal–Wallis with Dunn’s test for multiple testing; ns = not significant, ** < 0.005, error bars are standard error of the mean). Agarose droplets in oil with encapsulated S. pneumoniae, which have developed into compact microcolonies, ΔcpsC and ΔcpsD mutants develop similarly sized microcolonies compared to WT after 5 h. White arrow indicates microcolonies. Source data are available in the Source Data file.

Fig. 7 Capsule genes cpsC and cpsD are expendable in agarose droplets. a The volcano plot shows genome-wide fitness changes from a dTn-Seq experiment between droplet growth in glucose and droplet growth in 1% agarose. Significant genes are highlighted in color, which represent each gene’s functional category shown in the “Gene Category” figure key. b Wild-type (orange), ΔcpsC (green), and ΔcpsD (blue) were cultured in either liquid batch culture or agarose droplets. In each condition, the relative growth expansion was determined for each strain by counting the number of cells in the population at the beginning of the experiment and at the end after 5 h of culture. Growth expansion of the mutants is relative to the expansion of the wt strain within each growth environment (each experiment was performed at least four times). Growth is improved when the mutants are embedded in agarose (Kruskal–Wallis with Dunn’s test for multiple testing; ns = not significant, ** < 0.005, error bars are standard error of the mean). c Agarose droplets in oil with encapsulated S. pneumoniae, which have developed into compact microcolonies, ΔcpsC and ΔcpsD mutants develop similarly sized microcolonies compared to WT after 5 h. White arrow indicates microcolonies. Source data are available in the Source Data file.

Droplets enable bacterial cell and cell–host interactions. Besides the ability to separate and culture individual bacterial cells in isolation of the population, we also explored whether dTn-Seq can be used in applications to screen for phenotypes that are not directly the result of differences in growth rate. For instance, we were interested in whether interactions between bacterial cells or between bacteria and host cells can be explored, which are interactions that can critically affect bacterial population composition and/or survival38–40. Droplets force cells to grow in a confined space, and with the addition of agarose small spatial distances between cells can be maintained once the agarose is gelled. Agarose droplets, from which the oil has been removed, can then be easily switched between different treatments, visualized by microscopy, or sorted based on a specific fluorescently linked trait through fluorescence-activated cell sorting (FACS). Below we describe two examples in more detail that explore the potential to study cell–cell interactions utilizing agarose droplets by following: (1) competence signaling between S. pneumoniae strains through competence-stimulating peptide (CSP), and (2) the interaction between Yersinia pseudotuberculosis (Yptb) and bone marrow-derived macrophages (BMDMs).

CSP signaling in S. pneumoniae is part of a quorum-sensing process used to trigger competence mechanisms including uptake and recombination of the extracellular DNA, and can either induce or protect from fratricide, depending on the competence...
state of the cell\textsuperscript{41–43}. We generated the \emph{S. pneumoniae} strain sfCSPr that expresses GFP in response to CSP-1 was mixed with strain ADP112 that produces CSP-1 upon IPTG induction. A 40:1 mixture of ADP112:sfCSPr was encapsulated into agarose droplets, the oil removed, and then fluorescence and brightfield microscopy images were captured after 3-h culture in the absence (\textbf{a}) and the presence (\textbf{b}) of 1 mM IPTG (white arrows highlight GFP-expressing sfCSPr macrocolonies). Subsequent FACS analysis of agarose droplets accurately represents the predicted GFP signal frequency in the absence (\textbf{c}) and the presence (\textbf{d}) of 1 mM IPTG. \textbf{e}, \textbf{f} Gelled agarose droplets can be re-encapsulated into another droplet to form a second layer of agarose. Agarose droplets containing sfCSPr were re-encapsulated into media containing 1% agarose in the absence (\textbf{e}) or presence (\textbf{f}) of 560 ng/ml CSP-1. After 2 h of culture, the oil was removed and brightfield and fluorescence imaging revealed no GFP expression for the untreated sample, but positive GFP expression for the CSP-1 treated sample (black arrow highlights non-induced sfCSPr, white arrow highlights GFP-expressing sfCSPr, white asterisk indicates the inner droplet, black asterisk indicates the outer droplet layer). \textbf{g–j} Agarose/hydrogel droplets containing \emph{Yersinia pseudotuberculosis} (\textit{Yptb}) were exposed to murine bone marrow-derived macrophages (BMDMs) \textit{Yptb} strain IP2666 GFP + was grown in droplets overnight and visualized by fluorescence (\textbf{g}) and brightfield (\textbf{h}) microscopy. After a 1-h incubation, BMDMs can attach to oil-free empty droplets (\textbf{i}) or droplets containing \textit{Yptb} cells (\textbf{j}) (white arrows indicate \textit{Yptb} cells and black arrows indicate BMDMs).

\textbf{Fig. 8} Bacterial cell-cell and cell-host interaction models. \textbf{a–d} \emph{S. pneumoniae} strain sfCSPr that expresses GFP in response to CSP-1 was mixed with strain ADP112 that produces CSP-1 upon IPTG induction. A 40:1 mixture of ADP112:sfCSPr was encapsulated into agarose droplets, the oil removed, and then fluorescence and brightfield microscopy images were captured after 3-h culture in the absence (\textbf{a}) and the presence (\textbf{b}) of 1 mM IPTG (white arrows highlight GFP-expressing sfCSPr macrocolonies). Subsequent FACS analysis of agarose droplets accurately represents the predicted GFP signal frequency in the absence (\textbf{c}) and the presence (\textbf{d}) of 1 mM IPTG. \textbf{e}, \textbf{f} Gelled agarose droplets can be re-encapsulated into another droplet to form a second layer of agarose. Agarose droplets containing sfCSPr were re-encapsulated into media containing 1% agarose in the absence (\textbf{e}) or presence (\textbf{f}) of 560 ng/ml CSP-1. After 2 h of culture, the oil was removed and brightfield and fluorescence imaging revealed no GFP expression for the untreated sample, but positive GFP expression for the CSP-1 treated sample (black arrow highlights non-induced sfCSPr, white arrow highlights GFP-expressing sfCSPr, white asterisk indicates the inner droplet, black asterisk indicates the outer droplet layer). \textbf{g–j} Agarose/hydrogel droplets containing \emph{Yersinia pseudotuberculosis} (\textit{Yptb}) were exposed to murine bone marrow-derived macrophages (BMDMs) \textit{Yptb} strain IP2666 GFP + was grown in droplets overnight and visualized by fluorescence (\textbf{g}) and brightfield (\textbf{h}) microscopy. After a 1-h incubation, BMDMs can attach to oil-free empty droplets (\textbf{i}) or droplets containing \textit{Yptb} cells (\textbf{j}) (white arrows indicate \textit{Yptb} cells and black arrows indicate BMDMs).
single bacterium (Fig. 8)) also supported the adhesion of BMDMs to agarose droplets containing hydrogel. This setup could thereby enable screening for non-contact-mediated interactions between Yersinia and macrophages, which are known to affect virulence and clearance of the bacterium47.

d’Tn-Seq as a valuable complementary tool to Tn-Seq. In order to improve our understanding of how pathogenic bacteria cause disease or evolve antibiotic resistance, it is critical to develop approaches to determine how bacteria deal with or adapt to environmental stress, such as the host immune system, varying carbon sources, and antibiotics. In the past 10 years, the ease of use and high-throughput nature of Tn-Seq methods, such as Tn-Seq, IN-Seq, TraDIS, and HTS, have allowed for faster and easier strategies to obtain leads for gene function and increased insight into how bacteria cause disease. However, we hypothesized that these methods may fail to identify certain phenotypes, such as those caused by population effects within batch culture of the complex transposon libraries. Therefore, by combining Tn-Seq with droplet microfluidics, we developed d’Tn-Seq, a method which efficiently separates and cultures transposon mutants in their own compartment (a droplet), and enables identification of mutants whose fitness is affected by the presence of other (mutant) strains in the population. To illustrate the validity of d’Tn-Seq, we describe: (1) a novel role for lytB which contributes to controlling local hypercompetence and limiting cell death; (2) four genes (nagB, phosphosugar-binding transcriptional regulator, nanE, and nagA) that are involved in the community usage of host alpha-1-acid glycoprotein as an energy source; (3) an ion channel (mscl) that improves survival in the presence of neutrophil elastase; and (4) two capsule genes (cpsC and cpsD) that are necessary for planktonic culture, but are dispensable during microcolony formation. Moreover, we show that the droplet environment enables robust growth of Gram-positive and -negative species alike providing support that d’Tn-Seq can be implemented for any species for which Tn-Seq exists. We show that droplets can be imaged, that agarose droplets are sortable via FACS, and how d’Tn-Seq could be implemented to study phenotypes such as bacterial cell–cell and/or bacteria–host cell interactions.

Lastly, the droplet environment also potentially reduces the amount of compounds and chemicals needed to perform an experiment. For instance, elastase and AGP used in the experiments described above are expensive compounds, and can quickly increase experimental costs. While we normally perform Tn-Seq in ~8-ml cultures, an equivalent number of mutants can be screened with d’Tn-Seq in 400 µl, allowing for a ~20× reduction in volume and cost. While we recognize that syringe pumps (~$500) and microfluidic mold fabrication and chip production (~$200) have some start-up costs, these costs are quickly offset by: (1) a single mold can be used to generate many PDMS chips; (2) each PDMS chip contains 38 separate devices; and (3) each device can be re-used. Moreover, a more standard method to determine single-mutant phenotypes is through the use of an ordered array. While the construction of such an array is costly and time consuming, utilizing an array to perform experiments is also expensive, and would require even more material than a standard Tn-Seq, let alone d’Tn-Seq. For instance, screening ~1500 S. pneumoniae nonessential genes with an ordered mutant library in a 96-well format, requires a total culture volume of at least 225 ml (assuming ~150 µl per well). Thus, the significant reduction in volume, along with the inherent difficulty of generating and utilizing an ordered mutant library makes d’Tn-Seq an attractive alternative, or at the very least, complementary.

In conclusion, d’Tn-Seq is applicable to a wide variety of bacteria and can be used as an extension to any variation of Tn-Seq to uncover (complex) single-cell phenotypes due to population affects, environment size, or interactions with the extracellular microbial and/or host environment.

Methods
Bacterial strains, growth, and media. Sequencing and validation experiments were performed using Streptococcus pneumoniae strains TIG4 (NCBI Reference 518960, #NP_093028.3), and Y. pseudotuberculosis IN-Seq (NC-1#NP_092465.1, #NP_093028.3) to generate bacterial strains for the study for growth models were Yersinia pseudotuberculosis (IP3666), Yersinia pestis (KIM6, pCD1-negative and ppgm-negative), Escherichia coli (DH5α), Acinetobacter baumannii (ATCC 17978), Staphylococcus aureus (RNU, NR-45904), Klebsiella pneumoniae (UHKP37, NR-44537), Pseudomonas aeruginosa (PA114), Enterobacter aerogenes (NRRL B-115), and Enterobacter cloacae (NRRL B-442). Except for specific growth and selection experiments, the S. pneumoniae strains were cultured either statically in Todd Hewitt broth supplemented with yeast extract (THT) plus 5 µl/ml Oxystrepsa (Oxystrepsa, Inc.) and 150 µl/ml catalase (Worthington Bio Corp LS01896), or on Sheep’s blood agar plates at 37 °C in a 5% CO2 atmosphere. Y. pseudotuberculosis strains were cultured either statically in Todd Hewitt broth supplemented with yeast extract (THT) plus 5 µl/ml Oxystrepsa (Oxystrepsa, Inc.) and 150 µl/ml catalase (Worthington Bio Corp LS01896), or on Sheep’s blood agar plates at 37 °C in a 5% CO2 atmosphere. Y. pseudotuberculosis was cultured in 2XYT, and Y. pestis was cultured in brain heart infusion media or on blood agar while all other strains were cultured in Luria-Bertani (LB) broth or on LB agar at 37 °C. Unless otherwise noted, cells were cultured to the exponential phase before being washed in PBS and diluted down into the appropriate media. Growth curves were generated using a Tecan Infinite 200 PRO plate reader.

Microfluidic device production. Microfluidic device masks were designed using AutoCad 2016 software (AutoDesk) (Supplementary File 1), and photomasks made of acetate transparency film were ordered from CAD/Art Services, Inc. (Bandon, OR). The silicon mold and final microfluidic chip fabrication were performed at the Integrated Sciences Cleanroom and Nanofabrication Facility at Boston College. A master mold was fabricated by coating a silicon wafer with the negative photoresist SU-8 (MicroChem) using a spin coater (SU-8 1100) with a thickness of 40 µm, and set by baking at 95 °C. The photomask was aligned with the silicon wafer and UV exposed followed by a post-exposure bake ramping from 65 °C to 95 °C over 4 min. The mold was developed using SU-8 developer (MicroChem) per the manufacturer’s guidelines and rinsed with isopropanol and ethanol. The mold was then thoroughly dried with nitrogen gas. The channel sides of the PDMS chip was generated by mixing PDMS and curing agent (Dow Corning, Silgard 184) in a 10:1 ratio and added to the mold, degassed with a vacuum, and polymerized at 65 °C overnight. Polymerized PDMS was cut from the mold, and a biopsy punch (0.75 mm——Shiento Scientific) was used on top of a self-healing cutout mat to create ports for tubing (PE-Tubing—IV penids) just below the three star patterns for each device (Fig. 2a). PDMS slabs were bonded to glass (Corning —2947, 75 × 50 mm) at a clean room by first washing the glass with acetone and isopropanol in a sonicator bath while the PDMS was washed with isopropanol, followed by thorough drying with filtered nitrogen gas. The channel sides of the PDMS slab and the glass slide were treated with plasma (400 sccm flow; 400 watts; 45 s) using a faraday barrel screen. Plasma-treated surfaces were quickly brought into contact and pressed together and then placed at 65 °C for 10 min to completely bond.

Droplet production and culturing in droplets. Before droplet production, the device’s aqueous channel was primed with Aquapel (Aquapel #47100) and then flushed with fluorinated oil (Novec 7500 oil; 3 M #0212-2292-5) (Fig. 2a). Devices were used immediately or incubated overnight at 65 °C, covered in Scotch tape, and then stored in the dark for several weeks before use. To produce droplets, a 1-ml syringe (BD, 309628) was added to growth media and then filtered (0.45 µm) after which 3 µl/ml Oxyrase (Oxyrase, Inc.) and 150 U/ml catalase (Worthington Bio Corp LS01896) was added to Novec 7500 oil (Novec 7500 oil; 3 M #0212-2292-5) (Fig. 2a). This setup could thereby enable screening for non-contact-mediated interactions between Yersinia and macrophages, which are known to affect virulence and clearance of the bacterium47.
for liquid or agarose-droplet culture, a fraction of the droplet culture was collected and quickly broken open with a final working concentration of 16% 1H, 1H, 2H, 2H-perdeuterated D2O, which contained Tris-DNase (1.75 × 10⁶ cell/ml) and aqueous phases. For liquid droplets, the upper aqueous phase was added to a dounce homogenizer to break up the agarose and release cells for live cell plating. Live cell expansion was calculated by dividing live cell counts (CFU) at every time point by the CFU count at the beginning of each experiment.

Frequency of cell-doublet encapsulation in droplets. In order to generate droplets that contained single bacteria, the cell culture was first diluted based on droplet size according to a Poisson distribution¹⁸. Frequency of cell-doublets in live cell plating. Live cell expansion was calculated by dividing live cell counts was added to a dounce homogenizer to break up the agarose and release cells for liquid or agarose-droplet culture, a fraction of the droplet culture was collected and quickly broken open with a final working concentration of 16% 1H, 1H, 2H, 2H-perdeuterated D2O, which contained Tris-DNase (1.75 × 10⁶ cell/ml) and aqueous phases. For liquid droplets, the upper aqueous phase was added to a dounce homogenizer to break up the agarose and release cells for live cell plating. Live cell expansion was calculated by dividing live cell counts (CFU) at every time point by the CFU count at the beginning of each experiment.

Visualization of cells and droplets. Images of cells and droplets were captured with an Olympus IX83 inverted microscope. For planktonic batch culture, 10 μl of cells were mixed with green fluorescent S. pneumoniae (MK189) and red fluorescent streptococcus pneumoniae strains TIGR4 or Taiwan-19F. Each transposon library preparation method for Tn-Seq⁴,⁵ were performed depending on the amount of gDNA collected. Genomic DNA (gDNA) was extracted from S. pneumoniae using the DNeasy Blood & Tissue Kit according to the manufacturer’s guidelines for Gram-positive bacteria (Qiagen, 69506). DNA adaptor barcodes were made by mixing an equal volume of primers for liquid or agarose-droplet culture, a fraction of the droplet culture was collected and quickly broken open with a final working concentration of 16% 1H, 1H, 2H, 2H-perdeuterated D2O, which contained Tris-DNase (1.75 × 10⁶ cell/ml) and aqueous phases. For liquid droplets, the upper aqueous phase was added to a dounce homogenizer to break up the agarose and release cells for live cell plating. Live cell expansion was calculated by dividing live cell counts (CFU) at every time point by the CFU count at the beginning of each experiment.

Frequency of cell-doublet encapsulation in droplets. In order to generate droplets that contained single bacteria, the cell culture was first diluted based on droplet size according to a Poisson distribution¹⁸. Frequency of cell-doublets in live cell plating. Live cell expansion was calculated by dividing live cell counts as described. Transposon library construction and selection experiments. Library construction requires a transposon, Mag PCR Clean-up Kit, MAGPCRCL50) were mixed with 30 μl of magnetic beads (Axygen 11–μm magnetic beads) and 20 μl of alkaline phosphatase (NEB - M0290S Calf). After magnetic bead wash, 10 μl of magnetic beads plus 20 μl PEG solution were used to wash the sample followed by elution in 14.3 μl of dH₂O. (4) T4 DNA ligase (NEB M0202L) was used to ligate DNA adapter barcodes by adding 13.12 μl DNA to 1 μl of 1.5 diluted adapter, 1× T4 DNA ligation buffer and 400 units T4 DNA ligase, followed by incubation at 16 °C for 16 h, 65 °C for 10 min, and held at 10 °C. (7) In all, 10 μl magnetic beads plus 20 μl PEG solution were used to wash the sample followed by elution in 36 μl of dH₂O. (8) Adapter ligated DNA was then PCR amplified using Q5 high-fidelity DNA polymerase (NEB – M4491L) by adding 34 μl of 10X reaction buffer, 10 mM dNTPs, 0.45 μl of each primer (P1-M6-Gat-M-Mel; P2-ADPT-Tnseq-primer; Supplementary Data 5), one unit Q5 DNA polymerase, and incubated at 98 °C for 30 s, and 18–22 cycles of 98 °C for 10 s, 62 °C for 30 s, 72 °C for 15 s, followed by 72 °C for 2 min, and a 10 °C hold. (9) PCR products were gel purified and sequenced on an Illumina next generation system as described previously⁴⁷. Generated mutant strains and primers for marked deletions can be found in Supplementary Information (Supplementary Data 5, 6).

Co-culture assays. To validate genetic phenotypes associated with carbon utilization from AGP or stress from neutrophil elastase, single-gene mutants (mt) were co-cultured with their wild-type parental strain (wt) in a 1:20 ratio (mt:wt). Mutant and wt cell frequencies were calculated by live cell plating on blood agar plates with or without antibiotic selection for the mutant. Overall growth of the mutant is then represented either as fitness, which reflects the growth rate and is described above, or as percent survival which is calculated relative to the untreated control.

Gene expression analysis. Immediately after culture Streplococcus pneumoniae were pelleted and snap-frozen in an ethanol/dry-ice bath, followed by RNA isolation using RNeasy Mini Kit (Qiagen, 74106) according to the manufacturer’s guidelines. RNA was treated to remove genomic DNA with TURBO DNA-free kit (Ambion, AM1990T). cDNA was made from 400 ng of RNA-free DNA using iScript Reverse Transcription Supermix (Bio-Rad, 1708841). Primers for quantitave real-time PCR (qRT-PCR) were designed using Primer3 software⁴⁷. qRT-PCR was performed with iTaq SYBR Green Supermix (Bio-Rad, 1725124) using 2 μl of cDNA in a MyQ Real-Time PCR Detection System (Bio-Rad). Each sample was measured in three technical and biological triplicates and normalized to the 505 ribosomal gene SPT_2222 (rpl).
containing established Yptb colonies were then exposed to bone marrow-derived macrophages (BMDMs) collected from C57/BL6 mice. Droplets were then fixed with 4% PFA before visualization by brightfield and fluorescence microscopy.

**Double-layered agarose droplets.** The initial agarose droplets were generated using a 40 × 40 μm device as described in the “Droplet Production” method of this paper. Before the oil was removed with 16% PFO, droplets were incubated at 4 °C for 12 min with occasional shaking. Oil-free droplets were added to 37 °C pre-warmed media containing dissolved 1% Seaplaque agarose and then well vortexed. This mixture was then encapsulated into droplets using an 80 μm wide × 40 μm high device, which were designed without filters for the aqueous channel to prevent clogging caused by the initial agarose droplets. Resulting droplets were then gelled at 4 °C for 12 min before culture and visualization by microscopy.

**Fluorescence-activated cell sorting of agarose droplets.** After cell growth, the agarose droplets were incubated at 4 °C for 20 min with occasional shaking to ensure that droplets were gelled and stable enough for fluorescence-activated cell sorting (FACS). In total, 16% PFO was used to remove oil as described above, and then agarose droplets were washed several times in 1 ml of PBS by centrifugation at 500 × g. Immediately before running the samples on the flow cytometer, agarose droplets were thoroughly vortexed to reduce the clumping of droplets which would otherwise result in clogging of the flow cytometer nozzle (85 um). Acquisition was done using BD FACSAria II (Becton Dickinson, San Jose, CA), and the data were analyzed with FlowJo software (Becton Dickinson, San Jose, CA).

**Statistical analysis.** All statistical analyses were performed using GraphPad Prism version 8.1.2 Mac OS X (GraphPad Software, San Diego, California USA, www.graphpad.com). The specific statistical analyses used can be found in the figure legends. Data was tested for normality with a Kolmogorov–Smirnov test followed by an appropriate (non)parametric test as indicated. All error bars displayed are standard error of the mean.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All data and genetic material used for this paper are available from the authors on request. All sequence data can be found under the NCBI Sequence Read Archive accession SRP154922. Source data for Figs 2, 3, 5, 6, and Supplementary Figs 2 and 3 are available in the Source Data file.

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**Author contributions**

T.v.O., D.T., and P.J. conceived and worked out the idea. D.T., S.W., and P.J. designed, produced, and characterized microfluidics devices. D.T. and S.W. performed experiments. D.T., S.W., and T.v.O. performed data analyses. C.Q. and M.S. performed elastase validation experiments and data analysis. S.C. and R.I. performed and interpreted Yepl-BMDM interaction experiments. D.T. and T.v.O. wrote the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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