nature portfolio

Peer Review File

Chromosome segregation dynamics during the cell cycle of Staphylococcus aureus

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Remarks to authors:

The authors address a long-standing question of chromosome organization and dynamics in S. aureus. Here they adapt an established system for targeted fluorescent labelling of specific DNA loci, which allows the authors to track and visualize the key chromosomal loci (Ori, Ter, as well as chromosome arms) during different phases of S. aureus cell cycle. On top of that, the authors provide further supportive evidence of chromosomal organization, as well as differences in chromosomal organization in the presence and absence of key protein factors, using Hi-C, a benchmark technique that provided unprecedented insight into the chromosome organization of many bacterial species. The main novelty of this work is that S. aureus cells do not tightly time the initiation of chromosome replication to the cell division. Rather they initiate the replication before the previous cell division is finished. This results in the inheritance of multiple origins at the beginning of daughter cell cycle (P1 phase), which is contrary to the previous narrative of tight regulation between replication and division. The authors also update the full set *parS* chromosomal sites, including two newly discovered ParB-binding regions, which they experimentally confirm. ParB and SMC proteins are shown to be crucial for maintenance and efficient segregation of chromosomes to daughter cells, particularly by loading of SMC proteins to the chromosome. Commendably, the authors extend their findings to important proteins involved in chromosome integrity at the final segregation step, near the division septum Noc, SpoIIIE, FtsK, and XerC. Overall, this is an important and well-timed study for the bacterial field.

My most important comment is that results using FROS system (esp. LacO), but more importantly ParB/SMC deletions do not as accurately reflect the loci amount, a central part of this manuscript, and it should be supported using qPCR method (or a better alternative available to authors). The manuscript is well written and I provide minor points how it could be improved in the presentation of the results and discussion (see comment 8). The referencing of previous scientific work of others is largely missing, and should be included and appropriately discussed in relation to the current study.

Major comments:
1. FROS itself can affect the chromosome organization as it represents a long stretch of DNA bound by what is effectively a “roadblock” to moving DNA machinery (here, DNA replication). This is evident in Fig. S2 where switching tags from ori to ter can lead to a difference of ~40% in Ori/cell (light bins 1-2, from 1.5 to ~2.1), or ~20% in Ter/cell (dark bins 3-4, from ~1.2 to 1.0).

It is important to confirm the results of Ori/Ter ration with another technique that is not relying on fluorescence spot counting (this will be very important for Δsmc mutants, see comment #2). I advise qPCR with 2 primer pairs flanking **each** the Ori and the Ter region.

2. Investigating SMC defective mutants will result in post-replicative chromosomes whose origins are stuck together, due to the cohesive nature of ParB-clusters (due to self-self interaction and phase-separation). This convolutes the results from fluorescence imaging as multiple origins will appear as a single one. Related to comment #1, a qPCR confirmation of the ori-ter ratio in the wt, ΔparB, ΔscpAB and SMC^{stop}, is necessary to support your study and claims of the number of chromosomes, origins and termini.

Minor comments:

1. There are several very important studies missing to be referenced which should be incorporated. They are mentioned in the order of importance below:
- Critically, PMID26295962: this study shows all of the elements shown here, in B. subtilis, and is important to address and compare to results in the discussion. Most notably, ΔparB, Δsmc and the interpretation of the Hi-C maps' intra-arm hairpin (bow structure), which is also present in your Hi-C map and should be assessed for the presence of parS site.
- Line 80, then line 88 – "A second function of ParB is to load…": PMID: 36044845 – this study provides a most up-to-date model of ParB-SMC loading. Should be mentioned in the introduction.
- Line 105: - "Therefore, the existence of spherical bacteria which": PMID: 38548820 – this study shows a well-studied rod-shaped B. subtilis system converted to spherical cells, and the chromosome orientation ori and ter. Should be compared/mentioned in the introduction.
- As your ChIP-seq data and your introduction heavily mention ParB-CTP spreading model initial studies showing this pattern in ChIP seq (PMID: 17462018), in bulk with CTP (PMID: 31649139 and PMID:34562373) and in single-molecule (PMID: 34250901 and PMID:35767606) should be mentioned and referenced in the introduction.

Notably, while Wang and Pinho labs are indeed well-established and known experts on Hi-C and S. aureus biology, respectively, the work includes a total of 18 self-citations (9 each M.G.Pinho/X.Wang). As this work contains large sections focused on ParB and SMC proteins, where Thanbichler (Marburg, DE) and Gruber (Lausanne, CH) labs have several breakthrough works (esp on ParB CTPase activity and ParB-SMC loading) it is surprising that their key papers were not even mentioned. I advise reading into the literature of these two labs and improving on the introduction and discussion by including their benchmark studies that I had not mentioned above already.

2. "SMC molecules dimerize spontaneously, forming a V-shaped structure" – while many standardized cartoon representations show this, all more recent CryoEM and AFM (e.g., Nunez et al. Cell Reports (2021), Byung-Gil Lee et al, NSMB (2021); Higashi et al. eLife (2021), Bürmann et al. Mol Cell (2021)) studies have shown that SMC heads almost exclusively reside together in bacterial and eukaryotic SMCs. I suggest altering this claim to accompany new models (i.e., Shaltiel et al. Science 2022) and referencing the appropriate works.

3. "randomly loaded SMC-ScpAB molecules can compact and segregate the chromosome quite well" – there does not seem to be any direct evidence provided that SMC proteins are in fact loaded on the chromosome, and Hi-C maps do not show any long-extended loop structures. I suggest to alter this claim. This is repeated in the discussion, yet the paper does not provide experimental evidence of it.

4. "Presumably, this occurs because SMC is loaded onto the chromosome from the single parS2 locus, resulting in a specific subset of inter-arm interactions." This is addressed in Marbouty et al. Mol Cell (2015), Anchimiuk et al. eLife (2021) and Brandão et al. NSMB (2021). These works should be cited and discussed in this context.

5. Line 82: Reference 28 is not accurate in the context of this paragraph, as MukBEF is a completely different type of SMC, and does not need ParB loader, nor juxtaposes the chromosome arms.

6. The choice of Ref. 15 is a strange one. While the original studies are important to be acknowledged and mentioned, there are numerous more recent studies that in detail describe ori/ter ratios in B. subtilis which could be also cited. Further, in the initial paragraph of this referenced work it is clear that different strains of the same species (namely, W23 vs 168) show opposite conclusions when it comes to chromosome amount. This work however, shows everything on a single strain (S. aureus JE2). If readily available to the lab, authors could include another S. aureus strain to the qPCR analysis in order to show the universality of their conclusions, and if not, this limitation could be briefly mentioned in the discussion section.

7. Show n numbers in the legends of Fig. 4 and 5, as shown in Fig. 1D and 2E, as full numbers for conditions.

8. Showing bar graphs for a single number, without and underlying binning, is not the best way to show the trends here. This is apparent in Fig. 4B and 5B-C where the trends are extremely hard to track for the reader. Single points with error bars, eventually connected with a trend line, will be a much more intuitive way for the reader to track the trends that the authors are trying to show.

9. Figure S2: Showing error bars as SEM would be very beneficial to get the insight into how significant of an effect these tags really have.

10. Figure S2: Show exact n numbers on top/bottom of the chart instead of "n>1200 per condition".

11. Figure S5C: It strikes me that the curves for wt and ΔparB almost identically overlap. Just by eye the Hi-C map shows an increased contact near the origin region (as a darker square ~500kb around the ori), which is feature previously shown by Marbouty et al. as a condensed origin, and secondary hairpin occurring when a parS site is somewhat distant from the other parS sites. Is there an explanation how the Fig.S5C curve does not reflex what is clearly visible by eye? Is this due to a normalization/contrast?

12. Fig. S6: Show exact n numbers on top/bottom of the chart, or the legend

13. Marker frequency analysis requires more detailed explanation both in the main text, and I would advise in the methods too (not just the DNA prep, but also the logic behind the method), as it is not a widely adopted standard method in microbiology as Ref 98 is from 2025 (which is not referenced in the main text).

14. Figure S7: Add marker frequency analysis on JE2_FROS^{Ter}_ΔftsK so there is comparable insight on the effects on the chromosome shown in Fig. 5B.

15. Inference from marker frequency analysis should be very cautionary due to unwanted expression of prophages from the genome. While this might not directly affect the chromosome organization it likely severely affects the cell metabolism. I suggest not making strong claims based on data from Fig. S7, even if Fig. 5 supports it.

16. Full disappearance of Ter region in FtsK and SpoIIIE mutants is striking, and I suggest this to be confirmed and tested using qPCR as mentioned in major comments #1 and #2.

17. Table S2: plasmid “psav-mSc-I” construction should be either cited or described in the table/methods, rather than only names of people who constructed or supplied it.

18. Methods: Microscopy section: “Thermo Scientific” should likely be “Thermo Fisher Scientific”.

19. Methods: “lysostaphin” should be referenced the same in all four instances (Company, ascension number). In one instance it says (Sigma) in other (Sigma-Aldrich).

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
Adrian Izquierdo-Martinez et al. examine the spatio-temporal organization of the chromosome in Staphylococcus aureus, a spherical bacterium where chromosome segregation mechanisms remain incompletely understood. It is already known that S. aureus encodes ParB but lacks a ParA homolog, suggesting a non-canonical ParABS system. Additionally, prior studies have established that SMC plays a crucial role in chromosome segregation, though it is not essential. This study integrates these known aspects into a cohesive model by combining tracking of ori and ter loci using the FROS system with chromosome conformation capture assays and deep sequencing. As a result, this work is timely and represents a significant advance in the S. aureus field. While the study contributes to the broader understanding of chromosome organization in S. aureus, significant improvements are necessary before it is suitable for publication. I am particularly concerned about the lack of complementation assays, such as for the smc deletion mutant, to substantiate several claims made in this manuscript.

Major Comments:
1. There is no complementation for any deletion mutants in this manuscript. I would specifically like to see complementation for the smc- mutant, as its phenotype is difficult to interpret. The smc- (or scpAB-) strains produced approximately 17% anucleate cells, whereas parB- produced only 0.14% anucleate cells. Since ParB is suggested in this manuscript to be the loader of SMC, and the Hi-C maps of smc- and parB- are essentially identical, the manuscript proposes that in the parB- strain, SMC is still being loaded somewhere on the chromosome and hypothesizes that this is why the smc- strain produces a much higher fraction of anucleate cells. However, there is no experimental evidence in this manuscript that SMC is loaded elsewhere. The simplest explanation is that SMC in S. aureus has functions unrelated to chromosome organization (yet this possibility is not discussed). Another explanation is a polar effect caused by the smc deletion, necessitating a complementation assay to rule this out. Using a strain where an early stop codon is introduced into the smc coding sequence is not a foolproof way to rule out a potential polar effect.
2. Related to the above concern, the authors state, “Without ParB/parS, randomly loaded SMC-ScpAB molecules can compact and segregate the chromosome quite well, although the overall organization of the chromosome is altered.” This statement is not experimentally substantiated, and the two halves of the sentence contradict each other.
3. The manuscript lacks relevant literature citations and requires substantial improvement. While comprehensive reviews exist on S. aureus chromosome organization, key studies on ParB have been overlooked. The authors reference only one review from 2020, despite numerous studies (and reviews) contributing to the understanding of ParB as a CTPase since then. Two foundational papers identifying ParB as an enzyme are not cited. The discussion on SMC and Hi-C lacks proper citations, particularly for critical works such as Bock et al. and Marbouty (2015), which are essential for contextualizing the findings. Additionally, several relevant papers from the S. aureus field were omitted.
4. The use of the FROS system in this study raises some concerns due to its potential to introduce artifacts. Binding proteins to inserted sequences (48 copies) can create a roadblock for DNA-associated machinery, including DNA replication, which is a key marker analyzed in this study. Previous work has well-documented this issue, and the authors should acknowledge this limitation. A significant problem is evident in Figure S2, where changing the ori-ter labeling system results in strikingly different data—showing almost a 40% difference in ori per cell and a 20% difference in ter per cell. These discrepancies suggest that the labeling method influences the observed localization patterns, raising concerns about the reliability of the conclusions drawn from these data. Typically, ori labeling using the FROS system is confirmed by colocalizing it with a native marker. The authors claim that native ParB expression is weak but do not provide supporting data for this assertion beyond citing a single study. Even in that study, the native ParB focus appears usable. Additionally, Chan et al. (2020) demonstrated well-resolved images of ParB localization from a low-copy number plasmid, showing that ParB is not restricted to the cell's extreme periphery. This discrepancy should be acknowledged and discussed in the manuscript. To strengthen the reliability of their conclusions, I strongly recommend that the authors validate their ori-ter labeling using an alternative approach, such as: (i) generating a colocalization strain with the native ParB to confirm ori positioning at the cell

tip, (ii) using a different labeling system (e.g., parSPMT1-parSP1) to compare with the FROS system, or (iii) quantifying the relative abundance and validating spatial organization using a non-fluorescent approach, such as qPCR with primer pairs flanking the ori and ter regions.
5. A schematic representation showing the exact genomic locations of the FROS sequences relative to ori and ter would be very helpful. How far away from the actual origin of replication was the array inserted? Is this array next to a membrane protein-encoding gene? If so, is there a possibility that a translation-membrane insertion effect causes the ori FROS to be close to the membrane (but the ORI in general might not be)? Can the authors move the ori FROS labeling to another ori-located site to rule out this possibility?
6. Line 128 and beyond: If this is the first study stating that the ori is positioned at the tip of the hemisphere, then it should be noted that previous studies (e.g., Chan et al.) did not show tip-positioned ParB foci. How do the authors explain this discrepancy? How can they rule out the possibility that this is due to the FROS labeling system? Is it strain-specific?

Minor comments
1. Line 19: Reference for FROS?
2. Line 19: The authors state that "cell cycle and chromosome replication are not coupled." Since DNA replication is part of the cell cycle, clarification is needed regarding what exactly they mean.
3. Line 26: What is the distinction between "chromosome conformation" and "chromosome compaction" as referred to by the authors? The study monitors chromosome configuration using Hi-C but does not directly assess compaction. Without data on nucleoid-associated proteins (NAPs) or DNA content quantification, claims regarding compaction should be moderated.
4. Need to report the number of replicates for ChIP-seq and Hi-C experiments
5. The P1-2-3 stages are well established in the Staphylococcus literature but may not be familiar to all readers. It would be helpful to include some schematics alongside Figure 2D so that readers can better understand how the authors come to these conclusions from Figure 2B.
6. Line 46: The term "linear" chromosome arrangement is not widely accepted. It would be more appropriate to use "longitudinal," which has been established in the literature.
7. Line 47: Reference needed for "subpolar" chromosome positioning in M. xanthus.
8. I recommend discuss Pseudomonas chromosome organization as well, major knowledge in this area is gained through Hi-C work in this bacterium.
9. Line 48: References needed for ter positioning.
10. Line 53: The authors clearly distinguish between chromosome behaviour in slow- and fast-growing conditions in Bacillus, yet all their conclusions for S. aureus are based on a single growth condition.
11. Line 61: The E. coli chromosome is widely recognised as having a "transverse" orientation.
12. Lines 72–99: Incomplete referencing, particularly in the ParB field. No citations for ParB as a CTPase are included, and only one review from 2020 is cited, which is insufficient.
13. The authors fail to introduce or discuss Streptococcus pneumoniae, which is highly relevant given its similarities to S. aureus regarding the ParABS and SMC systems.
14. Line 125: Tracking the origin of replication with ParB is a well-established approach. The claim that this approach is flawed is not well-supported; it is one of the best native markers available for ori tracking.
15. Line 136: Relevant studies are missing citations.
16. Line 138: The claim that most cells have two to four origins contradicts Chan et al., where most cells have two origins (<62%), and four-origin cells are a minority.
17. The authors did not cite two extremely relevant papers for this study: Barbuti et al. and Gallay et al.
18. Line 153: Figure S2 is referenced vaguely; please specify which panel.
19. Figure S2A: The LacI-eCFP and TetR-eYFP ori markers largely overlap, but some cells clearly show a centrally positioned TetR-eYFP ori focus. These foci appear intense and possibly saturated.
20. Figure S2A (Ter labelling): Different labelling methods (TetR vs LacI) yield inconsistent results, with LacI-ter labelling often resembling ori. This suggests potential labelling artefacts (See Major Comment #2).
21. Figure S2B: No replicates?
22. Line 158: The authors switch to TetR-mNG, saying the expression conditions were better, presenting only a schematic in Figure 1A. Microscopy images should be provided alongside the schematic and heat maps or demographs for validation.
23. Lines 163–167: DnaN labelling is discussed without referencing a figure, only returning to Figure 1B later. The figure order should be improved for clarity. Additionally, the figure legend states that the bar charts in 1B represent the frequency of cells in a FROS-ori-DnaN-Halo strain, but this is unclear in the main text.
24. Showing bar graphs is not the best way to show the trend. This is visible throughout the paper, especially in Figures 4B and 5B-C. I suggest the authors use single points with stdv bars.
25. Line 169: The authors state that they “correlated that information with the cell cycle stage.” Can they explain precisely how this was done?
26. Figure S3 is enormous, and the text generally references it without explaining what the reader should focus on.
27. Figure 1B and Line 177: The percentages reported here differ from those in previous studies, such as Chan et al. Could the authors explain these discrepancies?
28. Line 183: The authors claim that colocalisation happens only in the P1 stage and cite Figure 1C. While Figure 1C provides the quantification, it would be preferable to see the actual colocalisation image or reference Figure 1D. If Figure 1D shows that, it should be presented before 1C. Additionally, the sample size for Figure 1D (n = 36) is quite small.
29. Figure 1D: The authors claim that ori and DnaN colocalise, which is unclear in Figure 1D; instead, it is a supplementary figure. 1D: Only the first image suggests possible colocalisation in the upper hemisphere. Furthermore, the colour labelling (green/red) should be explained directly within the images instead of relying solely on the figure legend.
30. Lines 199–200: The claim that "the whole chromosome is partially replicated" requires supporting data. A Ter-DnaN strain would be necessary to confirm this statement. Otherwise, I suggest rephrasing.
31. Lines 204-206: differences from the summary in the Barbuti review, which is not referenced. Can you clarify the

reasoning behind this? Is it condition-related or strain-specific?
32. Line 208: "Linear" should be replaced with "longitudinal."
33. Figure 2B: Without at least one or two ori-left or ter-right colocalisation experiments, the chromosome arrangements proposed by the authors lack strong support.
34. In Figure 2B, the heat map suggests a central positioning of the ter locus, but in the corresponding image above, the ter appears more peripheral. Could the authors clarify this discrepancy?
35. I strongly encourage the authors to generate a ter-DnaN strain and include time-lapse imaging to correlate these findings with ori-DnaN, providing a comprehensive overview of whole-chromosome dynamics.
36. Line 244: Again, the authors cite only one review for ParB in the entire paper, which is not ok given the extensive literature available.
37. Line 246: It would be beneficial for the authors to discuss in either this section or the discussion that Streptococcus pneumoniae also possesses ParB but not ParA. Many aspects of chromosome organisation in S. pneumoniae are strikingly similar to S. aureus, yet this is not mentioned anywhere.
38. Lines 252–254: The discussion of ParB spreading, bridging, and nucleation is presented without any supporting references, which must be corrected.
39. Line 263: "linear" should again be replaced with "longitudinal."
40. Line 264: Despite numerous studies describing this phenomenon, only one reference is provided.
41. Figure 3: The figure lacks panels and contains numerous graphs, making it difficult to follow in the main text.
42. Line 269: The impact of leaving only one parS sequence has been thoroughly investigated and explained in B. subtilis. However, no relevant references are provided here (Gruber and Marbouty should be cited).
43. Figure S5C and Figure 3: There is a clear difference between WT and ΔparB, yet the red and yellow curves in S5C appear identical. This analysis should be repeated to fix this issue.
44. Line 293: It would be helpful to see DAPI images next to the bar charts showing the percentage of anucleate cells in these mutants.
45. Line 306-307: But smc is not essential. The authors could discuss in greater detail, later on, the impact of other systems, such as NAPs or crowding effects, which are never mentioned.
46. Line 311: reference?
47. The SMC and ParB story in this study is quite similar to what has been described in Streptococcus pneumoniae, yet this work is not cited. In particular, Gallay et al. (2021), which discusses the CcrZ protein and even examines Staphylococcus, is highly relevant but not mentioned, even in the discussion.
48. Line 325: There are likely more relevant citations that could be added here to provide better context.
49. Figure 5: The claim that FtsK, SpoIII, and ori-ter labelling do not significantly impact chromosome spatial organisation is inconsistent with Figure 5A, where ori-ter labelling is visibly altered in ΔspoIII. This claim needs revision.
50. While the unwanted prophage expression might not directly affect the chromosome organisation, as it has not been tested, the authors cannot know if it affects something else in the cell. Please tone down the strong claims based on Figure S7.
51. Figure S7: The methodology behind this analysis is poorly explained in the figure legend and is entirely missing from the Materials and Methods section. Please clarify and include the relevant details.
52. Discussion, Line 370: Converting a rod-shaped cell into a spherical one and analysing ori-ter-left-right organisation has been previously studied in E. coli and Bacillus. However, the Bacillus study (Tišma et al., Nature Comm) is neither cited nor compared to these data. It would be valuable to draw a parallel between chromosomal organisation in spherical or forced-spherical Gram (+) bacteria.
53. Line 374: What do the authors expect would happen under slow-growth conditions?
54. Line 377: The analysis suggests ori and ter are opposite, but this is true only in averaged heat maps, not necessarily in individual cells. Could the authors explain why there isn't a colocalisation of ori-ter in the same strain?
55. Figure 6B mentions a DNA-free region, yet the authors never labelled DNA with DAPI or a similar stain when discussing chromosome positioning. This is particularly relevant given their claim that ori regions are located at the cell tips: are they also at the tip of the nucleoid? Some clarification or DAPI data on this point would be helpful.
56. Line 401: This would be an ideal place to cite Barbuti and Gallay, as they previously discussed the absence of visible multifork replication in Staphylococcus.
57. Line 451: Where exactly did the authors look for chromosome compaction? There is no figure quantifying chromosome condensation.
58. Line 452: The phrase "degradation of Ter" is unclear: what exactly do the authors mean?
59. Line 462: The statement that this is "the first comprehensive characterisation of chromosome positioning and dynamics" is an overstatement. The study includes only one live-cell imaging movie analysing ori-DnaN colocalisation, which is insufficient to characterise chromosome dynamics fully. Additionally, much of what is described about S. aureus chromosome organisation was already known. This claim should be toned down.
60. One of the strong claims throughout the paper is that ori is positioned at the periphery. Quantifying the distance between the membrane and the ori marker in WT and ΔparB–ΔSMC mutants would be valuable. If these proteins play a role in anchoring ori, a measurable shift in distance should be observed. In previous studies using the low-copy number plasmid ParB marker, ori was not positioned at the extreme tips as claimed here. It would strengthen the study if the authors recapitulated their findings using the native ParB marker and performed colocalisation with ori labelling, as mentioned earlier.

(Remarks on code availability)
I did not review the codes

Reviewer #3

(Remarks to the Author)
In this manuscript Izquierdo-Martinez et al. describe their extensive studies on chromosome segregation in the opportunistic pathogen Staphylococcus aureus. Chromosome segregation is a fundamental process in all bacteria, however, S. aureus challenges conventional models (usually rod-shaped bacteria) by operating in a spherical bacterium. This implies a highly constrained three-dimensional space without a clear polarity axis. Furthermore, the S. aureus genome lacks some canonical segregation proteins such as ParA. The authors employed a Fluorescent Repressor-Operator System (FROS) to precisely localize specific chromosomal loci within S. aureus cells. Using FROS, they observed the spatial and temporal organization of chromosomes during the S. aureus cell cycle. Based on the obtained data, the authors propose that the cell cycle and chromosome replication are not coupled and over-initiation of replication occurs at least under the tested growth conditions. The authors go on and show the binding of ParB to 5 ori-proximal parS sites and spreading of ParB away from these sides. The structural maintenance of chromosome (SMC) complex is likely loaded at these sites. Interestingly, it seems that the SMC complex plays a more critical role in chromosome segregation compared to ParB. Mutants lacking a functional SMC exhibited a significant increase in anucleate cells (~17%), whereas deletion of parB alone had only minimal impact on chromosome segregation. This suggests that in S. aureus, the SMC complex is essential for efficient chromosome segregation, and its recruitment by ParB to specific chromosomal sites enhances this process. Finally, they tested the effect of Noc, FtsK, SpoIIIE and XerC on the chromosome segregation process.
Overall this is a well written manuscript with plenty of interesting data. The experimental data are throughout excellent and the data are discussed based on the context of existing knowledge. As always with such comprehensive data sets, there are at places additional controls desirable.

Line 183: How where the cell cycle stages defined and reliably determined?

Line 372: What do the authors mean by "pseudo-diploid" - a merodiploidy due to partial replication? Maybe there is true diploidy? The authors checked chromosome loci in one medium (if I get this correct). The presented data show a vast majority of cells with 2 or more oriC foci. Would that also be the case under very slow growth conditions or in stationary phase?

Adding to the point before: true diploidy could be shown by the presence of at least two terC regions. Use of diffraction limited fluorescence microscopy is not suited to discriminate between one, two or even more FROS foci in close proximity. Therefore, a detailed marker frequency analysis of ori/ter ratios under varying growth conditions/replication states can give insights into this question.

It would have been nice to add time-lapse videos in which the dynamics of the origin and terminus regions could be followed. Ideally, these dynamics could be analyzed using kymographs.

Fig. 3. The HiC maps were performed with cell cultures that are not synchronized, which is likely impossible to do with S. aureus. However, the chromosome content could be tested to provide insights into the replication state of the cells (flow cytometry). It is interesting to note that the HiC maps do not show the segregation signal of the oriC region (which was clearly visible in those bacteria that employ a classical ParAB system to drive oriC segregation across the nucleoid). This could be discussed at appropriate places in the manuscript.

Lines 284-287: The authors state that the (diffuse) long range contacts seen in a parB deletion are absent in the deletion of the scpAB subunits of the SMC complex (Fig. 3). Therefore, they conclude that SMC would be involved in establishing long range contacts even without being loaded to the chromosome by ParB first. While this is clearly interesting, it still raises the question how SMC would be loaded onto the DNA in this case (absence of ParB). While there is a loading of MukBEF in E. coli that does not require a ParB, it has not been demonstrated for a classical SMC complex in Gram positive bacteria. Technically, this result could also stem from differences in the DNA amount (replication differences) and/or segregation differences.

ChiP data for SMC in absence of ParB would be ideal to see if there is still binding to specific places. Also a functional mutant (SMC(EQ) variant) that cannot migrate along the DNA could be used as an appropriate control.

Also to this point: I am not sure if I get the idea behind the cartoons in Fig. 3 underneath the HiC maps. The CIDs seen in the HiC maps would normally attribute to enhanced interaction in a given chromosomal region within one replichore arm. Therefore, loss of the second diagonal would open up the replichore. Differences in the CIDs would argue for more or less interaction within neighboring chromosomal regions. Are the CID boundaries coinciding with highly transcribed genes, operon structures or any other noticeable feature on the DNA?

Line 333: An ftsK deletion is described to give rise to an increased number of terC foci. Could it be that the termini are held together by an unknown mechanism that is partially relived in absence of FtsK? Again, the number of termini in a fluorescent spot is difficult to quantify.

Lines 342 onwards: Deletion of xerC is said to lead to degradation of the terminus region. Whole genome sequencing does indeed indicate prophage induction. However, terminus degradation could be tested by measuring DNA breaks in the otherwise circular chromosome (e.g. TUNEL-Assay, long range qPCR, fluorescent reporters etc.).

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have fully addressed my concerns and have significantly improved on manuscript readability, presentation and scientific rigor. Added control experiments, and analysis make this a compelling paper for the field of chromosome organization!

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
I am happy with this revision. No further concern.,

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors submitted now a thoroughly revised version of their manuscript entitled “Chromosome segregation dynamics during the cell cycle f Staphylococcus aureus”.
The revised version contains several new experiments, providing valuable data in support of their claims Particularly important are:
The revised version embraces the existing literature much better and allows a more comprehensive comparison of the findings.
Importantly, the authors provide a comprehensive marker frequency analysis to rule out that the FROS system interferes strongly with replication (new data in Fig. S3). Further control experiments show that FROS did not interfere with oriC positioning (new Fig. S7).
The authors also provide new data for oriC numbers at different growth rates (25°C and 37°C). As expected the replication rate was reduced at 25°C but subcellular organization of the chromosome remained the same as under fast growth conditions.
Complementation strains for smc and scpAB deletions are now shown and they readily complement the mutant phenotype (Fig. S11).
The concerns raised by myself and other referees were addressed comprehensively and the new data were integrated well into the manuscript. In my opinion, the revised form does not need any major revision. A few small point could be considered, before publication. I would like to congratulate the authors to their nice study on S. aureus chromosome dynamics.

Line 23: Maybe a clearer description of the relation of oriC towards the division plane would be good. How about: The two oriC foci are perpendicular to the plane of cell division?

(Remarks on code availability)

We are really thankful for the careful review of our manuscript and for the helpful suggestions to improve it.

We would like to explain that towards the end of the preparation of the manuscript we decided to shorten the introduction and discussion to make it easier to read and because we were above the word limit and the limit of citations of Nature Communications. However, we do agree with the reviewers' opinion that this led important references being missed, which were now reintroduced. We also thank the reviewers for pointing out additional references that we had missed, most of which have also been added to the manuscript.

We would also like to point out that, during the revision process we noticed that the JE2_FROS^{Ori}_Δ*ftsK* strain did not show the characteristic phenotype for FtsK mutants (delayed septum splitting). We therefore constructed the mutant again and replaced the data, which now shows a mild phenotype (Fig. 5A).

REVIEWER COMMENTS (black) and ANSWERS (blue)

Reviewer #1 (Remarks to the Author):

Remarks to authors:

The authors address a long-standing question of chromosome organization and dynamics in S. aureus. Here they adapt an established system for targeted fluorescent labelling of specific DNA loci, which allows the authors to track and visualize the key chromosomal loci (Ori, Ter, as well as chromosome arms) during different phases of S. aureus cell cycle. On top of that, the authors provide further supportive evidence of chromosomal organization, as well as differences in chromosomal organization in the presence and absence of key protein factors, using Hi-C, a benchmark technique that provided unprecedented insight into the chromosome organization of many bacterial species. The main novelty of this work is that S. aureus cells do not tightly time the initiation of chromosome replication to the cell division. Rather they initiate the replication before the previous cell division is finished. This results in the inheritance of multiple origins at the beginning of daughter cell cycle (P1 phase), which is contrary to the previous narrative of tight regulation between replication and division. The authors also update the full set *parS* chromosomal sites, including two newly discovered ParB-binding regions, which they experimentally confirm. ParB and SMC proteins are shown to be crucial for maintenance and efficient segregation of chromosomes to daughter cells, particularly by loading of SMC proteins to the chromosome. Commendably, the authors extend their findings to important proteins involved in chromosome integrity at the final segregation step, near the division septum Noc, SpoIIIE, FtsK, and XerC. Overall, this is an important and well-timed study for the bacterial field.

My most important comment is that results using FROS system (esp. LacO), but more importantly ParB/SMC deletions do not as accurately reflect the loci amount, a central part of this manuscript, and it should be supported using qPCR method (or a better alternative available to authors). The manuscript is well written and I provide minor

points how it could be improved in the presentation of the results and discussion (see comment 8). The referencing of previous scientific work of others is largely missing, and should be included and appropriately discussed in relation to the current study.

Major comments:
R1.1. FROS itself can affect the chromosome organization as it represents a long stretch of DNA bound by what is effectively a "roadblock" to moving DNA machinery (here, DNA replication). This is evident in Fig. S2 where switching tags from ori to ter can lead to a difference of ~40% in Ori/cell (light bins 1-2, from 1.5 to ~2.1), or ~20% in Ter/cell (dark bins 3-4, from ~1.2 to 1.0).

It is important to confirm the results of Ori/Ter ration with another technique that is not relying on fluorescence spot counting (this will be very important for Δsmc mutants, see comment #2). I advise qPCR with 2 primer pairs flanking **each** the Ori and the Ter region.

The FROS system has indeed been reported to cause replication roadblocks in *E. coli*, depending on the number of operators in the array and this limitation is now addressed in lines 175-182. To determine if this was also the case when using the FROS system in *S. aureus,* we performed marker frequency analysis (MFA) shown in Fig. S3.
MFA determines the copy number of every region of the chromosome, showing the replication profile of entire chromosome. This could be considered the equivalent of performing qPCR at every genomic locus, with the advantage that it is independent of variation caused by the use of different pairs of primers. Therefore, we think that MFA is more reliable for our questions than performing qPCR only for Ori and Ter, and have used this technique to address all reviewers' concerns related to possible roadblocks or that imply quantifying Ori:Ter ratios. We have expanded the MFA analysis to other mutants, including Δsmc (Fig. S11B).

Regarding possible roadblocks, FigS3 shows that JE2, JE2_FROS^{Ori} and JE2_FROS^{Ter} have very similar MFA profiles, indicating that the replication profile is not altered by the FROS system in *S. aureus*. Importantly, we do not see an abrupt drop in the number of reads downstream of the loci where arrays were introduced (notice that the drop seen at ~128Kb corresponds to the deletion of the spa gene). So, although we cannot rule out a minor effect from the introduction of the arrays, we can confirm that the overall replication profile of the labelled strains is not altered by the use of the FROS system.

R1.2. Investigating SMC defective mutants will result in post-replicative chromosomes whose origins are stuck together, due to the cohesive nature of ParB-clusters (due to self-self interaction and phase-separation). This convolutes the results from fluorescence imaging as multiple origins will appear as a single one. Related to comment #1, a qPCR confirmation of the ori-ter ratio in the wt, ΔparB, ΔscpAB and SMC^{stop}, is necessary to support your study and claims of the number of chromosomes, origins and termini.

We have now performed MFA analysis, which allows for Ori:Ter ratio quantification, on strains with the ΔparB, ΔscpAB and SMC^{stop} mutations (Fig. S11B). The results indicates

that the ΔparB mutant shows no difference when compared to the JE2 parental strain, while the ΔscpAB and SMC^{stop} mutants show a small reduction in their Ori Ter ratio (approx. 2 vs. 2.49 in JE2), suggesting that these mutants indeed are slightly under-replicating their DNA in comparison to the parental strain. However, the reduction in the number of cells with 3 or 4 Ori foci cannot be explained based solely on this under-replication, as the JE2 strain grown at 25°C also has an Ori/Ter ratio of 2 but presents a higher number of cells with 3 or 4 Ori foci than the SMC mutants (47% vs. 20%). Therefore, we conclude that the SMC complex plays an important role in the segregation of the Ori regions. These results are presented and discussed in lines 392-398.

Minor comments:

R1.3. There are several very important studies missing to be referenced which should be incorporated. They are mentioned in the order of importance below:

A- Critically, PMID26295962: this study shows all of the elements shown here, in B. subtilis, and is important to address and compare to results in the discussion. Most notably, ΔparB, Δsmc and the interpretation of the Hi-C maps' intra-arm hairpin (bow structure), which is also present in your Hi-C map and should be assessed for the presence of parS site.

B- Line 80, then line 88 – "A second function of ParB is to load…": PMID: 36044845 – this study provides a most up-to-date model of ParB-SMC loading. Should be mentioned in the introduction.

C- Line 105: - "Therefore, the existence of spherical bacteria which": PMID: 38548820 – this study shows a well-studied rod-shaped B. subtilis system converted to spherical cells, and the chromosome orientation ori and ter. Should be compared/mentioned in the introduction.

D- As your ChIP-seq data and your introduction heavily mention ParB-CTP spreading model initial studies showing this pattern in ChIP seq (PMID: 17462018), in bulk with CTP (PMID: 31649139 and PMID:34562373) and in single-molecule (PMID: 34250901 and PMID:35767606) should be mentioned and referenced in the introduction.

Notably, while Wang and Pinho labs are indeed well-established and known experts on Hi-C and S. aureus biology, respectively, the work includes a total of 18 self-citations (9 each M.G.Pinho/X.Wang). As this work contains large sections focused on ParB and SMC proteins, where Thanbichler (Marburg, DE) and Gruber (Lausanne, CH) labs have several breakthrough works (esp on ParB CTPase activity and ParB-SMC loading) it is surprising that their key papers were not even mentioned. I advise reading into the literature of these two labs and improving on the introduction and discussion by including their benchmark studies that I had not mentioned above already.

As stated above, at the end of the preparation of the manuscript we shortened the introduction and discussion which resulted in the removal of many references which we agree should not have been removed. All the suggested references, and other related

studies, have been added to the text at appropriate sections, with the exception of the paper describing rod-shaped *B. subtilis* system converted to spherical cells, which we did not include as we think may not reflect naturally spherical bacteria. Although we agree that it is a very interesting paper, the described spherical *B. subtilis* cells presented a block in DNA replication and have undergone size expansion of 3 times in volume, which is not representative of the constrains of the chromosome in living spherical bacteria.

R1.4. "SMC molecules dimerize spontaneously, forming a V-shaped structure" – while many standardized cartoon representations show this, all more recent CryoEM and AFM (e.g., Nunez et al. Cell Reports (2021), Byung-Gil Lee et al, NSMB (2021); Higashi et al. eLife (2021), Bürmann et al. Mol Cell (2021)) studies have shown that SMC heads almost exclusively reside together in bacterial and eukaryotic SMCs. I suggest altering this claim to accompany new models (i.e., Shaltiel et al. Science 2022) and referencing the appropriate works.

We thank the reviewer for this correction. We have removed the "V-shaped" description and introduced the recommended citations regarding the structure of SMC/MukBEF complexes (now lines 87-89).

R1.5. "randomly loaded SMC-ScpAB molecules can compact and segregate the chromosome quite well" – there does not seem to be any direct evidence provided that SMC proteins are in fact loaded on the chromosome, and Hi-C maps do not show any long-extended loop structures. I suggest to alter this claim. This is repeated in the discussion, yet the paper does not provide experimental evidence of it.

The experimental evidence for this comes from 1) the major difference in Hi-C maps between the WT, Δ*parB* and Δ*scpAB*, the latter one showing a loss of long-range interactions, and 2) from the contact probability curves (Fig. S10C) where the loss of long-range contacts is seen in the Δ*scpAB* mutant, but not in the Δ*parB* mutant. We have now clarified this in the results section (lines 404-414). Additionally, we have added a new figure panel (Figure S10D) showing that in the absence of ParB there is an increase of long-range contacts in areas outside the second diagonal, supporting that SMC is still able to load onto the chromosome. We have discussed these ideas in the revised manuscript, also citing the work F. P. Bock *et al*. 2022, where it is suggested that in *B. subtilis* SMC complexes can load onto the chromosome in the absence of ParB, although at low efficiency (lines 366-368).

R1.6. "Presumably, this occurs because SMC is loaded onto the chromosome from the single parS2 locus, resulting in a specific subset of inter-arm interactions." This is addressed in Marbouty et al. Mol Cell (2015), Anchimiuk et al. eLife (2021) and Brandão et al. NSMB (2021). These works should be cited and discussed in this context.

The citations have been included in line 345-347

R1.7. Line 82: Reference 28 is not accurate in the context of this paragraph, as MukBEF

is a completely different type of SMC, and does not need ParB loader, nor juxtaposes the chromosome arms.

We agree and that reference has been removed.

R1.8. The choice of Ref. 15 is a strange one. While the original studies are important to be acknowledged and mentioned, there are numerous more recent studies that in detail describe ori/ter ratios in B. subtilis which could be also cited. Further, in the initial paragraph of this referenced work it is clear that different strains of the same species (namely, W23 vs 168) show opposite conclusions when it comes to chromosome amount. This work however, shows everything on a single strain (S. aureus JE2). If readily available to the lab, authors could include another S. aureus strain to the qPCR analysis in order to show the universality of their conclusions, and if not, this limitation could be briefly mentioned in the discussion section.

We have added two additional references besides ref 15.
The FROS constructs used in this work are quite laborious to make, so they were made only in strain JE2. Our previous observations using ParB as proxy for Ori localization in an MSSA strain NCTC8325-4 suggest that there are no major differences between the two strains, although this analysis in mostly qualitative (and therefore not included in the manuscript). Given that we mention strain JE2 only throughout the entire result section, we think it is clear for the reader that the work was done using a single strain.

R1.9. Show n numbers in the legends of Fig. 4 and 5, as shown in Fig. 1D and 2E, as full numbers for conditions.

n numbers have been added to all figures.

R1.10. Showing bar graphs for a single number, without and underlying binning, is not the best way to show the trends here. This is apparent in Fig. 4B and 5B-C where the trends are extremely hard to track for the reader. Single points with error bars, eventually connected with a trend line, will be a much more intuitive way for the reader to track the trends that the authors are trying to show.

We tested the representation suggested by the reviewer, but the trends remained hard to discern, as lines would cross each other in many spots. So, instead, we decided to change the organization of the bars of the bar charts in figures 4 and 5. Now the bars are grouped per strain, showing the distribution of cells in relation to the number of foci for each strain. We think that results in graphs much clearer and easier to compare than the ones we had included in the original submission.

R1.11. Figure S2: Showing error bars as SEM would be very beneficial to get the insight into how significant of an effect these tags really have.

Initially, the purpose of Fig S2 was only to showcase that our FROS system can accommodate dual loci labelling. However, following the suggestions of the reviewers

we have added replicates to figure S2, allowing us to add error bars, and we discuss in the text (lines 165-168) the potential sources of variability of these data.

R1.12. Figure S2: Show exact n numbers on top/bottom of the chart instead of “n>1200 per condition”.

n numbers have been added to all figures.

R1.13. Figure S5C: It strikes me that the curves for wt and ΔparB almost identically overlap. Just by eye the Hi-C map shows an increased contact near the origin region (as a darker square ~500kb around the ori), which is feature previously shown by Marbouty et al. as a condensed origin, and secondary hairpin occurring when a parS site is somewhat distant from the other parS sites. Is there an explanation how the Fig.S5C curve does not reflex what is clearly visible by eye? Is this due to a normalization/contrast?

We agree that visually, wt and ΔparB Hi-C maps look y different (Figure 3), but the Pc(s) curves are very similar (i.e. nearly overlapping). These curves calculate the averaged contact frequency as a function of distance. These curves indicate that the two strains have the same level of chromosome contacts on average, but in the WT these contacts are specific (i.e-, approx. the same in every cell), while in the ΔparB mutant the contacts are not specific (i.e. vary from cell to cell). Therefore, the Hi-C maps of the two strains appear different while the Pc curves are more similar, given that non-specific contacts are not seen in the HiC maps, but are taken in account in Pc curves.

R1.14. Fig. S6: Show exact n numbers on top/bottom of the chart, or the legend

n numbers have been added to all figures.

R1.15. Marker frequency analysis requires more detailed explanation both in the main text, and I would advise in the methods too (not just the DNA prep, but also the logic behind the method), as it is not a widely adopted standard method in microbiology as Ref 98 is from 2025 (which is not referenced in the main text).

We have extended our explanation in the methods (lines 758-760) and results (lines 175-182) section and added a new figure (Fig. S3). We have also included a reference of a recent publication that also uses this technique to assess replication impairments in another bacterium (MV. Mazzuoli et al., 2025), as well as a previous work that uses it in *S. aureus* to investigate changes in DNA replication rate (Pang T. et al., 2017).

R1.16. Figure S7: Add marker frequency analysis on JE2_FROS^{Ter}_ΔftsK so there is comparable insight on the effects on the chromosome shown in Fig. 5B.

We have added the marker frequency analysis of the JE2_FROS^{Ter}_ΔftsK (Fig. S12, lines 433-435). In brief, the MFA shows no significant differences between the

JE2_FROS^{Ter}_ΔftsK strain and its parental strain JE2_FROS^{Ter}, indicating that it does not have a defect in chromosome replication (nor it is over-replicating).

R1.17. Inference from marker frequency analysis should be very cautionary due to unwanted expression of prophages from the genome. While this might not directly affect the chromosome organization it likely severely affects the cell metabolism. I suggest not making strong claims based on data from Fig. S7, even if Fig. 5 supports it.

We have modified the text in lines 446-448 and 586-588 to reduce the strength of the claim. For example, in the discussion (lines 633-636) we now state: "*Furthermore, our data suggest that the deletion of xerC could give rise to alterations (or even in DNA degradation) in the Ter region…*"

R1.18. Full disappearance of Ter region in FtsK and SpoIIIE mutants is striking, and I suggest this to be confirmed and tested using qPCR as mentioned in major comments #1 and #2.

We think there was a typo and the reviewer is referring to XerC and SpoIIIE mutants, which are the ones for which a disappearance of the terminus was observed in a fraction of the cells. MFA was done for these strains, exactly because of this unexpected phenotype (Fig S12B and lines 445-456).
Nevertheless, to answer a previous comment from the reviewer we have now performed MFA for JE2_FROS^{Ter}_ΔftsK and show that data in figure S12A and discussed the results in lines 433-435.

R1.19. Table S2: plasmid "psav-mSc-I" construction should be either cited or described in the table/methods, rather than only names of people who constructed or supplied it.

We have added a sentence in the table explaining that the plasmid contains the gene encoding mScarlet fluorescent protein and that we used the plasmid merely as a template to amplify this sequence.

R1.20. Methods: Microscopy section: "Thermo Scientific" should likely be "Thermo Fisher Scientific".

This has been corrected

R1.21. Methods: "lysostaphin" should be referenced the same in all four instances (Company, ascension number). In one instance it says (Sigma) in other (Sigma-Aldrich).

This has been corrected

Reviewer #2 (Remarks to the Author):

Adrian Izquierdo-Martinez et al. examine the spatio-temporal organization of the

chromosome in Staphylococcus aureus, a spherical bacterium where chromosome segregation mechanisms remain incompletely understood. It is already known that S. aureus encodes ParB but lacks a ParA homolog, suggesting a non-canonical ParABS system. Additionally, prior studies have established that SMC plays a crucial role in chromosome segregation, though it is not essential. This study integrates these known aspects into a cohesive model by combining tracking of ori and ter loci using the FROS system with chromosome conformation capture assays and deep sequencing. As a result, this work is timely and represents a significant advance in the S. aureus field. While the study contributes to the broader understanding of chromosome organization in S. aureus, significant improvements are necessary before it is suitable for publication. I am particularly concerned about the lack of complementation assays, such as for the smc deletion mutant, to substantiate several claims made in this manuscript.

Major Comments:
R2.1. There is no complementation for any deletion mutants in this manuscript. I would specifically like to see complementation for the smc- mutant, as its phenotype is difficult to interpret. The smc- (or scpAB-) strains produced approximately 17% anucleate cells, whereas parB- produced only 0.14% anucleate cells. Since ParB is suggested in this manuscript to be the loader of SMC, and the Hi-C maps of smc- and parB- are essentially identical, the manuscript proposes that in the parB- strain, SMC is still being loaded somewhere on the chromosome and hypothesizes that this is why the smc- strain produces a much higher fraction of anucleate cells. However, there is no experimental evidence in this manuscript that SMC is loaded elsewhere. The simplest explanation is that SMC in S. aureus has functions unrelated to chromosome organization (yet this possibility is not discussed). Another explanation is a polar effect caused by the smc deletion, necessitating a complementation assay to rule this out. Using a strain where an early stop codon is introduced into the smc coding sequence is not a foolproof way to rule out a potential polar effect.

Similarly to the reviewer, we were also worried about possible polar effects. This is why we constructed two different mutants, *smc*^{STOP} (where a stop codon was introduced instead of a deletion to reduce the probability of polar effects) and a Δ*scpAB* mutant. Importantly, *scpAB* is located in a different region of the chromosome than *smc*, therefore if there would be polar effects, these would be on different genes. Reassuringly, both *smc*^{STOP} and Δ*scpAB* show similar number of anucleate cells

Nevertheless, we agree with the reviewer that the most informative way of ruling out polar effects is a complementation assay, which we now have done. Fig. S11 now includes complementation strains for both the *smc*^{STOP} and Δ*scpAB* mutants, showing that the fraction of anucleate cells revert to wild type (negligible) levels.

We would also like to point out that the Hi-C maps of the Δ*parB* and Δ*scpAB* mutants are not identical, as most contacts outside of the main diagonal have been abolished in the Δ*scpAB* mutant but not in the Δ*parB* mutant. This can be clearly seen in the Pc curves (Fig. S10C). We have re-written the text (lines 411-414) to make these points clearer. Additionally, we have included a map of the ratio between the JE2 WT and Δ*parB*

mutant (Fig. S10D), showing that in the absence of ParB, there is an increase of long-range contacts in areas outside of the secondary diagonal, meaning that SMC is still able to load and bridge these interactions (in agreement with Fig. S10C).

R2.2. Related to the above concern, the authors state, "Without ParB/parS, randomly loaded SMC-ScpAB molecules can compact and segregate the chromosome quite well, although the overall organization of the chromosome is altered." This statement is not experimentally substantiated, and the two halves of the sentence contradict each other.

We thank the reviewer for bringing to our attention that the text was not clear and we have now re-written it to improve clarity (lines 411-414). As mentioned in the previous comment, we think our data indicates that SMC can load onto the chromosome in the absence of ParB/*parS*. We want to note that in B. subtilis, it has also been observed that SMC can load to some extent onto the chromosome in the absence of ParB, which is now discussed in the revised manuscript (lines 555-557).

R2.3. The manuscript lacks relevant literature citations and requires substantial improvement. While comprehensive reviews exist on S. aureus chromosome organization, key studies on ParB have been overlooked. The authors reference only one review from 2020, despite numerous studies (and reviews) contributing to the understanding of ParB as a CTPase since then. Two foundational papers identifying ParB as an enzyme are not cited. The discussion on SMC and Hi-C lacks proper citations, particularly for critical works such as Bock et al. and Marbouty (2015), which are essential for contextualizing the findings. Additionally, several relevant papers from the S. aureus field were omitted.

As mentioned above, at the end of the preparation of the manuscript we shortened the introduction and discussion which resulted in the removal of many references which we agree should not have been removed. We have now reverted this situation and included 30 additional citations, which has improved the quality of the manuscript.

R2.4. The use of the FROS system in this study raises some concerns due to its potential to introduce artifacts. Binding proteins to inserted sequences (48 copies) can create a roadblock for DNA-associated machinery, including DNA replication, which is a key marker analyzed in this study. Previous work has well-documented this issue, and the authors should acknowledge this limitation. A significant problem is evident in Figure S2, where changing the ori-ter labeling system results in strikingly different data—showing almost a 40% difference in ori per cell and a 20% difference in ter per cell. These discrepancies suggest that the labeling method influences the observed localization patterns, raising concerns about the reliability of the conclusions drawn from these data. Typically, ori labeling using the FROS system is confirmed by colocalizing it with a native marker. The authors claim that native ParB expression is weak but do not provide supporting data for this assertion beyond citing a single study. Even in that study, the native ParB focus appears usable. Additionally, Chan et al. (2020)

demonstrated well-resolved images of ParB localization from a low-copy number plasmid, showing that ParB is not restricted to the cell's extreme periphery. This discrepancy should be acknowledged and discussed in the manuscript. To strengthen the reliability of their conclusions, I strongly recommend that the authors validate their ori-ter labeling using an alternative approach, such as: (i) generating a colocalization strain with the native ParB to confirm ori positioning at the cell tip, (ii) using a different labeling system (e.g., parSPMT1-parSP1) to compare with the FROS system, or (iii) quantifying the relative abundance and validating spatial organization using a non-fluorescent approach, such as qPCR with primer pairs flanking the ori and ter regions.

We have modified the text to acknowledge the possibility that FROS systems can cause roadblocks (lines 175-182). However, we think the system we are using does not cause a roadblock, as that would be seen in MFA analysis as a drop in the frequency of sequences downstream of the array. We have included an additional figure with extended MFA analyses (Fig. S3) showing that the constitutive FROS system used in this work does not affect the profile of chromosome replication. We have also modified the text (lines 165-168) to address the differences observed in Fig. S2 between strains and induction times (we note that these strains were not used in the rest of the experiments).
We agree that our language regarding ParB foci may have been overly negative and we have rephrased that text (lines 142-147). The relevant point we wanted to make is that the foci produced by the FROS system are more condensed and clearer to distinguish than those produced by ParB (Fig. S7).

Additionally, we have followed the reviewer´s recommendation and colocalized ParB with the FROS Ori marker (new Fig. S7, which also includes ParB localization in a background without FROS system). These experiments show that the FROS signal and ParB largely colocalize, and they both tend to localize in the cell poles.

Regarding the data in H. Chan *et al.*, 2020, we would like to point out that it is not in contradiction with ours, as their figure 3a also shows ParB foci towards the cell periphery. However, there is no membrane dye in that figure, so it does not allow the direct evaluation of how close to the membrane the ParB foci are.
Furthermore, due to the almost perfectly spherical shape of *S. aureus*, cells can lay on the microscopy slide in different orientations. For this reason, a focus at the cell pole can look like a focus in the middle of the cell if the pole is facing the objective. Therefore, the localization pattern of ParB foci only clearly emerges by examining hundreds of cells in a systematic way, as done in our manuscript.
In any case, we have modified the text to compare our results with those from H. Chan *et al.*, 2020 in lines 142-145, 549-550.

R2.5. A schematic representation showing the exact genomic locations of the FROS sequences relative to ori and ter would be very helpful. How far away from the actual origin of replication was the array inserted? Is this array next to a membrane protein-encoding gene? If so, is there a possibility that a translation-membrane insertion effect causes the ori FROS to be close to the membrane (but the ORI in general might not be)?

Can the authors move the ori FROS labeling to another ori-located site to rule out this possibility?

Thank you for bringing this to our attention. The location of the arrays is described in the strain list. In particular the tetO array for the Ori labelling was inserted between the genes SAUSA300_2631-SAUSA300_2632, 14 Kb from the Origin (we have added the distance to the table and we have depicted their location in Fig. S3A). SAUSA300_2632 is indeed predicted to be a membrane protein. According to unpublished transcriptomic data from our lab this is not a highly transcribed gene. However, we are confident that the Ori localization near the membrane is not an artifact for two main reasons: (i) ParB localization in a strain lacking the FROS system is also at the cell periphery (Fig. S7), (ii) in a screening unrelated to this work, we identified a protein that tethers the Ori to the membrane. In a mutant lacking this protein (which is encoded far away from the Ori), the membrane-proximal localization of the Ori is lost, even when the Ori is labeled using the FROS system described in this study (manuscript under preparation).

R2.6. Line 128 and beyond: If this is the first study stating that the ori is positioned at the tip of the hemisphere, then it should be noted that previous studies (e.g., Chan et al.) did not show tip-positioned ParB foci. How do the authors explain this discrepancy? How can they rule out the possibility that this is due to the FROS labeling system? Is it strain-specific?

As mentioned in the previous answers, we do not think that there a discrepancy between our data and that of H. Chan *et al*., 2020 or others, since the localization of ParB or the Ori was never analysed in *S. aureus* in a quantitative manner. We have updated the text to reflect this (142-147).

Minor comments
R2m.1. Line 19: Reference for FROS?

The mentioned line is in the abstract, where no citations can be included. The reference for the FROS system is given in line 148.

R2m.2. Line 19: The authors state that "cell cycle and chromosome replication are not coupled." Since DNA replication is part of the cell cycle, clarification is needed regarding what exactly they mean.

The text has been modified to improve clarity (line 20).

R2m.3. Line 26: What is the distinction between "chromosome conformation" and "chromosome compaction" as referred to by the authors? The study monitors chromosome configuration using Hi-C but does not directly assess compaction. Without data on nucleoid-associated proteins (NAPs) or DNA content quantification, claims regarding compaction should be moderated.

We inferred compaction from the Pc(s) curves, which calculate the averaged contact frequency as a function of distance. We modified line 357 to make this clearer and

added a relevant citation. Nevertheless, to avoid over-interpreting our data we have removed most mentions to compaction.

R2m.4. Need to report the number of replicates for ChIP-seq and Hi-C experiments

Number of replicates has been added to the figure legends.

R2m.5. The P1-2-3 stages are well established in the Staphylococcus literature but may not be familiar to all readers. It would be helpful to include some schematics alongside Figure 2D so that readers can better understand how the authors come to these conclusions from Figure 2B.

We have modified the figure 2 accordingly.

R2m.6. Line 46: The term "linear" chromosome arrangement is not widely accepted. It would be more appropriate to use "longitudinal," which has been established in the literature.

Thank you for pointing this out. This has been corrected throughout the text.

R2m.7. Line 47: Reference needed for "subpolar" chromosome positioning in M. xanthus.

The reference has been added.

R2m.8. I recommend discuss Pseudomonas chromosome organization as well, major knowledge in this area is gained through Hi-C work in this bacterium.

Thanks for calling our attention to this. Discussion on this topic was removed to shorten the manuscript before submission, but is now included again.

R2m.9. Line 48: References needed for ter positioning.

Relevant references have been added.

R2m.10. Line 53: The authors clearly distinguish between chromosome behaviour in slow- and fast-growing conditions in Bacillus, yet all their conclusions for S. aureus are based on a single growth condition.

We agree that this was a limitation of our study and following reviewers' recommendations we have now included data on Ori localization and number for *S. aureus* growing at a slower rate (growth at 25°C) and also in stationary phase (Figures S8 and S9,) as well as marker frequency analysis in these conditions (Fig. S3). These results are discussed in lines 287-297.

R2m.11. Line 61: The E. coli chromosome is widely recognised as having a "transverse" orientation.

Thanks for pointing this out, it has been corrected.

R2m.12. Lines 72–99: Incomplete referencing, particularly in the ParB field. No citations for ParB as a CTPase are included, and only one review from 2020 is cited, which is insufficient.

Thanks for calling our attention to this. Discussion on this topic was removed to shorten the manuscript before submission, but is now included again (lines 79-83), together with relevant references.

R2m.13. The authors fail to introduce or discuss Streptococcus pneumoniae, which is highly relevant given its similarities to S. aureus regarding the ParABS and SMC systems.

We have expanded in the text references to *S. pneumoniae*, included additional citations, and made additional comparisons with data from this bacterium (for example lines 537-537 or 604-609).

R2m.14. Line 125: Tracking the origin of replication with ParB is a well-established approach. The claim that this approach is flawed is not well-supported; it is one of the best native markers available for ori tracking.

We agree that ParB localization is a good proxy for Ori localization, and indeed it is one we have used in the past. Our intention was to highlight the imaging advantages of the FROS system, which produces much brighter and sharper foci. However, we may have used overly strong language, and we have now revised the text so now lines 145-147 state: "*We noticed that fluorescently tagged versions of the native S. aureus ParB often produce diffuse foci, hindering precise quantitative analysis*.". Additionally, a direct comparison between the ParB and FROS signal is made in Fig. S7 and we discuss ParB localization in lines 257-263, where we conclude that ParB and our FROS system agree regarding Ori localization.

R2m.15. Line 136: Relevant studies are missing citations.

We have cited Chan et al., 2020, Veiga et al 2011 and Saraiva et al 2020 which, to the best of our knowledge, are the most recent works that have reported on ParB localization in *S. aureus*. If the reviewers point to additional references that should be included, we are happy to do so.

R2m.16. Line 138: The claim that most cells have two to four origins contradicts Chan et al., where most cells have two origins (<62%), and four-origin cells are a minority.

The referenced data from in H. Chan *et al.*, 2020 refers a quantification of SMC foci (not ParB), which is not widely accepted as a marker of the origin, although it can colocalize with ParB. In agreement with this, the cited work does not claim that the SMC foci are indicative of the number of Ori loci in the cell. Furthermore, in H. Chan *et al.*, 2020 the images were not acquired in multiple planes to pick up all of the foci, while our data

comes from projections of 3 planes in order to capture all foci. Still in Figure 3a from the H. Chan *et al*., 2020 paper, out of 9 cells shown, one has four ParB foci and another three cells have at least three ParB foci.

We have made changes in the text to further comment and compare the data presented in H. Chan *et al*., 2020 with that of our study (lines 142-145 and 549-550).

R2m.17. The authors did not cite two extremely relevant papers for this study: Barbuti et al. and Gallay et al.

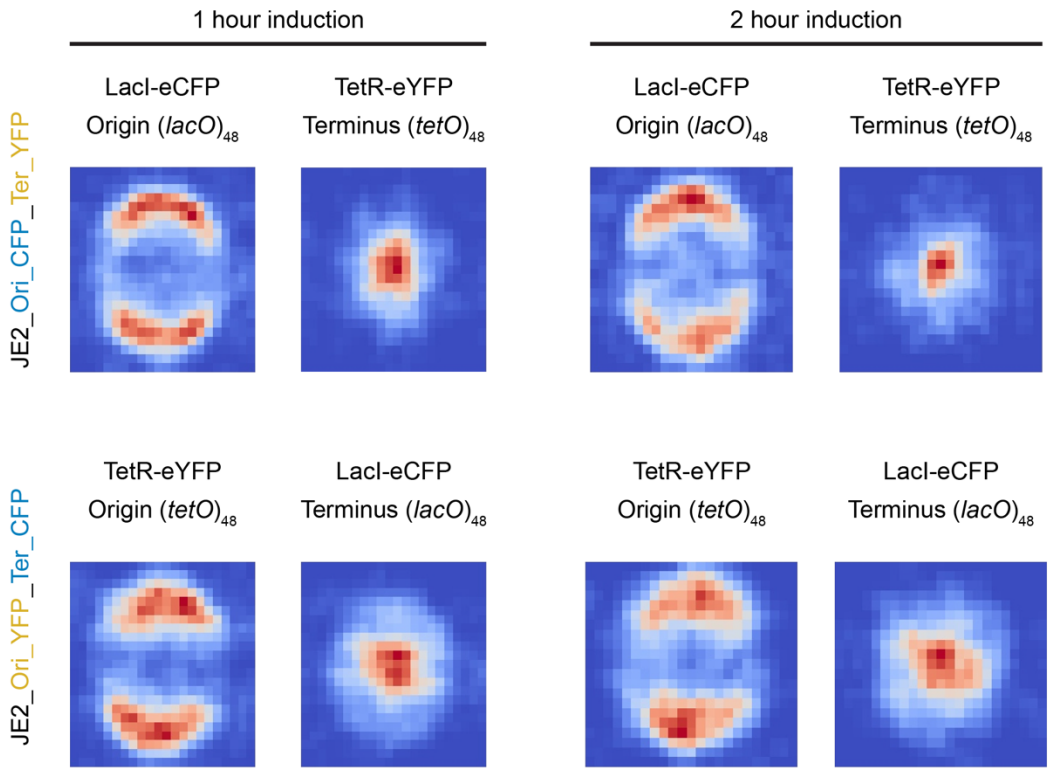
We have now added these references.

R2m.18. Line 153: Figure S2 is referenced vaguely; please specify which panel.

We have changed the text to address this.

R2m.19. Figure S2A: The LacI-eCFP and TetR-eYFP ori markers largely overlap, but some cells clearly show a centrally positioned TetR-eYFP ori focus. These foci appear intense and possibly saturated.

Given that *S. aureus* is spherical, depending on the cell orientation foci in the cell periphery can appear in the cell center too. We have confirmed using heatmaps that these strains show the same average localization of the Ori and Ter regions as that described with the constitutive FROS system (see image below). Other regions of the microscopy image have been selected to reflect better the localizations.



R2m.20. Figure S2A (Ter labelling): Different labelling methods (TetR vs LacI) yield inconsistent results, with LacI-ter labelling often resembling ori. This suggests potential labelling artefacts (See Major Comment #2).

We have repeated these experiments to include replicates and the possible causes of the discrepancies between conditions are now are addressed in the lines 165-168. As mentioned above, using heatmaps, we show that Ter and Ori localization in these strains is, on average, similar to that observed in with the constitutive FROS system

R2m.21. Figure S2B: No replicates?

Our original aim was just to illustrate that Ori and Ter could be labelled in the same strain. Nevertheless, replicates have now been added.

R2m.22. Line 158: The authors switch to TetR-mNG, saying the expression conditions were better, presenting only a schematic in Figure 1A. Microscopy images should be provided alongside the schematic and heat maps or demographs for validation.

The reason why we choose to use the TetR-mNG is that it eliminates potential variation due to differences in cadmium concentration, induction times or cellular responses to the presence of this heavy metal on the media (the TetR-mNG system works constitutively, without need to add cadmium, relying only on the leaky expression of the TetR-mNG fusion). The text has been changed to make this clearer (lines 171-174).

R2m.23. Lines 163–167: DnaN labelling is discussed without referencing a figure, only returning to Figure 1B later. The figure order should be improved for clarity. Additionally, the figure legend states that the bar charts in 1B represent the frequency of cells in a FROS-ori-DnaN-Halo strain, but this is unclear in the main text.

We have clearly stated the name of the strain in the text to make it clear that we refer to the JE2_FROS^{Ori}_DnaN-Halo strain (line 196).

R2m.24. Showing bar graphs is not the best way to show the trend. This is visible throughout the paper, especially in Figures 4B and 5B-C. I suggest the authors use single points with stdv bars.

Thanks for pointing this out. Reviewer 1 also suggested a similar approach. We plotted the data as suggested, but we found that it did not improve clarity. We then decided to plot the bars grouped according to strain which we think makes the trends much easier to follow and compare (Figs 4 and 5).

R2m.25. Line 169: The authors state that they "correlated that information with the cell cycle stage." Can they explain precisely how this was done?

Text has been modified in line 189 to clarify that we refer to the absence/presence of a septum.

R2m.26. Figure S3 is enormous, and the text generally references it without explaining what the reader should focus on.

We agree that this figure has a lot of information that is not easy to digest. This is why in the main text, in Figure 1, we present aggregated data from this figure in a form that is easier to understand. However, we wanted to make all the data available, in case a reader was interested in a more detailed analysis.

R2m.27. Figure 1B and Line 177: The percentages reported here differ from those in previous studies, such as Chan et al. Could the authors explain these discrepancies?

As we mention in an answer to a previous comment (please see reply to R2m.16), the mentioned percentual data from H. Chan *et al.*, 2020 refers a quantification of SMC foci, not ParB or any other Ori marker.

R2m.28. Line 183: The authors claim that colocalisation happens only in the P1 stage and cite Figure 1C. While Figure 1C provides the quantification, it would be preferable to see the actual colocalisation image or reference Figure 1D. If Figure 1D shows that, it should be presented before 1C. Additionally, the sample size for Figure 1D (n = 36) is quite small.

Thank you for your feedback, we agree that it would be a good idea to show this in the main figure, so we have added examples of cells showing DnaN and Ori colocalization or no colocalization (Fig 1C). Regarding the n number, since the data was obtained in triplicate, in total there are about 100 observations. This number is lower than for other experiments because it requires time consuming manual inspection of time-lapses, to identify the exact point at which cells divide.

R2m.29. Figure 1D: The authors claim that ori and DnaN colocalise, which is unclear in Figure 1D; instead, it is a supplementary figure. 1D: Only the first image suggests possible colocalisation in the upper hemisphere. Furthermore, the colour labelling (green/red) should be explained directly within the images instead of relying solely on the figure legend.

Fig 1D is not trying to show colocalization for all cells, we say in the text "*Additionally, around 70% of cells had an assembled replisome that did not colocalize with the origins, indicating that cells are typically born with a partially replicated chromosome (Fig. 1D).*" We have now added examples of the colocalization in panel 1C.
We also explain the color code in the figure.

R2m.30. Lines 199–200: The claim that "the whole chromosome is partially replicated" requires supporting data. A Ter-DnaN strain would be necessary to confirm this statement. Otherwise, I suggest rephrasing.

We agree with this suggestion, so we have now included data from a FROS^{Ter}_DnaN-Halo strain (Fig. S5), where we show that around 95% of newborn cells have a single Ter focus. The implications of these observations are also discussed (lines 217-226).

R2m.31. Lines 204-206: differences from the summary in the Barbuti review, which is not referenced. Can you clarify the reasoning behind this? Is it condition-related or strain-specific?

The number of origins and chromosomal state depicted in the mentioned review are just part of a model, they are not based on experimental data, nor is a citation provided. As far as we know, ours is the first work that uses a quantitative approach to report on the ploidy and chromosomal organization of *S. aureus*.
We ourselves used to draw the cell cycle of S. aureus starting with one origin only, until we did the quantitative analysis that became possible with the FROS system.

R2m.32. Line 208: "Linear" should be replaced with "longitudinal."

Thank you for pointing this out, we have corrected it.

R2m.33. Figure 2B: Without at least one or two ori-left or ter-right colocalisation experiments, the chromosome arrangements proposed by the authors lack strong support.

Since we are using as a reference point the membrane labelling and the longer axis of *S. aureus* cells, we think simultaneously labelling the two loci would not add additional information to separately labelling them. Particularly because given the spherical shape of the cells and the consequent variation in their positioning on the slide, strong conclusions can only be taken from the analysis of a large number of cells, not from the direct comparison, in each cell, of the localization of foci.

R2m.34. In Figure 2B, the heat map suggests a central positioning of the ter locus, but in the corresponding image above, the ter appears more peripheral. Could the authors clarify this discrepancy?

The heatmap data reflects the average of thousands of cells. Where referring to the localization of chromosomal loci, we describe their tendency to localize in specific locations. However, there are cells that show foci in other locations, like the mentioned example. The heatmaps reflect the locations with highest probability for a locus. We have revised the text to make sure that this is clear. We have also replaced the mentioned image to one more representative of the Ter overall localization.

R2m.35. I strongly encourage the authors to generate a ter-DnaN strain and include time-lapse imaging to correlate these findings with ori-DnaN, providing a comprehensive overview of whole-chromosome dynamics.

We have now included data from a FROS^{Ter}_DnaN-Halo strain (Fig. S5), where we show that around 95% of newborn cells have a single Ter focus. The implications of these observations are also discussed (lines 217-226), where we conclude that overall this supports that most cells are born with a hemi-replicated chromosome.

R2m.36. Line 244: Again, the authors cite only one review for ParB in the entire paper, which is not ok given the extensive literature available.

We agree. To shorten the manuscript before submission we reduced some sections on ParB and eliminated important references. We have now included additional citations and expanded the relevant sections (lines 79-83, 345-347, 549-552).

R2m.37. Line 246: It would be beneficial for the authors to discuss in either this section or the discussion that Streptococcus pneumoniae also possesses ParB but not ParA. Many aspects of chromosome organisation in S. pneumoniae are strikingly similar to S. aureus, yet this is not mentioned anywhere.

We have now included comparisons to *S. pneumoniae* in the results and discussion sections (see our answer to comment R2m.13.), and this particular point of the lack of ParA is now discussed in lines 604-609.

R2m.38. Lines 252–254: The discussion of ParB spreading, bridging, and nucleation is presented without any supporting references, which must be corrected.

We have now included additional citations regarding ParB, as well as the ParB nucleocomplex formation (lines 315-318).

R2m.39. Line 263: "linear" should again be replaced with "longitudinal."

Thank you for pointing this out, we have corrected it.

R2m.40. Line 264: Despite numerous studies describing this phenomenon, only one reference is provided.

We have now added additional references (lines 334-335)

R2m.41. Figure 3: The figure lacks panels and contains numerous graphs, making it difficult to follow in the main text.

We have now divided the figure into panels, making it easier to cite in the text.

R2m.42. Line 269: The impact of leaving only one parS sequence has been thoroughly investigated and explained in B. subtilis. However, no relevant references are provided here (Gruber and Marbouty should be cited).

Thank you for pointing this out, we have added relevant references describing this phenomenon in other organisms (lines 345-347).

R2m.43. Figure S5C and Figure 3: There is a clear difference between WT and ΔparB, yet the red and yellow curves in S5C appear identical. This analysis should be repeated to fix this issue.

Please see our response to Reviewer 1's point 13 above, answering the same question.

R2m.44. Line 293: It would be helpful to see DAPI images next to the bar charts showing the percentage of anucleate cells in these mutants.

We have now added representative images of DNA labelled cells to Figure S11.

R2m.45. Line 306-307: But smc is not essential. The authors could discuss in greater detail, later on, the impact of other systems, such as NAPs or crowding effects, which are never mentioned.

The mentioned line says that "*the SMC complex plays a key role in the spatial organization and segregation of the S. aureus chromosome*" which we think correctly conveys the idea that this complex activity is required for the correct chromosome organization (as shown by the differences in Ori and Ter localization in Fig. 4 and the HiC data in Fig. 3) and segregation (as shown by the generation of anucleate cells in Fig. S11), even if the complex is not essential. We agree that there are other factors that can also contribute to this process, and we have added this idea to the text, together with a mention to the NAPs (lines 558-563).

R2m.46. Line 311: reference?

We have modified the text (lines 386-389) to make it clearer that this is just a hypothesis from our side, not a statement backed by previous research.

R2m.47. The SMC and ParB story in this study is quite similar to what has been described in Streptococcus pneumoniae, yet this work is not cited. In particular, Gallay et al. (2021), which discusses the CcrZ protein and even examines Staphylococcus, is highly relevant but not mentioned, even in the discussion.

We have now added when relevant comparisons with *S. pneumoniae* data (for example, lines 537-539 and 604-609), and expanded our references to studies on this organism, including the work of Gallay *et al*. (2021).

R2m.48. Line 325: There are likely more relevant citations that could be added here to provide better context.

We have added a more recent and very complete review that we think can provide the reader with a good overview on the FtsK family of proteins. In the following sentences relevant works on FtsK/SpoIIIE are also cited.

R2m.49. Figure 5: The claim that FtsK, SpoIII, and ori-ter labelling do not significantly impact chromosome spatial organisation is inconsistent with Figure 5A, where ori-ter labelling is visibly altered in ΔspoIII. This claim needs revision.

We agree that our previous wording stating that "*Ori and Ter foci remained similar*" in these strains could lead the reader to think their localization was identical. We have modified the text to clarify that the Ter positioning is less constricted to the cell center (lines 460-462) and thus, that these proteins may have a role in Ter positioning.

R2m.50. While the unwanted prophage expression might not directly affect the chromosome organisation, as it has not been tested, the authors cannot know if it affects something else in the cell. Please tone down the strong claims based on Figure S7.

We have changed the text accordingly, for example in lines 446-448 we now state: "*Thus, we hypothesize that the absence of XerC can cause severe alterations, or even DNA degradation, in the terminus region*").

R2m.51. Figure S7: The methodology behind this analysis is poorly explained in the figure legend and is entirely missing from the Materials and Methods section. Please clarify and include the relevant details.

We have extended our explanation in the methods (lines 758-760) and results (175-182) section, added a new figure (Fig. S3) and also included a reference of a recent publication that also uses this technique to assess replication impairments in another

bacterium (MV. Mazzuoli et al., 2025) as well as a previous work that uses it in *S. aureus* to investigate changes in DNA replication rate (Pang T. et al., 2017).

R2m.52. Discussion, Line 370: Converting a rod-shaped cell into a spherical one and analysing ori-ter-left-right organisation has been previously studied in E. coli and Bacillus. However, the Bacillus study (Tišma et al., Nature Comm) is neither cited nor compared to these data. It would be valuable to draw a parallel between chromosomal organisation in spherical or forced-spherical Gram (+) bacteria.

Although interesting, we think converting a rod into a spherical cell is not an ideal model to study segregation in spherical cells, as all the proteins involved in segregation in a cylinder are present. Moreover, the cells in that study had undergone a replication block and a threefold volume increase, which is not representative of the constrains ruling chromosome conformation in living spherical bacteria.

R2m.53. Line 374: What do the authors expect would happen under slow-growth conditions?

We agree that this is an interesting question. We expected that the cells would maintain a similar cycle as we described for 37°C though as at a slower path. To investigate whether this was correct we have performed the experiments and included data on Ori number and localization of cells growing at 25°C (Fig. S8, lines 287-292).

R2m.54. Line 377: The analysis suggests ori and ter are opposite, but this is true only in averaged heat maps, not necessarily in individual cells. Could the authors explain why there isn't a colocalisation of ori-ter in the same strain?

The strains that allow co-localization are available and shown in Fig. S2 (see also our answer to the comment R2m.19.), where it can be observed that in general the Ter in center and Ori in periphery. For the reasons stated in lines (171-174) most of the work was done with strains with a single locus labelled. In brief, the single-locus labelling system does not need any inducer, eliminating any possible variation due to variations in the induction time/concentration, or due to the cellular response to the presence of cadmium.

R2m.55. Figure 6B mentions a DNA-free region, yet the authors never labelled DNA with DAPI or a similar stain when discussing chromosome positioning. This is particularly relevant given their claim that ori regions are located at the cell tips: are they also at the tip of the nucleoid? Some clarification or DAPI data on this point would be helpful.

We have eliminated the mention to the DNA-free region, since what we meant was that the inter-chromosome area is the only region where the septum formation can happen without guillotining the chromosomes This is supported by previous study on the Noc system in *S. aureus* (H. Veiga *et al.*, 2011), which is cited in the following sentence. Regarding the second point, the nucleoid occupies most of the cell volume in S. aureus (as can be seen in images showing DNA staining of Fig. S11), thus being at in the cell

periphery does indicate that the Ori are indeed in the outer area of the nucleoid, similar to other organisms where the Ori has a polar localization.

R2m.56. Line 401: This would be an ideal place to cite Barbuti and Gallay, as they previously discussed the absence of visible multifork replication in Staphylococcus.

Thanks for pointing this out, we have that citation to discuss this point (now line 275-278).

R2m.57. Line 451: Where exactly did the authors look for chromosome compaction? There is no figure quantifying chromosome condensation.

Although we do not measure the condensation of the chromosome, the Pc curves (Fig. S10C) report on the contacts between loci of the chromosome in function on the distance and can be used as an indirect measure of the nucleoid compaction (which now explicitly stated in line 357). Nevertheless, the mention to compaction has been limited to statements in relation to figure S10C.

R2m.58. Line 452: The phrase “degradation of Ter” is unclear: what exactly do the authors mean?

The text has been rephrased to avoid over -interpreting our data and now says “*Furthermore, our data suggest that the deletion of xerC could give rise to alterations (or even in DNA degradation) in the Ter region, potentially explaining why a subset of cells lack a Ter focus*” (lines 586-588).

R2m.59. Line 462: The statement that this is "the first comprehensive characterisation of chromosome positioning and dynamics" is an overstatement. The study includes only one live-cell imaging movie analysing ori-DnaN colocalisation, which is insufficient to characterise chromosome dynamics fully. Additionally, much of what is described about S. aureus chromosome organisation was already known. This claim should be toned down.

In our opinion our work can be described as *the first comprehensive characterization* as it addresses the following points:

1) As we mention in lines 142-145, previous works had shown origin localization through ParB, however not in a quantitative manner. This is the first work that examines through a FROS system (and, in the revised version using ParB too) the positioning of the Ori in a population level. Moreover, we also employed the FROS system to investigate the positioning of the Ter region and loci in the left and right arms, which had never been shown before in this bacterium. We also investigated the localization of the Ori and Ter loci depending on the cell cycle phase, providing information in a temporal manner.
2) Although previous works had quantified the Ori/Ter ratios of *S. aureus* there was no quantitative information regarding the ploidy number in this bacterium. Our quantification of Ori and Ter regions in the overall population and also in newborn cells, together with the MFA analyses clarify that, in general, *S. aureus*

cells are born with a partially-replicated chromosome. This, together with the previous point, provides for the first time a data-based model of *S. aureus* chromosome spatial organization and replication cycle.

3) Previous works had investigated the role of SMC in chromosome segregation in *S. aureus* and its relationship with ParB, but not their influence in chromosome positioning or chromosome conformation. By including mutants lacking the ScpAB proteins (and now the added complementation strains), we clarify that the lack of a complete SMC complex leads to the production of 17% of anucleate cells in *S. aureus* JE2 strain. Moreover, we directly show that the SMC complex is a key component for Ori positioning and segregation in *S. aureus*. Furthermore, our HiC data demonstrates that ParB determines the inter-arm alignment of S. aureus chromosome, however it is not required for the loading of SMC onto the chromosome. Altogether, our results clarify the role that each of these systems (ParB-*parS* nucleocomplex / SMC complex) play in *S. aureus* chromosome segregation and conformation.
4) On the topic of ParB, this work is the first one to provide experimental data on the identity of the *parS* sites of S. aureus, which can be the starting point of any study aiming to characterize the functioning of ParB-*parS* in this bacterium.
5) Although we did not study in depth the role of FtsK, SpoIIIE, XerC or Noc, we reported on how their absence causes perturbations on the *S. aureus* chromosomal biology using the FROS system, which we think can be valuable additions to our knowledge about these factors in *S. aureus*.

R2m.60. One of the strong claims throughout the paper is that ori is positioned at the periphery. Quantifying the distance between the membrane and the ori marker in WT and ΔparB–ΔSMC mutants would be valuable. If these proteins play a role in anchoring ori, a measurable shift in distance should be observed. In previous studies using the low-copy number plasmid ParB marker, ori was not positioned at the extreme tips as claimed here. It would strengthen the study if the authors recapitulated their findings using the native ParB marker and performed colocalisation with ori labelling, as mentioned earlier.

Regarding the first part of the comment, deletion of ParB or SMC does not affect the peripheral localization of the origin as shown in heatmaps from Fig. 4A. Notice that the analysis to produce the heatmaps uses a membrane dye to determine the limits of the cell, so it allows the assessment of proximity to the membrane.

Regarding the second point, as we mention in answers to previous comments (R2.6, R2m.16.), in H. Chan et al., 2020, the ParB localization was not quantified in the population, so we cannot make direct comparisons. Furthermore, in H. Chan *et al.*, 2020 the images were not acquired in multiple planes to pick up all of the foci, while our data comes from projections of 3 planes in order to capture all foci.

Regarding the use of a ParB native marker, we have followed the reviewer´s suggestion and have now included ParB localization as well as the colocalization of ParB and FROS labelled Ori, and also the localization of ParB in a strain without FROS labelling (Fig. S7). Ori localization using both markers is identical.

Reviewer #3 (Remarks to the Author):

In this manuscript Izquierdo-Martinez et al. describe their extensive studies on chromosome segregation in the opportunistic pathogen Staphylococcus aureus. Chromosome segregation is a fundamental process in all bacteria, however, S. aureus challenges conventional models (usually rod-shaped bacteria) by operating in a spherical bacterium. This implies a highly constrained three-dimensional space without a clear polarity axis. Furthermore, the S. aureus genome lacks some canonical segregation proteins such as ParA. The authors employed a Fluorescent Repressor-Operator System (FROS) to precisely localize specific chromosomal loci within S. aureus cells. Using FROS, they observed the spatial and temporal organization of chromosomes during the S. aureus cell cycle. Based on the obtained data, the authors propose that the cell cycle and chromosome replication are not coupled and over-initiation of replication occurs at least under the tested growth conditions. The authors go on and show the binding of ParB to 5 ori-proximal parS sites and spreading of ParB away from these sides. The structural maintenance of chromosome (SMC) complex is likely loaded at these sites. Interestingly, it seems that the SMC complex plays a more critical role in chromosome segregation compared to ParB. Mutants lacking a functional SMC exhibited a significant increase in anucleate cells (~17%), whereas deletion of parB alone had only minimal impact on chromosome segregation. This suggests that in S. aureus, the SMC complex is essential for efficient chromosome segregation, and its recruitment by ParB to specific chromosomal sites enhances this process. Finally, they tested the effect of Noc, FtsK, SpoIIIE and XerC on the chromosome segregation process.
Overall this is a well written manuscript with plenty of interesting data. The experimental data are throughout excellent and the data are discussed based on the context of existing knowledge. As always with such comprehensive data sets, there are at places additional controls desirable.

R3.1 Line 183: How where the cell cycle stages defined and reliably determined?

The cell cycle classification is based on septa formation, so the P1 cells mentioned in the cited line are cells without any visible septa, nascent or complete. In order to asses this, we use a membrane dye and, in the section referenced, we visually inspect the cells and manually classify them (text changed to make this clearer in line 189). In further sections (Fig. 2, 4, 5) the classification is done automatically by the eHooke software (Saraiva B. et al., 2021) trained to use both membrane dye and DNA dye signal to classify the cells between P1, P2 and P3.

R3.2 Line 372: What do the authors mean by "pseudo-diploid" - a merodiploidy due to partial replication? Maybe there is true diploidy? The authors checked chromosome loci in one medium (if I get this correct). The presented data show a vast majority of cells with 2 or more oriC foci. Would that also be the case under very slow growth conditions or in stationary phase?
Adding to the point before: true diploidy could be shown by the presence of at least two

terC regions. Use of diffraction limited fluorescence microscopy is not suited to discriminate between one, two or even more FROS foci in close proximity. Therefore, a detailed marker frequency analysis of ori/ter ratios under varying growth conditions/replication states can give insights into this question.

Thank you for bringing this up, we agree that the term “pseudo-diploid” may not be clear, and we have removed it from the text,

Regarding slow growth, we agree that this is an interesting question and have now performed those experiments. We included MFA and Ori foci quantitative data from cells grown at lower temperature (Fig. S8, lines 287-292), and also in stationary phase (Fig. S9, lines 293-397). In brief, cells growing at 25°C still have 2-4 Ori foci (>90%), but there are more cells with a lower Ori foci number when compared to growth at 37°C. At 25C cells also have a lower Ori/Ter ratio (Fig. S3). As for stationary phase cells, they usually have 1 or 2 Ori foci, and the MFA shows that they have greatly reduced DNA replication rate.

R3.3 It would have been nice to add time-lapse videos in which the dynamics of the origin and terminus regions could be followed. Ideally, these dynamics could be analyzed using kymographs.

Thank you for your suggestion. We actually had tried to look at Ori dynamics, but did not include these data as tracking the Ori is very challenging: This is because 1) Ori foci move around or “wobble” in all cell cycle stages, and this movement is in three dimensions (and thus hard to capture in a kymograph, as can be seen in the image below), and 2) origins on the same half of the cell can overlap several times per cell cycle when observed in timelapses (making the tracking of individual spots impossible for more than a few minutes). We are trying to develop ways to track and quantify the Ori movements, but we do not have any reliable solution that we could apply now.

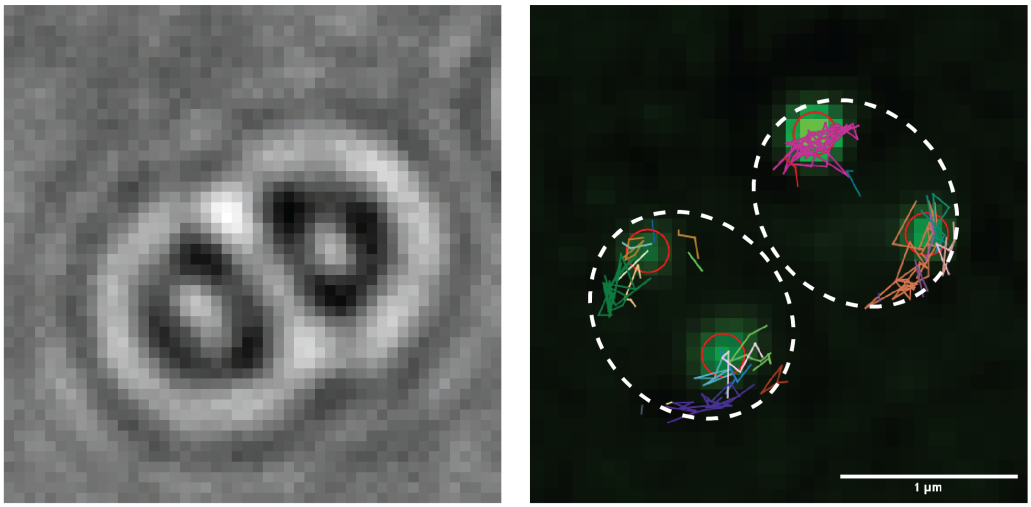


Figure legend: Tracking spots (red circunferences) and lines (various colours) generated with Trackmate from a timelapse with 30s intervals for 30 min of the strain JE2_FROS^{Ori} (TetR-mNG signal in green). Cell outlines indicated as white dashed lines, brightfield image on the left as reference. Both images correspond to the first frame of the timelapse.

R3.4 Fig. 3. The HiC maps were performed with cell cultures that are not synchronized, which is likely impossible to do with S. aureus. However, the chromosome content could be tested to provide insights into the replication state of the cells (flow cytometry). It is interesting to note that the HiC maps do not show the segregation signal of the oriC region (which was clearly visible in those bacteria that employ a classical ParAB system to drive oriC segregation across the nucleoid). This could be discussed at appropriate places in the manuscript.

Thank you for the suggestion.
Regarding the segregation signal, we have now discussed its absence in lines 498-505. Regarding chromosome content, optimizing FACS analysis would be very time consuming and, although interesting, would severely delay the publication of this work. We´ll be very honest and say that this is because the FACS we can use for bacteria is out of order and instead of repairing it, the institute has decided to buy a new one, but this will take a few months. It is not easy to convince other facilities to let us inject pathogenic bacteria into their systems. Given that this is not a critical experiment, we would prefer not to do it.

R3.5 Lines 284-287: The authors state that the (diffuse) long range contacts seen in a parB deletion are absent in the deletion of the scpAB subunits of the SMC complex (Fig. 3). Therefore, they conclude that SMC would be involved in establishing long range contacts even without being loaded to the chromosome by ParB first. While this is clearly interesting, it still raises the question how SMC would be loaded onto the DNA in this case (absence of ParB). While there is a loading of MukBEF in E. coli that does not require a ParB, it has not been demonstrated for a classical SMC complex in Gram positive bacteria. Technically, this result could also stem from differences in the DNA amount (replication differences) and/or segregation differences.

ChiP data for SMC in absence of ParB would be ideal to see if there is still binding to specific places. Also a functional mutant (SMC(EQ) variant) that cannot migrate along the DNA could be used as an appropriate control.

Regarding the first point, please see our response to Reviewer 1's point 13 above. In *B. subtilis*, F.P. Bock *et al*., 2022 showed that SMC can load to the chromosome without ParB (we have included this in the results and discussion, for example lines 555-557). The MFA plots showed that DNA replication profiles of WT and Δ*parB* are very similar (Fig. S11B); our microscopy results showed that the localization pattern and number or anucleate cells of Δ*parB* and WT are very similar. Since S. aureus Δ*scpAB/smc*^{STOP} mutants have a much stronger phenotype than Δ*parB*, we reasoned that the SMC complex must be able to load on the chromosome without ParB (Fig. S11). Moreover, without *parS* sites, SMC loaded presumably in a random manner on the chromosomes provided the same level of chromosome contacts on average, despite that the specific regions of these DNA contacts are different from those of wt as indicated by the Hi-C maps (Fig. S10C, D).

Regarding your suggestion of performing ChIP-seq on SMC, we have already performed those experiments using tagged variants of SMC. However, we have been unsuccessful so far. We would perhaps need to generate antibodies against the native protein and further optimize the ChIP-seq experiments, which is not doable within the time-frame for this revision.

R3.6 Also to this point: I am not sure if I get the idea behind the cartoons in Fig. 3 underneath the HiC maps. The CIDs seen in the HiC maps would normally attribute to enhanced interaction in a given chromosomal region within one replichore arm. Therefore, loss of the second diagonal would open up the replichore. Differences in the CIDs would argue for more or less interaction within neighboring chromosomal regions. Are the CID boundaries coinciding with highly transcribed genes, operon structures or any other noticeable feature on the DNA?

Thanks for pointing this potentially confusing point. We have modified the figure legend to clarify that the schematics are illustrating the inter-arm interactions and do not represent CIDs boundaries. We have added a sentence to explain CIDs boundaries (Lines 325-328).

R3.7 Line 333: An ftsK deletion is described to give rise to an increased number of terC foci. Could it be that the termini are held together by an unknown mechanism that is partially relived in absence of FtsK? Again, the number of termini in a fluorescent spot is difficult to quantify.

This is indeed a possible explanation, and we have added it to the discussion section (lines 576-579).

R3.8 Lines 342 onwards: Deletion of xerC is said to lead to degradation of the terminus region. Whole genome sequencing does indeed indicate prophage induction. However, terminus degradation could be tested by measuring DNA breaks in the otherwise circular chromosome (e.g. TUNEL-Assay, long range qPCR, fluorescent reporters etc.).

We agree that our data is not robust enough to strongly suggest that there is DNA degradation, and therefore we have toned down our claims regarding this in the results and discussion sections, for example in lines 586-588: "*Furthermore, our data suggest that the deletion of xerC could give rise to alterations (or even in DNA degradation) in the Ter region…".*
We think that the phenomenon we observed is very interesting, but studying it in detail is beyond the scope of this manuscript.

Reviewer #1 (Remarks to the Author):

The authors have fully addressed my concerns and have significantly improved on manuscript readability, presentation and scientific rigor. Added control experiments, and analysis make this a compelling paper for the field of chromosome organization!

We thank the reviewer for their words

Reviewer #2 (Remarks to the Author):

I am happy with this revision. No further concern.

We thank the reviewer for their words

Reviewer #3 (Remarks to the Author):

The authors submitted now a thoroughly revised version of their manuscript entitled “Chromosome segregation dynamics during the cell cycle f Staphylococcus aureus”. The revised version contains several new experiments, providing valuable data in support of their claims Particularly important are:
The revised version embraces the existing literature much better and allows a more comprehensive comparison of the findings.
Importantly, the authors provide a comprehensive marker frequency analysis to rule out that the FROS system interferes strongly with replication (new data in Fig. S3).
Further control experiments show that FROS did not interfere with oriC positioning (new Fig. S7).
The authors also provide new data for oriC numbers at different growth rates (25°C and 37°C). As expected the replication rate was reduced at 25°C but subcellular organization of the chromosome remained the same as under fast growth conditions.
Complementation strains for smc and scpAB deletions are now shown and they readily complement the mutant phenotype (Fig. S11).
The concerns raised by myself and other referees were addressed comprehensively and the new data were integrated well into the manuscript. In my opinion, the revised form does not need any major revision. A few small point could be considered, before publication. I would like to congratulate the authors to their nice study on S. aureus chromosome dynamics.

Line 23: Maybe a clearer description of the relation of oriC towards the division plane would be good. How about: The two oriC foci are perpendicular to the plane of cell division?

We thank the reviewer for his words and suggestion. However, given that foci can not be perpendicular to a plane, we would have to mention a line connecting the two Oris which would be perpendicular to the division plane, which may not be straightforward to understand. So we opted to leave the abstract as it was.