Response to Reviewer 2 Comments

Please see below for our point-by-point responses (in blue).

Point 1: Line 199-203 (and throughout). The authors use the term "injection" when adding CSP in the microfluidics system. I find this a bit confusing. As far as I understand "injection" in this context means to change the inlet to a well with CSP? Can the author rephrase or clarify this?

Response 1: Reviewer 2 is right. CSP induction is achieved by switching the flow from one well containing growth medium to another one containing growth medium with CSP. We changed the word "injection" to "perfusion" throughout the text.

Point 2: Line 60-66. As the authors state, most of the information about pneumococcal competence and transformation comes from population data, and single cell studies with methods such as those presented in this manuscript would be highly valuable go get more insights into the process. However, I think the authors should also include information from single cell, real time, time-lapse data that are already available, including their own, Kurushima et al (2020) and other studies such as Berge et al. (2013), Moreno-Gamez et al. (2017), Kjos et al. (2016).

Response 2: We added this information with corresponding references in the introduction:

> **Lines 67-72:** "More recently single cell analyses using fluorescence microscopy have been used to detect competent cells [16–20], to localize transformation proteins [12,21,15], to analyze the binding of transforming DNA at the surface of competent cells and to visualize transformants [12,15]. The development of the droplet microfluidic technology to study transformation also allowed the characterization of the genetic outcome of individual cell-cell interactions."

Point 3: Related to the previous point. In the discussion, the author should compare their results from the microfluidics setup with results obtained from time-lapse imaging of competence development and transformation without a microfluidics setup (ie on agar pads), including those mentioned above)

Response 3: We modified the discussion as follows:

> **Lines 530-549:** "Earlier reports, based on the measurement of the luciferase reporter enzyme activity, have shown that the time to initiate transcription of early *com* genes following the addition of saturating amounts of CSP in bulk cultures is about 2 min [33]. Similar measurements are not possible with cells grown on an agarose pad. Indeed, the very short time window necessary to activate *com* genes would make it technically challenging to observe the first cells entering competence without being able to introduce the CSP during the microscopy observation. Here, we overcame this difficulty but we found that the time required to detect the first cells expressing GFP under the control of an early competence promoter was more than 8 min (Figures 2 and S2). The larger delay measured in our experiments can be explained by several factors including the time for the CSP to reach the microfluidic chamber and the maturation rate of the GFP fluorescent protein. In contrast, the interval between the expression of early and late *com* genes is consistent with values recorded by other methods, including RNA-seq analyses [9,10],

and the use of luciferase or beta-galactosidase reporters [7,8,33]. In addition, we found that the first transformants exhibiting an FtsZ-GFP signal appeared about 35 minutes after the onset of CSP perfusion. Considering that competence develops shortly after medium exchange with CSP, and knowing that DNA uptake is maximal 10 min after competence induction [12], the kinetics for the phenotypic expression of transformants are in line with results obtained from transformation assays [34]. Interestingly however, and in agreement with the fact that microfluidic systems are better suited to continuous imaging than agarose pads, transformants were detected in the microfluidic chamber about 15 minutes earlier than in agarose pad-based experiments [12]."

Point 4: 1 and Fig. S1. Fig. S1a (quantification of generation times) could be moved into the main manuscript as part of Fig. 1. I believe this plot provides important information that should be shown next to the micrographs.

Response 4: We agree with Rewiever 2 and added the panel (**a**) of former Figure S1 to Figure 1.

Point 5: 2A. Is the competence development uniform in cells across the entire image frame – or is there any cell-to-cell variability with regard to timing or signal intensity?

Response 5: The fluorescence intensity is broadly heterogeneous suggesting cell-to-cell variation of P_E-*gfp*/*mCherry*/*mTurquoise* expression. We cannot exclude that the CSP peptide does not diffuse homogeneously in the microfluidic chamber. However, cell-to-cell variation of the fluorescence intensity of cells expressing *gfp* under the control of a competence promoter is also observed in batch cultures (our and other's previous work: Martin *et al.* Molecular Microbiology 2010 doi/10.1111/j.1365-2958.2010.07071.x, Slager *et al.* Cell 2014 doi/10.1016/j.cell.2014.01.068, Bergé *et al.* Nature Communications 2017 doi/10.1038/s41467-017-01716-9).

Point 6: 2B. Please explain exactly how the FtsZ-signal was used as a marker for cell division. How do the authors know that the FtsZ-gfp fusion is functional, as stated in the legend? Any reference for that?

Response 6: FtsZ-GFP fusions are functional in otherwise wild-type pneumococcal cells (van Raaphorst *et al.* PNAS 2017 doi/10.1073/pnas.1620608114, Perez *et al.* PNAS 2019 doi/10.1073/pnas.1816018116). Here, we used an assay previously described to monitor the doubling time of single cells containing an FtsZ-GFP fusion (Bergé *et al.* Nature Communications 2017). We modified the text (Materials and Methods, Section 2.4, **Lines 176 to 180**) to clarify this point as follows:

"To measure doubling times, we manually tracked single cell lineages up to seven generations using phase-contrast time-lapse microscopy images. Alternatively, we used overlaid phase contrast and fluorescence images of cells harboring a functional FtsZ-GFP fusion as a marker for cell division, as previously described [21]. A minimum of 50 cell lineages were analyzed per experiment and the experiments were repeated 2 to 4 times."

Point 7: 4. Is the DprA-GFP fusion (fully) functional? Could the use of this strain influence the competence and transformation dynamics compared to wild-type?

Response 7: The DprA-GFP fusion has no effect on competence induction or on the transformation efficiency. Western blots indicate that the fusion protein is produced in competent cells at the same time as the wild-type DprA protein. We did not present these data here because they are part of another manuscript currently submitted elsewhere, which describes the DprA-GFP protein and its dynamic localization in competent cells. A preprint version of this manuscript is available in bioRxiv (Johnston *et al*. https://www.biorxiv.org/content/10.1101/2020.06.12.147637v1).

Point 8: 5 and line 341: It should be made clear that the R3708 contains the construct ftsZ-stop-gfp (stated in the legend, but should also be made clear in the text). Why did the authors not attempt to make a dual labelled strain, where both competence development and transformation could be visualized in the same strain?

Response 8: We modified the text as follows:

> **Line 433:** "This assay consists of the transformation of a recipient cell (strain R3708, *ftsZ*-stop-*gfp*), with a '*ftsZ-gfp*' donor DNA fragment carrying a mismatch of 3 bp and allowing expression of an FtsZ-GFP fusion upon integration into the chromosome (see section 2.5)."

We first thought of making a dual labelled strain containing the P_E-*mCherry* reporter to follow competence induction and the *ftsZ*-stop-*gfp* construct as suggested by the reviewer. However, we added strain R4256 (P_E-*mCherry*) in the transformation experiment shown in Figure 5 only as a positive control to verify that competence is induced in the microfluidic chamber. We thus felt that a dual labelled strain was not essential for this experiment.

Point 9: Line 108 (section about microfluidics methodology): were the cells always trapped in the same "height" of the microfluidics plates?

Response 9: Pneumococcal cells were preferentially trapped in subchamber 5 with a trap height of 0.7 µm. We modified the text (Materials and Methods, Section 2.3, **Lines 136 to 137**) to add this information as follows:

> "We found that pneumococcal cells were preferentially immobilized into the fifth trap (trap height of 0.7 µm)."

Point 10: Line 119 "…OD550 = 0.1." Approximately how many cells/µl is this. Is this within the range of cell concentrations mentioned in the instructions for this microfluidics plate?

Response 10: This is about 10^8 cells/mL, ten times more concentrated than recommended by the manufacturer (1–20 × 10^6 cells/mL). We added this information in the text (Materials and Methods, Section 2.3, **Lines 132 to 136**):

> "Exponential growing cultures (OD550 0.3) were diluted 50-fold in C+Y medium complemented or not with catalase (300 U/mL) and incubated at 37°C to an OD550 of 0.1 (~10^8 cells/mL). Cells were subsequently loaded into the microfluidic chamber according to manufacturer's protocol. Note however that the concentration of the cell suspension used for loading was about 10 times higher than the concentration recommended by the manufacturer, which is 1 to 20 x 10^6 cells/mL."

Point 11: Line 41. Consider replacing "and" with a comma: "…pathogenicity islands, antibiotic resistance …"

Response 11: We made the modification as suggested.

Point 12: 5. In the timeline ADN should be changed to DNA.

Response 12: corrected.