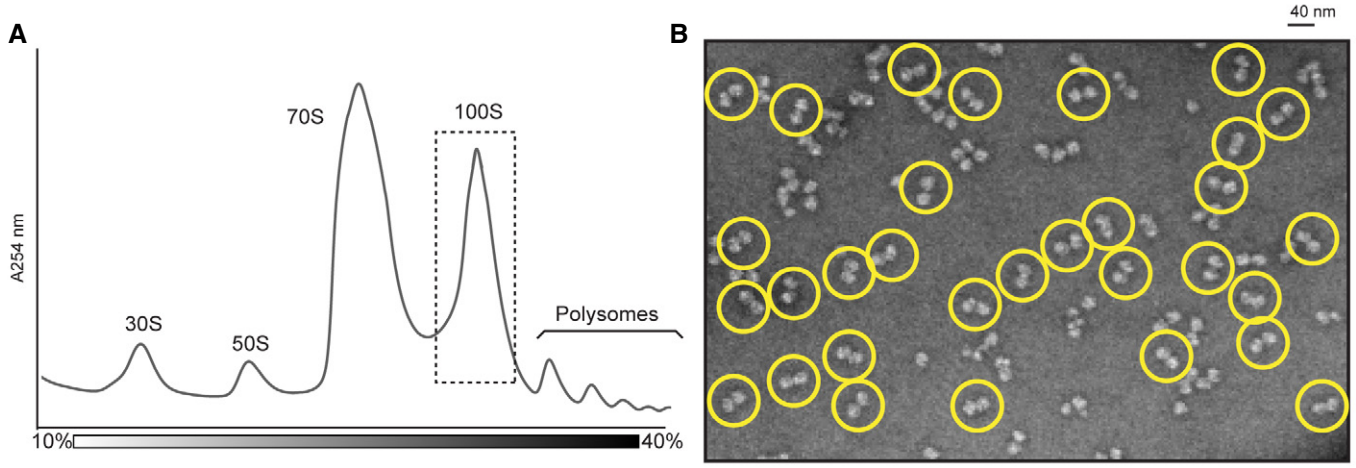
Expanded View Figures



C

```
Conservation:    9  99 9 9 9 9 99   9  9  999 999      9               9
EcHPF         1  MQLNITGNNVEITEALREFVTAKFAKLEQYFDRINQVYVVLKVEKV---THTSDATLHVNGGEIHASAEG   67
4V8H.pdb      1  MQLNITGNNVEITEALREFVTAKFAKLEQYFDRINQVYVVLKVEKV---THTSDATLHVNGGEIHASAEG   67
BsNTD_HPF     1  MNYNIRGENIEVTPALKDHVERKIGKLERYFDHSVDADVNVNLKFYNDKESKVEVTIPMTDLALRSEVHN   70
Consensus_aa:    MphNIpGpNlElT.AL+-@Vp.KhtKLEpYFD+.sph.Vslplcbh...ppps-hTl.hss..l+tphcs
Consensus_ss:     eeeeeeeeee  hhhhhhhhhhhhhhhhh    eeeeeeeee       eeeeeeee    eeeeeee

Conservation:      999 999    99 99  999 9
EcHPF         68  QDMYAAIDGLIDKLARQLTKHKDKLKQH----------   95
4V8H.pdb      68  QDMYAAIDGLIDKLARQLTKHKDKLKQH----------   95
BsNTD_HPF     71  EDMYNAIDLATNKLERQIRKHKTKVNRKFREQGSPKYLL  109
Consensus_aa:    pDMYsAID.hhsKL.RQlpKHKsKlpp+..........
Consensus_ss:      hhhhhhhhhhhhhhhhhhhhhhhhhhh
```

D

```
Conservation:                 99  999  999  999 9 9  99999999 9   9  9999 9 99 999999
3KA5 chainA  1    ----------EIVKTKRFAIKPMSEEEAVLEMELLGHNFFVFQNGDSNEVNVVYKRKDGNYGLIEPE-   57
BsCTD        121  VQDDIEEEESLDIVRQKRFNLKPMDSEEAILQMNMLGHNFFVFTNAETNLTNVVYRRNDGKYGLIEPTE  189
Consensus_aa:     ...........-IV+pKRFslKPMspEEAlLpMphLGHNFFVFpNt-oNbhNVVY+RpDGpYGLIEPp.
Consensus_ss:                eeeeeeee     hhhhhhhhh     eeeee      eeeeeee    eeeeee
```

Figure EV1. Isolation of *Bacillus subtilis* 100S and sequence alignments of *Bs*HPF with *Ec*HPF-NTD and *Ca*CTD.

A Sucrose density gradient profile of *B. subtilis* extract from late log phase cells, with 30S, 50S, 70S, 100S, and polysome peaks indicated.

B Negative stain electron microscopy images of purified *Bs*100S from (A), with selected 70S dimers circled in yellow.

C PROMALS3D (Pei *et al*, 2008) sequence alignment of *Bs*HPF-NTD with *Escherichia coli* HPF (PDB 4V8H)(Polikanov *et al*, 2012) that was used to generate the homology model for *Bs*HPF-NTD.

D PROMALS3D (Pei *et al*, 2008) sequence alignment of *Bs*HPF-CTD with *Clostridium acetobutylicum* HPF-CTD (*Ca*CTD; PDB ID 3KA5) that was used to generate the homology model for *Bs*HPF-CTD.

Data information: In (C) and (D), fully conserved residues are indicated with "9" and are bold in the Consensus_aa, whereas similar residues are indicated with a "+". Consensus_ss indicates β-sheet (e) and helical (h) regions.

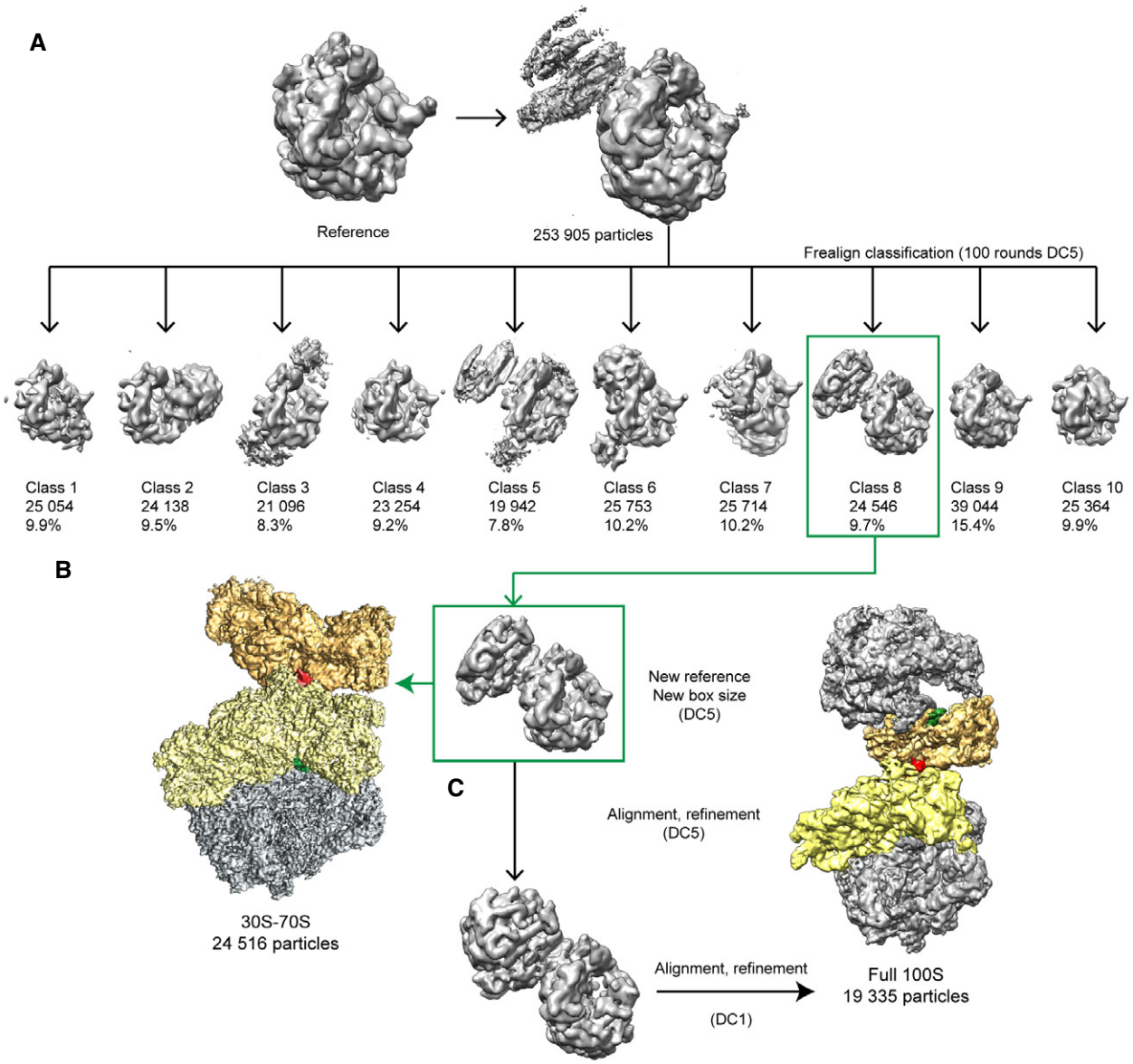


Figure EV2. *In silico* sorting and refinement scheme for the *Bs*70S-30S subcomplex and complete *Bs*100S.

A–C 253,905 particles were sorted into 10 classes. Class 8 had the most defined density for the 70S-B and was taken for further refinement using (B) a box size that includes the 70S-A ribosome and the 30S part of the 70S-B, and (C) a larger box size that encompasses both the 70S-A and 70S-B ribosomes.

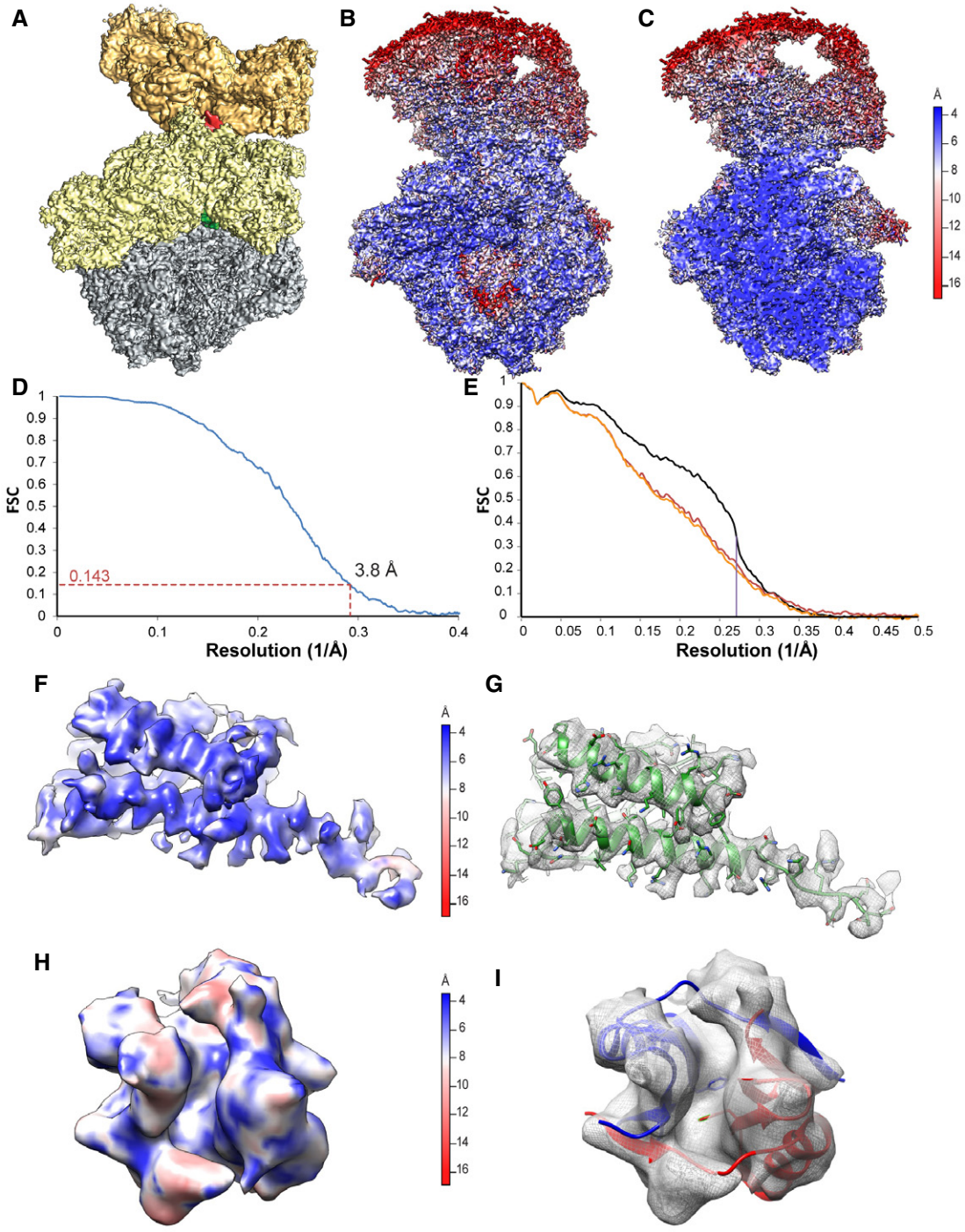


Figure EV3. Resolution of 70S-A in the *Bs*70S-30S subcomplex.

A Overview of the *Bs*70S-30S subcomplex with 30S-A (yellow), 50S-A (gray), and 30S-B (orange), as well as *Bs*HPF-NTD (green) and *Bs*HPF-CTD (red).

B, C Overview (B) and transverse section (C) of the *Bs*70S-30S subcomplex colored according to the local resolution, as calculated using ResMap (Kucukelbir *et al*, 2014).

D Fourier-shell correlation curve of the refined cryo-EM map, indicating the average resolution of 70S-A in the *Bs*70S-30S subcomplex is 3.8 Å.

E Fit of models to maps. FSC curves calculated between the refined model and the final map (black), with the self- and cross-validated correlations in orange and red, respectively. Information beyond 4 Å was not used during refinement and preserved for validation.

F–I Map density for the (F, G) *Bs*HPF-NTD and (H, I) *Bs*HPF-CTD, which are (F, H) colored according to the local resolution, as calculated using ResMap (see Materials and Methods), or (G, I) shown as a gray mesh with molecular models (G) for *Bs*HPF-NTD (green) or (I) *Bs*HPF-CTD for 70S-A (red) and 70S-B (blue), using the same respective view as in (F, H).

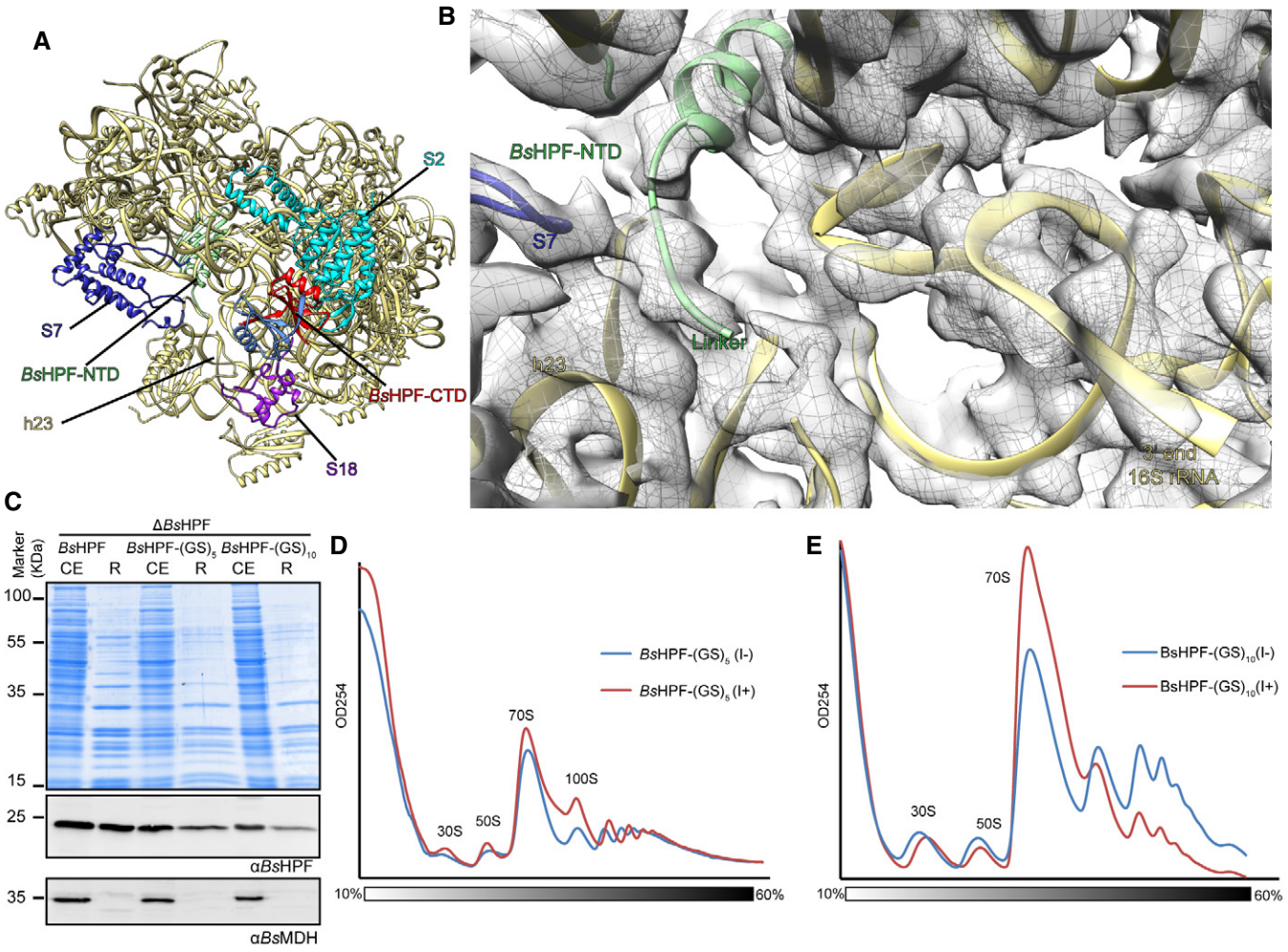


Figure EV4. *Bs*HPF linker region approaches the 30S platform cavity.

A Overview of the 30S cavity region showing *Bs*HPF-NTD (green) and *Bs*HPF-CTD (red) and 30S (yellow), except S2 (cyan), S7 (blue), and S18 (purple).

B Zoom of (A), showing map density (gray mesh) for the N-terminal part of the linker region of *Bs*HPF (green) as well as for the 3′ end of the 16S rRNA.

C Coomassie (upper panel) and Western blot of cell extracts (CE) and ribosome pelleted fractions (R) of the wild-type *Bs*168 (wt) strain or the Δ*Bs*HPF strains expressing either wild-type *Bs*HPF, *Bs*HPF-$(GS)_5$, or *Bs*HPF-$(GS)_{10}$.

D, E Sucrose gradient profiles of cell extracts from the (D) *Bs*168 Δ*Bs*HPF amyE::*Bs*HPF-$(GS)_5$ strain and (E) *Bs*168 Δ*Bs*HPF amyE::*Bs*HPF-$(GS)_{10}$ strain, in the absence (I−) or presence (I+) of IPTG.

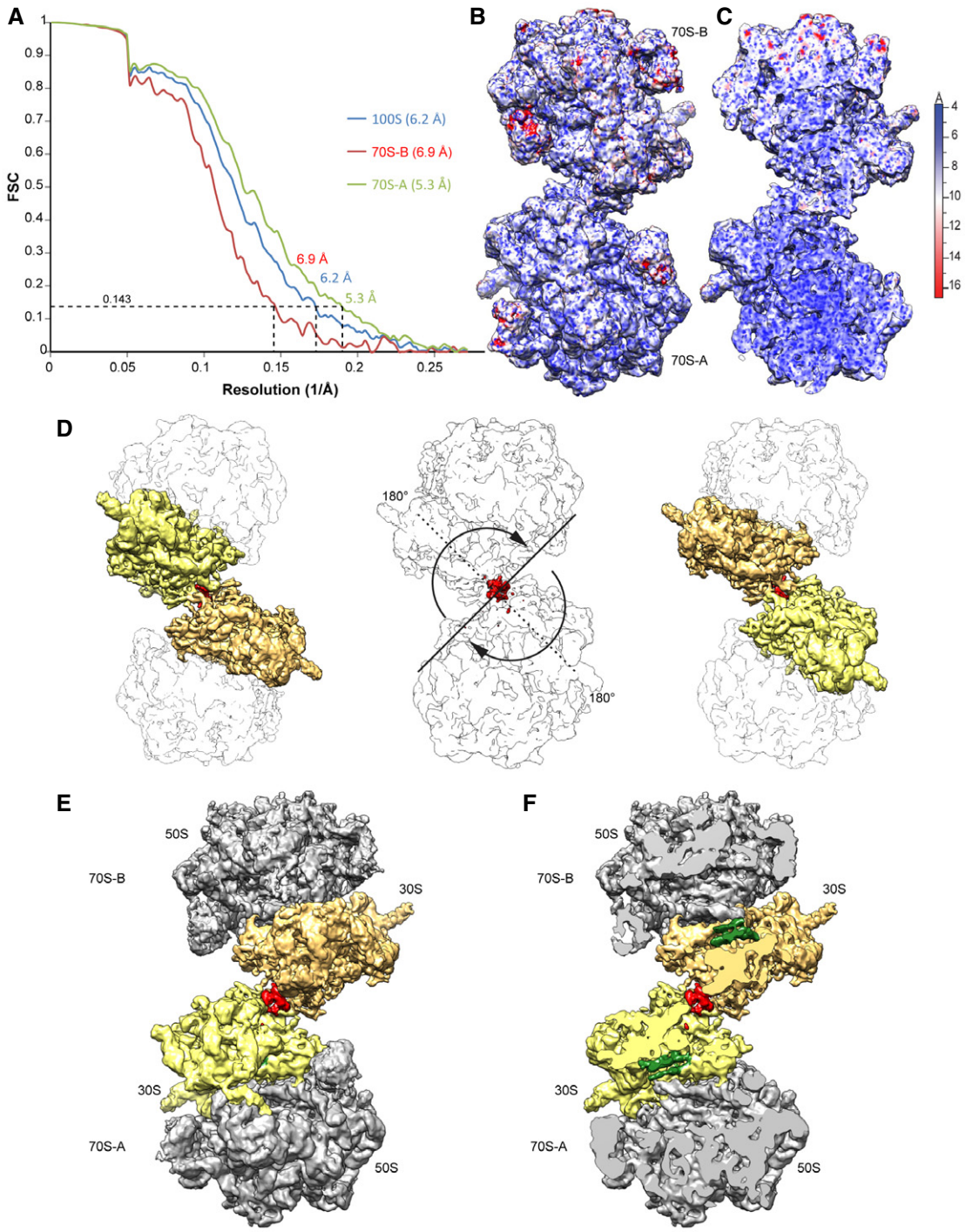


Figure EV5. Resolution of the complete dimeric *Bs*100S.

A Fourier-shell correlation curve of the refined cryo-EM map, indicating the average resolution of 70S-A, 70S-B, and the complete *Bs*100S is 5.3, 6.9 and 6.2 Å, respectively.

B, C Cryo-EM map of the dimeric *Bs*100S colored according to local resolution showing (B) overview and (C) transverse section of the complete 100S disome.

D The 70S-A and 70S-B monomers in the Bs100S are related by rotational symmetry of ~180°.

E, F Cryo-EM map of the (E) dimeric *Bs*100S with 30S-A (yellow), 30S-B (orange) and 50S (gray), and (F) transverse section of (E) highlighting the densities for the *Bs*HPF-NTD (green) and *Bs*HPF-CTD (red).