Nucleoid size scaling and intracellular organization of translation across bacteria

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Summary

The scaling of organelles with cell size is thought to be exclusive to eukaryotes. Here, we demonstrate that similar scaling relationships hold for the nucleoid in bacteria. Despite the absence of a nuclear membrane, nucleoid size strongly correlates with cell size, independent of changes in DNA amount and across various nutrient conditions. This correlation is observed in diverse bacteria, revealing a near-constant ratio between nucleoid and cell size for a given species. As in eukaryotes, the nucleocytoplasmic ratio in bacteria varies greatly among species. This spectrum of nucleocytoplasmic ratios is independent of genome size, and instead appears linked to the average cell size of the population. Bacteria with different nucleocytoplasmic ratios have different biophysical properties of the cytoplasm, impacting the mobility and localization of ribosomes. Together, our findings identify new organizational principles and biophysical features of bacterial cells, implicating the nucleocytoplasmic ratio and cell size as determinants of the intracellular organization of translation.

Keywords

Introduction

The spatial organization of the cell has a profound effect on various cellular processes from bacteria to humans (Bisson-Filho et al., 2018; Diekmann and Pereira-Leal, 2013; Harold, 2005; Surovtsev and Jacobs-Wagner, 2018). In eukaryotic cells, a distinctive feature of intracellular organization is the nucleus, a membrane-enclosed organelle that harbors most of the cell's genetic material. The nuclear envelope hereby spatially confines the genetic material and physically separates transcription and translation. While the sizes of cells and nuclei vary considerably among species and tissues, there is a remarkable linear size scaling relationship between the cell and the nucleus for a given cell type, which was first reported over 100 years ago (Conklin, 1912; Woodruff, 1913). Correlations between cell size and nuclear size are not only widespread among eukaryotic cells but also robust to genetically- and nutritionally-induced cell size perturbations (Jorgensen et al., 2007; Neumann and Nurse, 2007). This scaling property results in a constant ratio between nuclear and cellular volumes, also known as the karyoplasmic or nucleocytoplasmic (NC) ratio (Wilson, 1925). Why cells maintain a specific NC ratio is generally not well understood, though alterations in NC ratios have been associated with aging and diseases such as cancer (Capell and Collins, 2006; Chow et al., 2012; Prokocimer et al., 2009; Zink et al., 2004). The sizes of other cellular components such as vacuoles, mitotic spindles, centrosomes and mitochondria have also been shown to scale with cell size in various eukaryotic cell types (Levy and Heald, 2012; Marshall, 2015; Reber and Goehring, 2015). As such, these scaling properties are believed to be unique to eukaryotes.

In bacteria, the chromosomal DNA typically occupies a subcellular region called the nucleoid (Kellenberger et al., 1958; Mason and Powelson, 1956). Recently, we showed that the average size of the nucleoid scales with the average size of the cell across ~4,000 gene-deletion mutants of *Escherichia coli* (Campos et al., 2018). In addition, nucleoid size and cell size in *E. coli* correlate at the single-cell level, at least under specific growth conditions (Junier et al., 2014; Paintdakhi et al., 2016). An intuitive explanation for these observations may be linked to differences in DNA amount. Even under nutrient-poor conditions, DNA replication happens during a large part of the cell cycle, such that bigger cells tend to contain more DNA. This is exacerbated under nutrient-rich conditions under which *E. coli* displays overlapping DNA replication cycles (Cooper and Helmstetter, 1968). This leads to a continuous increase in DNA content from cell birth to division

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71 (Cooper and Helmstetter, 1968). Recent work with mutants of altered cell widths further suggests

72 that the amount of DNA in such rapidly growing cells is directly coupled to cell volume (Shi et al.,

73 2017). However, whether the scaling of nucleoid size with cell size is exclusively linked to changes

74 in DNA content remains to be established. It is also currently unclear whether a scaling

75 relationship between nucleoid and cell size is robust across growth conditions or widespread

76 among bacteria. At the same time, it is unclear whether the size of the nucleoid or the volume

77 fraction it occupies within the cell has any physiological consequence. We address all of these

78 unknowns below.

79

Results

Nucleoid size scaling is robust across a wide range of cell sizes in *E. coli*

82 Given that different nutrient conditions give rise to cells of different sizes (Pierucci, 1978;

83 Schaechter et al., 1958), we used phase contrast and fluorescence microscopy to examine how

84 cell size variation in exponentially growing *E. coli* may affect nucleoid size across 30 nutrient

85 conditions (M9 medium supplemented with different carbon sources ± casamino acids and

86 thiamine, see Table S1). Cell contours were detected and curated in an automated fashion using

87 the open source software package Oufti (Paintdakhi et al., 2016) and a support vector machine

88 model (see STAR Methods). For each condition, the areas of thousands of cells were quantified

89 from the cell contours (Figure 1A, Figure S1A). DAPI-stained nucleoids were detected using the

90 objectDetection module of Oufti, from which we extracted the total area occupied by the DAPI

91 signal (Figure 1A). Since estimation of the nucleoid area can vary with the chosen Oufti

92 parameters (e.g., contour rigidity, relative signal threshold), we used the same parameter values

93 across growth conditions.

94

95 Using this methodology, we observed a strong correlation (Kendall correlation $\tau \geq 0.77$) between

96 the cell area and nucleoid area of individual cells within all 30 tested growth conditions (Figure

97 1B). These results show that the nucleoid size scaling property is robust across a wide range of

98 growth rates, with doubling times varying from ~40 min to ~4 h (Figure S1B). For each condition,

99 the nucleocytoplasmic (NC) ratio (nucleoid area divided by cell area) was independent of the total

100 or normalized intensity of the DAPI signal per cell (Figure S2), and was therefore unaffected by

101 variations in DAPI staining efficiency. Moreover, we observed identical scaling relationships

102 between nucleoid and cell area for nucleoids labeled with an mCherry or CFP fusion to a subunit

103 of the nucleoid-associated HU complex (Figure S3A-C). The scaling between the cell area and the

104 total nucleoid area was preserved in filamentous cells obtained by treatment with cephalexin

105 (Figure S3D), a drug that inhibits cells division without affecting growth and DNA replication

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106   (Boye and Lobner-Olesen, 1991; Rolinson, 1980). The scaling relationship in these filamentous

107   cells was almost indistinguishable from that in untreated cells (Figure S3D). These observations

108   indicate that nucleoid size scaling occurs independently of cell division and persists across a wide

109   range of cell sizes and growth conditions.

110

111   At the population level, we also observed a strong correlation ($\tau = 0.86$) between the mean cell

112   area and the mean nucleoid area of untreated cells across the tested 30 growth conditions (Figure

113   1C). This relationship was not perfectly linear, as the average NC ratio slightly decreased with

114   increasing average cell size (Figure 1C, inset). This small decrease, which will be addressed later,

115   was not a consequence of differences in growth medium osmolality (Figure S1C), which can cause

116   variations in nucleoid morphology (Cagliero and Jin, 2013).

117

Nucleoid size scaling is independent of DNA replication

119   We next investigated whether changes in DNA content underlie the scaling of nucleoid size with

120   cell size by using nutrient-poor growth conditions. In such environments, *E. coli* cells display

121   discrete cell cycle periods, known as the B, C, and D periods, corresponding to cell-cycle phases

122   before, during, and after DNA replication, respectively (Cooper and Helmstetter, 1968). If DNA

123   replication was solely responsible for nucleoid size scaling, we would expect to observe a

124   correlation between nucleoid and cell size only during the C period, and not during the B and D

125   periods when the DNA amount does not change. As cell size and the DAPI signal intensity did not

126   provide sufficient resolution to distinguish between cells in the B, C, and D periods (Figure S4A),

127   we used a strain producing a SeqA-mCherry fusion. SeqA associates with newly replicated DNA

128   by transiently binding hemi-methylated GATC sites (Brendler et al., 1995; Lu et al., 1994; Slater

129   et al., 1995). When fluorescently tagged, SeqA forms bright fluorescent foci that trail the

130   replication forks during DNA replication (C period). In the absence of DNA replication (B and D

131   periods), SeqA-mCherry displays diffuse nucleoid-associated fluorescence (Adiciptaningrum et

132   al., 2015; Helgesen et al., 2015; Molina and Skarstad, 2004; Wallden et al., 2016) (Figure 2A). By

133   quantifying the relative area of the SeqA-mCherry signal and combining this information with cell

134   area measurements, we were able to identify three distinct groups of cells—corresponding to the

135   B, C, and D cell cycle periods—in populations growing under various nutrient-poor conditions

136   (Figure 2A and Figure S4B-D). Surprisingly, we found a strong correlation of nucleoid area with

137   cell area for all three periods (Figure 2B and Figure S4D). The correlations and slopes were the

138   strongest in the C period under all 11 tested nutrient-poor conditions, but both remained

139   significant during the B and D periods (Figure 2B and Figure S4B-C). Apart from these small

140   differences between cell cycle periods, we observed similar average NC ratios for each growth

4

141   condition (Figure S4B). These results indicate that the scaling between nucleoid and cell sizes

142   occurs independently of DNA replication.

143

144   To confirm this unexpected conclusion, we used temperature-sensitive *dnaC2* mutant cells

145   producing an HU-mCherry fusion to visualize the nucleoids. At restrictive temperatures, these

146   cells are unable to initiate new rounds of DNA replication, but continue to grow without dividing

147   (Carl, 1970). We found that 90 min after the temperature shift to 37 °C, the average cell size of

148   the population began increasing at which time we measured the size of both cells and nucleoids

149   at regular intervals for 210 min. Remarkably, the size of the nucleoid increased with cell size over

150   an almost 4-fold range before reaching a plateau in long cells (Figure 2C-D). Before reaching this

151   limit, the scaling relationship in the absence of DNA replication was similar to that observed

152   under the permissive temperature (30 °C) when DNA replication occurs (Figure 2C-D). Together,

153   these observations demonstrate that nucleoid size scaling occurs irrespective of changes in DNA

154   content.

155

156   **The nucleoid size scaling property is conserved in *Caulobacter crescentus*, but with**

157   **a different NC ratio**

158   To examine whether a scaling relationship between nucleoid and cell size is observed in other

159   bacteria, we imaged DAPI-stained *C. crescentus* cells expressing CFP-labeled DnaN. DnaN is the β

160   sliding clamp of the DNA polymerase, which, when fluorescently labeled, forms foci during DNA

161   replication but otherwise displays a disperse distribution (Arias-Cartin et al., 2017; Collier and

162   Shapiro, 2009; Fernandez-Fernandez et al., 2013). By quantifying the signal area of DnaN-CFP, we

163   were able to readily identify cells in distinct cell-cycle periods (Figure 3A). As with *E. coli* (Figure

164   2B), we observed a strong scaling relationship between nucleoid size and cell size in cells in the

165   B and D periods (Figure 3B), indicating that nucleoid size scaling occurs even in the absence of

166   DNA replication. As in *E. coli*, nucleoid size determination in *C. crescentus* was independent of

167   DAPI signal intensity (Figure S5A), and insensitive to the nucleoid labeling method (Figure S5B).

168   Scaling was maintained in defined (M2G) and complex (PYE) growth media (Figure S5C) as well

169   as in mutants with altered cell sizes and morphologies (Figure S5D-E), such as FtsZ-depleted,

170   Δ*rodZ* and Δ*hfq* cells (Alyahya et al., 2009; Irnov et al., 2017; Wang et al., 2001).

171

172   Nucleoid size scaled with cell size in both *E. coli* and *C. crescentus*. However, their NC ratios were

173   very different (Figure 3C). This is consistent with observations that the nucleoid spreads through

174   most of the cell in *C. crescentus* whereas *E. coli* displays DNA-free regions (Jensen and Shapiro,

175   1999; Kellenberger et al., 1958). The large NC ratio in *C. crescentus* was not due to PopZ-mediated

176   attachment of the chromosome to the cell poles (Bowman et al., 2008; Ebersbach et al., 2008), as

177   it was maintained in the Δ*popZ* mutant (Figure S5E-F).

178

Nucleoid size scaling across bacterial phyla reveals a continuum of NC ratios

180   The scaling relationship between nucleoid and cell sizes is likely a common bacterial feature, as

181   we observed it in over 35 species from different phyla or classes (Figure 4A and Figure S6A). Each

182   species investigated displayed a constant, specific NC ratio (Figure 4B). To avoid measurement

183   biases, we used the same Oufti parameters to identify the nucleoid contour of all cells in this

184   dataset. As with *E. coli* and *C. crescentus*, we confirmed that the NC ratio was not affected by the

185   intensity of the DNA signal (Figure S7A-B). We also observed no correlation between the average

186   DNA signal intensity and the average NC ratio (Figure S7C). The various species were generally

187   grown in complex media described in the literature or recommended by the provider. In some

188   cases, we examined different growth conditions. For example, we imaged *Bacteroides*

189   *thetaiotaomicron* (*B. theta*) grown *in vitro* in both complex (TYG) and defined (GMM) media, or *in*

190   *vivo* in mono-associated gnotobiotic mice. For the latter, the samples were obtained from the

191   cecum and feces. These different growth conditions revealed differences in cell sizes but, in all

192   cases, nucleoid size scaled with cell size at the single-cell level (Figure 4A).

193

194   The name "nucleoid" (nucleus-like) comes from the early observation that the bacterial

195   chromosome occupies a distinct intracellular region (Kellenberger et al., 1958; Mason and

196   Powelson, 1956), as exemplified by the organization of the γ-proteobacterium *E. coli* (Figure 1A).

197   The near-cell-filling organization of the chromosome in the α-proteobacterium *C. crescentus* is

198   usually ignored or thought of as an exception (Campos and Jacobs-Wagner, 2013; Surovtsev and

199   Jacobs-Wagner, 2018). Analysis of the average NC ratios of our panel of diverse species revealed

200   that high average NC ratios, i.e., near-cell-filling nucleoids, can be found not only in other α-

201   proteobacteria but also in some Bacteroidetes (Figure 4B). Furthermore, there was no

202   subdivision of the analyzed bacteria into discrete lower and higher NC ratio categories. Instead,

203   we observed a continuum of average NC ratios across species (Figure 4B).

204

205   While sorting species based on their average NC ratios revealed some phylogenetic clustering

206   (Figure 4B), phylum association was not necessarily predictive of NC ratio. For example, α-

207   proteobacteria generally had a higher NC ratio than proteobacteria from the β, γ, or δ classes

208   (Figure 4B). Bacteroidetes provided a striking example of distinct chromosome organization

209   within a phylum. *Cytophaga hutchinsonii* displayed a high NC ratio, characteristic of cell-filling

210   DNA, whereas *Parabacteroides distasonis* exhibited a considerably lower NC ratio and clear DNA-

211   free regions (Figure 4B and Figure S6A). These results indicate that the intracellular organization

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212   of the chromosome is an evolvable feature that varies significantly between species without strict

213   phylogenetic determinants.

214

The average NC ratio negatively correlates with the average cell size

216   Given this surprisingly large spectrum of average NC ratios among bacteria, we wondered

217   whether certain cellular characteristics are associated with a given NC ratio. We found no

218   correlation between genome size and average NC ratio (or average nucleoid area, or cell volume),

219   despite a ~3-fold difference in genome size between the included species (Figure S6B). Growth

220   rate was also a poor predictor of NC ratios. Fast-growing species such as *E. coli* (in LB), *Bacillus*

221   *subtilis* (in LB) and *B. theta* (in TYG medium), which have doubling times of ~20 to ~30 min at 37

222   °C (Eley et al., 1985; Taheri-Araghi et al., 2015; Weart et al., 2007) displayed a wide range of NC

223   ratios, whereas the NC ratio of the slower-growing *Myxococcus xanthus* (in CYE medium), which

224   has a doubling time of ~4 h (Sun et al., 1999), was similar to that of *E. coli* growing in LB.

225

226   We did, however, observe a striking, seemingly exponential relationship between the average cell

227   volume and the average NC ratio of bacteria (Figure 5A). The exponential relationship was

228   particularly apparent upon plotting the mean NC ratio versus the logarithm of the mean cell

229   volume (Figure 5A, inset), with a Kendall correlation τ = -0.70. This strong correlation indicates

230   that the average cell volume of a species is highly predictive of the average NC ratio. We also

231   observed strong relationships between other morphological descriptors and the NC ratio (Figure

232   5B). Further underscoring the validity of these relationships is the fact that the *E. coli* data from

233   cultures grown under 30 different nutrient conditions (Figure 1C) overlapped almost perfectly

234   with the curve obtained with the different bacterial species (Figure 5A, inset). The negative

235   relationship between average NC ratio and average cell size observed in these experiments

236   (Figure 1C) thus appears to be a consequence of the more general relationship between these two

237   cellular characteristics.

238

The cytoplasm of bacteria with different NC ratios displays different biophysical
properties

241   What are the physiological implications of a high or low NC ratio? We speculated that DNA might

242   affect the dynamics, and thereby the organization, of large cellular components whose diffusion

243   may be impeded by the DNA meshwork. In bacteria with low NC ratios like *E. coli*, large objects

244   may be able to more freely diffuse in DNA-free regions. In contrast, motion may be limited in

245   bacteria with high NC ratios like *C. crescentus* where the DNA spreads throughout most of the

246   cytoplasm. To test this idea, we conducted experiments using genetically-encoded GFP-μNS

247   particles expressed in *E. coli* and *C. crescentus*. We previously showed that GFP-μNS particles are

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248   useful to probe the biophysical properties of the bacterial cytoplasm (Parry et al., 2014). These
249   probes derive from a mammalian reovirus protein that assembles into spherical objects
250   (Broering et al., 2005; Broering et al., 2002). Once fused to GFP, they form fluorescent particles
251   that increase in signal intensity and absolute size with increased GFP-μNS synthesis (Parry et al.,
252   2014). We tracked GFP-μNS particles from three bins of particles of similar intensity (and,
253   consequently, size) in both *E. coli* and *C. crescentus* growing at a similar rate (Figure 6A and Movie
254   S1-2). Comparison of the ensemble-averaged mean squared displacements (MSDs) for particles
255   belonging to these bins revealed drastic differences in probe dynamics between the two species
256   (Figure 6B). GFP-μNS particles in *C. crescentus*, independent of their size range, displayed
257   significantly lower mobility than in *E. coli* (Figure 6B)*.* Diffusion measurements of free GFP are
258   similar in these two species (Elowitz et al., 1999; Montero Llopis et al., 2012), indicating that a
259   difference in cytoplasmic viscosity cannot explain these observations. Instead, these observations
260   support the notion that different NC ratios can lead to different biophysical properties of the
261   cytoplasm that affect the mobility of large cytoplasmic objects.
262
263   **Ribosome dynamics differ in bacteria with different NC ratio**
264   What large cytoplasmic components may be impacted by differences in NC ratio? Under the
265   conditions we used, GFP-μNS particles have reported sizes between 50 and 200 nm (Parry et al.,
266   2014), a similar size range as polysomes (Brandt et al., 2009), which are mRNAs loaded with
267   multiple ribosomes (Miller et al., 1970; Warner et al., 1962). If polysome mobility is impacted by
268   the DNA meshwork and the fraction of cellular space it occupies, it may explain a currently
269   unresolved discrepancy in mRNA localization in the literature. Fluorescence *in situ* hybridization
270   (FISH) microscopy experiments on several mRNAs in *C. crescentus* suggest that these mRNAs
271   remain close to their corresponding gene loci throughout most of their lifetime (Montero Llopis
272   et al., 2010). In contrast, a genome-wide FISH study in *E. coli* reveals no spatial enrichment of
273   mRNAs near the corresponding chromosomal regions (Moffitt et al., 2016). Because translation
274   starts on nascent mRNAs, polysomes are expected to form within the nucleoid. However, in *E. coli*,
275   the low NC ratio creates DNA-free regions in which polysomes can more freely diffuse once they
276   escape the DNA meshwork, leading to their dispersion. Conversely, the high NC ratio of *C.*
277   *crescentus* would prevent the escape of polysomes from the DNA meshwork.
278
279   To test this hypothesis, we used photoactivated localization microscopy to track ribosomes in
280   both *E. coli* and *C. crescentus*. In *E. coli*, we labeled ribosomes using a fusion of ribosomal subunit
281   protein S22 with mEos2 (Wang et al., 2011). In *C. crescentus*, we tracked L1-Dendra2-tagged
282   ribosomes (Lim et al., 2014). In both cases, the fusion replaced the wild-type copy of the
283   ribosomal gene at its native chromosomal locus (Lim et al., 2014; Wang et al., 2011). Importantly,

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284 we acquired data at five different frame intervals (between 5 and 100 ms) and constructed
285 ensemble MSDs for each frame interval (> 8900 trajectories per frame interval, Figure 6C and
286 Movie S3-4). We reasoned that polysomes diffusing in a DNA meshwork may experience caging
287 and uncaging behaviors, as observed for probes diffusing in gels (Brangwynne et al., 2009; Cai et
288 al., 2011; Guo et al., 2014; Wong et al., 2004). Tracking at multiple timescales may reveal such
289 non-linear dynamics in MSDs. As the majority of ribosomes (~75-80%) are engaged in translation
290 in both organisms (Forchhammer and Lindahl, 1971; Lin et al., 2004; Montero Llopis et al., 2012;
291 Phillips et al., 1969; Varricchio and Monier, 1971), most of our trajectories likely reflected
292 polysome dynamics.

293

294 The MSDs indeed revealed non-linear dynamics, with polysomes in *C. crescentus* displaying lower
295 mobility than those in *E. coli*, especially at the longer (subsecond) timescales (Figure 6C). The
296 difference in MSDs was not due to polysomes "experiencing" cell membrane confinement sooner
297 in *C. crescentus* because of its smaller size than *E. coli*, as higher MSD values were obtained in both
298 organisms following treatment with the transcription initiation inhibitor rifampicin (Figure 6D).
299 Rifampicin treatment results in mRNA depletion, thus converting all polysomes into smaller, and
300 therefore faster, free ribosomes (Blundell and Wild, 1971) that explore more cellular space in the
301 same amount of time (Figure 6D). This finding demonstrates that at the subsecond timescale, cell
302 size does not limit polysome mobility in either organism, and that cell confinement is not
303 responsible for the observed mobility difference between the two species.

304

305 The non-linear dynamics of ribosomes became particularly apparent when we calculated the
306 apparent diffusion coefficient (D_a) and the anomalous exponents (α) from the MSDs. The value
307 for D_a is commonly extracted from the slope of the first few time lags of the MSD curve using the
308 equation $MSD = 4D_a t$ (Michalet, 2010). Anomalous exponents were obtained from the slope of the
309 first three points of the MSD vs. time (as anomalous diffusion in the cytoplasm is characterized
310 by a power law scaling: $MSD(t) \propto t^\alpha$ (Bouchaud and Georges, 1990)). Generally, in biological
311 studies, D_a and α are assumed to be constant over time, such that most single-molecule tracking
312 experiments are done using only a single time frame. However, our analysis revealed a striking
313 dependency of D_a and α on the timescales at which the measurements were made (Figure 6E-F).
314 In both organisms, D_a decreased with longer timescales, while α increased. Furthermore, the
315 difference in D_a between *E. coli* and *C. crescentus* increased with increasing time intervals (Figure
316 6E), and the α value was consistently lower for ribosomes in *C. crescentus* than in *E. coli* (Figure
317 6F). These differences indicate that ribosomes, the majority of which is contained within
318 polysomes, are much more confined in the high NC ratio bacterium *C. crescentus* than in the low
319 NC ratio bacterium *E. coli*.

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320

321 In single-molecule tracking experiments, the frame rate is usually under 100 ms to ensure
322 accurate localization determination. However, the lifetime of most bacterial mRNAs is on the
323 minute timescale (Chen et al., 2015; Kristoffersen et al., 2012; Moffitt et al., 2016; Redon et al.,
324 2005). Given the time-dependency of ribosome dynamics, we anticipated that the difference in
325 spatial exploration of ribosomes between *E. coli* and *C. crescentus* would be even more apparent
326 at the physiologically relevant timescale of minutes. This is indeed what we observed in
327 fluorescence recovery after photobleaching (FRAP) microscopy experiments (Figure 6G). To
328 minimize the effects of cell geometry and photobleaching location on the observed fluorescence
329 recovery, we used filamentous cells that were unable to divide due to cephalexin treatment (*E.*
330 *coli*) or FtsZ depletion (*C. crescentus*), as routinely done (Elowitz et al., 1999; Montero Llopis et
331 al., 2012). In these filamentous cells, the NC ratio remained the same as in normal sized cells
332 (Figure S3D and S5D-E). Ribosomes were labeled using a RplA-GFP fusion in both species. RplA
333 is the 50S ribosomal subunit protein L1, previously used to examine ribosome localization in *B.*
334 *subtilis* (Mascarenhas et al., 2001). Due to the heterogenous distribution of the ribosomal signal
335 in *E. coli*, we were unable to quantify ribosome mobility with a simple one or two-state diffusion
336 model, but we did observe clear qualitative differences in ribosomal recovery between the two
337 species (Figure 6G, Movie S5-6). *E. coli* cells showed nearly complete fluorescence recovery at the
338 photobleaching location after 450 s while *C. crescentus* cells often recovered less than 20% of
339 their prebleached fluorescence intensity.

340

341 **Intracellular organization of translation is associated with the NC ratio and cell size**
342 The decreased mobility of polysomes in *C. crescentus* is consistent with the notion that the cell-
343 filling nucleoid impedes polysome motion in this species. In *E. coli*, on the other hand, polysomes
344 display higher mobility likely because they can diffuse more freely and accumulate in DNA-free
345 regions once they escape the DNA meshwork. This raises the intriguing possibility that the
346 difference in NC ratio and its impact on ribosome mobility contribute to the striking difference in
347 spatial organization of ribosomes and thus translation between these two organisms. In *E. coli*, as
348 in other bacteria with low NC ratios like *B. subtilis* and *Lactococcus lactis*, ribosomes are enriched
349 in the nucleoid-free regions of the cytoplasm (Azam et al., 2000; Bakshi et al., 2012; Lewis et al.,
350 2000; Robinow and Kellenberger, 1994; van Gijtenbeek et al., 2016), resulting in partial
351 segregation of transcription and translation. In *C. crescentus* and *Sinorhizobium meliloti*, two
352 bacteria with high NC ratios, a large physical separation of ribosomes and DNA is not observed,
353 as both are found throughout the cytoplasm (Bayas et al., 2018; Montero Llopis et al., 2010).

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355   If the NC ratio does indeed affect the spatial organization of translation, we may expect to already

356   see changes in ribosome localization in *E. coli* cells grown in different nutritional environments

357   that lead to small variations in NC ratios (Figure 1C, inset). To test this expectation, we used an *E.*

358   *coli* strain carrying a mEos2 fusion to a ribosomal protein (Sanamrad et al., 2014) and grew this

359   strain under 12 growth conditions that result in slightly varying NC ratios. Although nucleoid

360   exclusion of ribosomes was observed for each growth condition, the exclusion was more

361   pronounced in cells with smaller average NC ratios. This is exemplified in Figure 7A showing a

362   comparison between cells in a nutrient-rich medium (M9gluCAAT, average NC ratio = 0.53) and

363   cells in nutrient-poor medium (M9gly, average NC ratio = 0.58). We quantified the average

364   nucleoid exclusion of ribosomes by calculating the signal correlation factor (SCF), a metric that

365   measures the correlation between two fluorescent signals (see STARS Method). An SCF of 1, 0 and

366   -1 indicates that the two signals display perfect co-localization, independent localization and

367   exclusion, respectively. We restricted the calculation of SCF to a specific "correlation area" within

368   individual cells (Figure S8A) to minimize the effects of cell size and geometry on the correlation

369   (see STAR methods). This quantification across 12 growth media with varying NC ratios

370   confirmed the gradual increase in ribosome exclusion with decreasing NC ratio (increasing

371   average cell size), as evidenced by the more negative average SCF values (Figure 7B).

372

373   Given the continuum of NC ratios among diverse species (Figure 4B), we may also expect to see

374   differences in ribosome localization among species with varying NC ratios. To examine this

375   possibility, we performed fluorescence *in situ* hybridization (FISH) microscopy on 10 different

376   species using a Cy5-labeled EUB338 probe complementary to the 5' domain of 16S rRNA (Amann

377   et al., 1990). This probe is complementary to the majority of eubacterial species sequenced and

378   provides a method to visualize bulk ribosome localization in diverse species. As a control, we first

379   performed SCF quantification for an *E. coli* strain producing fluorescently labeled ribosomes. This

380   test revealed that cell fixation, a necessary step of the FISH procedure, slightly affects ribosome

381   and DNA localization, thereby artificially increasing the SCF value (Figure S8B). Despite this

382   caveat, we still observed nucleoid exclusion of ribosomes and strong colocalization between the

383   ribosome signals obtained from the fluorescent labeling (using RlpA-GFP) and the FISH

384   procedure (using Cy5-EUB338) at the single-cell and population levels (Figure 7D-E), validating

385   our FISH method. For the 10 species tested, we found that the SCF obtained by FISH correlates

386   with their average NC ratio (Figure 7F). Given the spectrum of NC ratios among diverse species

387   (Figure 4B) and the correlation between the average NC ratio and the average cell size (Figure 5),

388   our results also suggest a relationship between nucleoid exclusion of ribosomes and average cell

389   size. Indeed, we found a strong negative correlation (τ = -0.82) between the average SCF and the

390   average cell size across the tested species (Figure 7G). In other words, the bigger the average size

11

391   of the species, the smaller its average NC ratio and the more ribosomes were excluded from the

392   nucleoid. Altogether, our findings suggest a continuum of ribosome organization across bacteria

393   and identify the average NC ratio and cell size of a species in a given growth medium as good

394   predictors of how this bacterium spatially organizes translation.

395

Discussion

397   Although the first reports of scaling relationships in eukaryotes between the size of subcellular

398   components and that of the cell date back more than 100 years (Conklin, 1912; Marshall, 2015;

399   Wilson, 1925; Woodruff, 1913), this phenomenon has remained largely unexplored in bacteria.

400   Here, we demonstrate that nucleoid size strongly scales with cell size in exponentially growing

401   cultures across a wide range of cell sizes and a diverse panel of bacterial species (Figures 1, 2, 3,

402   4, S1, S3D, S4, S5 and S6). Despite the apparently conserved nature of nucleoid size scaling, we

403   found a continuum of NC ratios across species (Figure 4B), which can be predicted from the

404   average cell size of the bacterial population (Figure 5). We highlight important biological

405   implications of having a different NC ratio for the mobility and localization of larger particles such

406   as polysomes (Figure 6), thereby implicating the NC ratio as an important determinant of the

407   intracellular organization of bacterial translation (Figure 7).

408

409   Using the model bacteria *E. coli* and *C. crescentus*, we show that the scaling of nucleoid size with

410   cell size occurs in the absence of changes in DNA content (Figure 2B-D and 3B). This is in line with

411   findings in yeast cells in which increases in the amount of DNA do not directly lead to increases

412   in nuclear size (Jorgensen et al., 2007; Neumann and Nurse, 2007). In eukaryotes, nuclear

413   structural components, nucleocytoplasmic transport and nuclear envelope expansion have all

414   been implicated in regulating nuclear size (Hara and Merten, 2015; Jevtic et al., 2014; Kume et al.,

415   2017; Levy and Heald, 2010). The fact that the scaling property extends to bacteria, which lack a

416   nuclear envelope, makes it even more remarkable. It highlights an intrinsic property of the DNA

417   and the cell that predates the development of membrane-enclosed organelles such as the nucleus.

418   The fact that it arises regardless of the way the genome is packaged into the cell (independently

419   of nuclear membrane or histones) suggests that it is an ancient and basic cellular feature.

420

421   Although nucleoid size scaling is widespread among bacteria, the resulting NC ratios vary

422   considerably (Figure 4B). Here again, this is similar to what is observed in eukaryotes where the

423   NC ratio varies greatly among cell types (Ganguly et al., 2016; Jevtic and Levy, 2015; Jorgensen et

424   al., 2007; Kume et al., 2017; Neumann and Nurse, 2007; Novakova et al., 2016; Su Lim et al., 2015).

425   We found no link between NC ratio and chromosome size or growth rate of a given species (Figure

426   S6). Instead, we discovered a remarkable relationship between the average cell size of a

12

427   population and its average NC ratio, as the latter strongly correlated with morphological metrics
428   that reflect average cell size (i.e., average cell volume, length, width, area and surface area to
429   volume ratio) (Figure 5). Although the relationship is strongest for the average cell volume, the
430   strong correlations with other size-related variables currently preclude us from associating the
431   NC ratio with a specific morphological feature. It is important to note that while the relationship
432   between average cell size and average NC ratio has predictive value at the population level, it does
433   not extend to the single-cell level. This is evident from the maintenance of the NC ratio over the
434   course of a cell cycle (Figure 1) and is further exemplified by the fact that an overlap in cell size
435   between *C. crescentus* and *E. coli* does not lead to an overlap in NC ratio at the single-cell level
436   (Figure 3C). These findings indicate that although a general relationship between average cell size
437   and the NC ratio exists, the latter is controlled by factors other than cell size at the single-cell level.
438
439   Differences in NC ratio among species and across growth conditions (Figures 1C and 4B) have
440   physiological implications. By comparing the motion of ribosomes in *E. coli* and *C. crescentus*, we
441   found that their mobility is significantly decreased in cells with high NC ratios. Given that
442   cytoplasmic viscosity is similar in *E. coli* and *C. crescentus* based on GFP diffusion measurements
443   (Elowitz et al., 1999; Montero Llopis et al., 2012), this reduction likely arises because the diffusion
444   of polysomes is impeded by the DNA meshwork. This difference was most pronounced on longer
445   timescales due to the time-dependent properties of ribosome movement (both in terms of D_a and
446   α). These non-linear dynamics of polysomes inside the bacterial cytoplasm reveal that the DNA
447   affects the biophysical properties of the bacterial cytoplasm. Our data suggest that polysomes and
448   other similarly sized objects experience local caging when they encounter the DNA mesh. An
449   implication of such non-linear dynamics is that direct comparisons of diffusion coefficients
450   without considering physiologically relevant timescales and differences in α can be misleading.
451   For example, at short frame rates, polysomes may not diffuse far enough to be "aware" that they
452   are within a DNA meshwork. As a result, the D_a values in *E. coli* and *C. crescentus* are relatively
453   close to each other, consistent with previous determinations (Bakshi et al., 2012; Bayas et al.,
454   2018; Sanamrad et al., 2014). This could lead to the interpretation that ribosome dynamics are
455   the same in these organisms. We show that this is true only at the millisecond timescale, a
456   timescale at which polysomes primarily experience protein crowding, which is similar in the two
457   species (Elowitz et al., 1999; Montero Llopis et al., 2012). As the timescale increases, the D_a values
458   decrease as polysomes increasingly experience the DNA mesh. This highlights the non-linear
459   biophysical properties of the bacterial cytoplasm and stresses the importance of making diffusion
460   measurements at different length and time scales.
461

13

462   The decrease in D_a values over time is most dramatic in *C. crescentus* (Figure 5E) where, unlike in
463   *E. coli*, polysomes cannot escape the DNA meshwork because it fills most of the cell. By
464   themselves, the differences in ribosome mobility in *E. coli* and *C. crescentus* could be attributed to
465   other factors (e.g., fraction of nascent vs. mature mRNAs) than a difference in NC ratio between
466   the two species. However, the decreased mobility of genetically encoded GFP-µNS particles in *C.*
467   *crescentus* (in comparison to *E. coli*) supports our interpretation that different NC ratios give rise
468   to different physical properties of the cytoplasm and have widespread implications for larger
469   cellular components and their associated processes. The reduction of polysome mobility in *C.*
470   *crescentus* explains why mRNAs remain in close proximity to their corresponding gene loci in this
471   organism (Montero Llopis et al., 2010). In *E. coli*, on the other hand, polysomes would be able to
472   escape the nucleoid due to the lower NC ratio, after which their increased mobility would lead to
473   a more dispersed mRNA localization, as recently shown (Moffitt et al., 2016). Based on this
474   interpretation, we anticipate that the NC ratio of a given bacterium, together with the lifetime of
475   the mRNA, will dictate whether protein synthesis from this mRNA primarily occurs near the gene
476   locus where the mRNA was transcribed, or away from it.

477

478   In eukaryotes, the term cytosol is used to designate the part of the cytoplasm that is not held by
479   organelles. We propose that a similar distinction can be made in bacteria. Even without a
480   membrane-enforced separation, the nucleoid (organelle) provides a distinct biophysical
481   environment from the DNA-free region of the cytoplasm (cytosol). The spectrum of NC ratios
482   across species and growth conditions suggests that the cytosolic fraction of a bacterial cell is far
483   from fixed, and is instead an evolvable feature (Figure 4B). Although the NC ratio depends on the
484   growth conditions for a given species, the actual fluctuations between conditions are small in
485   comparison to the entire spectrum observed across species (Figures 1C and 5B). This observation
486   may reflect unappreciated evolutionary constraints on intracellular organization and cell size for
487   a given bacterial species.

488

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Author contributions

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505 Investigation, W.T.G. and S.K.G.; Data Curation, W.T.G., S.K.G., Y.X. and B.R.P.; Writing – Original
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Declaration of Interests

511 The authors declare no competing interests.
512

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844 **Figure legends**

845 **Figure 1. Nucleoid size scaling is robust across a wide range of *E. coli* cell sizes.**

846 A. Phase contrast and DAPI images of *E. coli* cells (CJW6324) grown in liquid cultures of M9

847 medium supplemented with 0.2% mannose (M9mann) or 0.2% glucose, 0.1% casamino acids and

848 1µg/ml thiamine (M9gluCAAT) at 37 °C. The images were processed with Oufti to identify the

849 contours of the cells (green) and nucleoids (purple, insets).

850 B. Density contour plots showing the strong correlation between cell area and nucleoid area for

851 individual CJW6324 cells grown in the indicated growth media (for a full description of the

852 growth media, see Table S1). The contour lines represent the 0.10, 0.25, 0.50 and 0.75 probability

853 envelopes of the data.

854   C. Scatter plot of the average cell area versus the average nucleoid area for the indicated growth

855   conditions. Inset: scatter plot of the average cell area versus the average NC ratio for the same

856   growth conditions. Error bars indicate 95% confidence intervals.

857   See also Figures S1-3.

858

859   **Figure 2. Nucleoid size scaling with cell size does not depend on DNA replication.**

860   A. Density scatter plot (left) and density contour plot (right) of cell area versus the relative SeqA-

861   mCherry signal area of *E. coli* cells (CJW6324) grown in M9 medium supplemented with 0.2%

862   glycerol. The gray scale in the density scatter plot indicates the relative density of dots (cells) in

863   a given area of the chart. This plot was used to identify cells in the B, C and D cell cycle periods

864   found under these growth conditions. The contour lines represent the 0.10, 0.25, 0.50 and 0.75

865   probability envelopes of the data. Insets: representative images of the subcellular SeqA-mCherry

866   signal in a specific cell cycle period.

867   B. Density contour plots of cell area versus nucleoid area for cells in B, C and D periods based on

868   the analysis shown in A. The contour lines represent the 0.10, 0.25, 0.50 and 0.75 probability

869   envelopes of the data. The nucleoid was detected by DAPI staining. See also Figure S4.

870   C. Representative fluorescence images of *dnaC2* cells (CJW6370) producing HU-mCherry at

871   different time points after a shift to a restrictive temperature (37 °C).

872   D. Plot showing the average nucleoid area per cell area bin for HU-mCherry-labeled *dnaC2* cells

873   at 37 °C. Cells (n = 12268) from different time points following temperature shifts were combined

874   into one dataset and grouped into bins based on their cell areas. Shown are the average nucleoid

875   area and standard deviation (SD) of each cell area bin. The solid yellow line indicates the expected

876   relationship between nucleoid and cell area based on the scaling observed under permissive

877   conditions (30 °C). The dotted lines indicate the 99% confidence interval (CI) of the fit.

878

879   **Figure 3. Nucleoid size scaling is also observed in *C. crescentus*, a bacterium with a different**

880   **NC ratio.**

881   All contour lines represent the 0.10, 0.25, 0.50 and 0.75 probability envelopes of the data.

882   A. Density contour plot of cell area versus the relative DnaN-CFP signal area of *C. crescentus* cells

883   producing a DnaN-CFP fusion (CJW5969) and grown in M2 medium supplemented with 0.2%

884   glucose. This plot was used to identify cells in the B, C, and D cell cycle periods. Insets:

885   representative images of the subcellular DnaN-CFP signal in a specific cell cycle period.

886   B. Density contour plots of cell area versus nucleoid area for cells in panel A. The nucleoid was

887   detected by HU-mCherry labeling.

888   C. Density contour plots of cell area versus NC ratio for *E. coli* (CJW6324) and *C. crescentus*

889   (CJW5969) cells grown in M9 medium supplemented with 0.2% glycerol and M2 medium

890   supplemented with 0.2% glucose, respectively. The nucleoid was detected by DAPI staining for *E.*

891   *coli* and by HU-mCherry labeling for *C. crescentus.*

892   See also Figure S5.

893

894   **Figure 4. Nucleoid size scaling across bacterial species from different phyla reveals a**

895   **continuum of NC ratios.**

896   A. Density contour plots of cell area versus nucleoid area for fixed cell populations from different

897   bacterial species. The contour lines represent the 0.10, 0.25, 0.50 and 0.75 probability envelopes

898   of the data. When different growth conditions were examined for the same species, the growth

899   medium is indicated next to the species name. Contours of the same color indicate affiliation to

900   the same phylum or class. The DNA dye used for nucleoid labeling for each species is detailed in

901   the STAR methods.

902   B. Average NC ratio (with error bars representing the standard deviation) for all the included

903   species.

904   See also Figures S6-7.

905

906   **Figure 5. The average NC ratio is linked to the average cell size.**

907   For all plots, error bars indicate 95% confidence intervals.

908   A. Scatter plot of average cell volume versus average NC ratio for all the included species.

909   Abbreviated species names are indicated next to the corresponding datapoint; see Key Resources

910   Table for a full name description. Inset: same relationship with average cell volume on a

911   logarithmic scale.

912   B. Scatter plot of average NC ratio versus average cell length, average cell width, average cell area

913   or average surface area to volume ratio.

914

915   **Figure 6. The intracellular mobility of large objects displays non-linear dynamics and is**

916   **different between *E. coli* and *C. crescentus*.**

917   *E. coli* cells (CJW6723) were grown in M9 medium supplemented with 0.2% glycerol and *C.*

918   *crescentus* cells (CJW6917) in M2 medium supplemented with 0.2% glucose, resulting in similar

919   doubling times of ~120 min.

920   A. Frequency distributions of GFP-μNS spot intensities in *E. coli* (n= 2279) and *C. crescentus* (n =

921   2019) cells. Three bins of GFP-μNS particles with similar intensities and thus sizes are indicated

922   in color.

923   B. Ensemble-averaged MSDs of GFP-μNS particles (belonging to the intensity bins highlighted in

924   panel A) in *E. coli* cells (n = 1208, 600 and 200 for bins 1, 2 and 3, respectively) and *C. crescentus*

925   cells (n = 837, 984 and 374 for bins 1, 2 and 3, respectively). Error bars indicate 95% confidence

926   intervals.

927   C. Ensemble-averaged MSDs of fluorescently-labeled ribosomes in *E. coli* (SX289) and *C.*

928   *crescentus* (CJW5156) at different acquisition frame intervals. For each frame interval, > 8900

929   trajectories were collected. Only the first four points of the MSDs are shown for each frame

930   interval. Error bars indicate 95% confidence intervals.

931   D. Same as panel C, except that ensemble-averaged MSDs from rifampicin-treated cells (200

932   µg/ml, 2 h) were added for comparison to the results shown in panel A. For each frame interval,

933   > 3700 trajectories were collected in rifampicin-treated cells. For these MSDs, only the first six

934   points are shown for the 5 ms and 30 ms frame intervals. The color scheme for the frame intervals

935   is the same is in C. Error bars indicate 95% confidence intervals.

936   E. Plot showing the apparent diffusion coefficients calculated from the aforementioned MSDs as

937   a function of the frame interval. Error bars indicate 95% confidence intervals.

938   F. Same as E, but for the anomalous exponent.

939   G. Representative plots showing the evolution of the ribosomal fluorescence recovery over time

940   (up to 450 s) along the length of a cephalexin-treated *E. coli* cell (CJW4677) and of an FtsZ-

941   depleted *C. crescentus* cell (CJW3821) following photobleaching of about half of the cell. The

942   dotted line shows the fluorescence profile prior to bleaching.

943   See also Figure S8.

944

945   **Figure 7. The spatial organization of ribosomes in bacteria is linked to the average NC ratio**

946   **and cell size.**

947   A. Top, representative fluorescence images of *E. coli* cells (CJW6769) grown in M9 medium

948   supplemented with 0.2% glycerol (M9gly) or 0.2% glucose, 0.1% casamino acids and 1µg/ml

949   thiamine (M9gluCAAT). Bottom, fluorescence intensity profiles of DAPI and RpsB-mEos2 signals

950   for these cells.

951   B. Scatter plot of average SCF versus average NC ratio for *E. coli* cells (CJW6769) grown in the

952   indicated growth media (for a full description of the growth media, see Table S1). The SCF was

953   calculated by comparing the correlation between the DAPI and the RpsB-mEos2 signals for the

954   indicated species. Error bars indicate 95% confidence intervals.

955   C. Representative phase contrast and fluorescence images of *E. coli* cells (CJW4677) after the FISH

956   procedure, highlighting the correspondence between the use of RplA-GFP and FISH (targeting

957   16S ribosomal RNA with the Cy5-labeled EUB338 probe) for visualizing ribosome localization.

958   Cells were grown in M9 medium supplemented with 0.2% glycerol, 0.1% casamino acids, and

959   1µg/ml thiamine.

960 D. Fluorescence intensity profiles of DAPI, RplA-GFP, and rRNA FISH (Cy5-EUB338) signals of *E.*

961 *coli* cells (CJW4677) indicated in C, grown in M9 medium supplemented with 0.2% glycerol, 0.1%

962 casamino acids, and 1μg/ml thiamine.

963 E. Frequency distributions of SCF values between the rRNA FISH (Cy5-EUB338), RplA-GFP, and

964 DAPI signals.

965 F. Scatter plots of average SCF versus average NC ratio (left) or versus average cell area (right).

966 The SCF was calculated by comparing the correlation between the DAPI and the rRNA FISH (Cy5-

967 EUB338) signals for the indicated species. Error bars indicate 95% confidence intervals.

968 See also Figure S8.

969

970 ## STAR Methods

971

972 ### CONTACT FOR REAGENT AND RESOURCE SHARING

973 Further information and requests for resources and reagents should be directed to and will be

974 fulfilled by the Lead Contact, Christine Jacobs-Wagner (Christine.Jacobs-Wagner@yale.edu).

975

976 ### EXPERIMENTAL MODEL AND SUBJECT DETAILS

977 ***Bacterial strains and growth conditions***

978 Construction of strains and plasmids is detailed in Table S2.

979

980 To obtain steady-state growth conditions, cells were first inoculated in the appropriate growth

981 medium and grown to stationary phase in culture tubes. Cells were subsequently re-inoculated

982 into fresh medium by diluting them 1/10000 or more, and grown until they reached an optical

983 density at 600 nm (OD_{600}) of 0.1-0.3 (depending on the growth medium and organism) before

984 sampling for microscopy.

985

986 *E. coli* cells were grown in LB medium (30 °C), gut microbiota medium (GMM; 30 °C) (Goodman

987 et al., 2011) or M9 medium (37 °C) supplemented with 0.2% carbon source and, in certain

988 instances, with 0.1% casamino acids and 1 μg/ml thiamine (CAAT). *C. crescentus* cells were grown

989 at 30 °C either in PYE medium or M2G medium. *Sinorhizobium meliloti* (30 °C), *Psuedomonas*

990 *syringae* (30 °C), *Janthinobacterium lividum* (25 °C), and *Burkholderia thailandensis* (30 °C) were

991 grown in LB medium. *Rhizobium leguminosarum*, *Agrobacterium tumefaciens*, *Asticcacaulis*

992 *excentricus*, *Chryseobacterium indologenes*, *Brevundimonas subvibrioides*, *Brevundimonas*

993 *bacteroides,* and *Brevundimonas diminuta* were grown at 30 °C in PYE medium. *Vibrio harveyi* and

994 *Vibrio fischeri* were grown at 30 °C in LBS medium. *Myxococcus xanthus* and *Flavobacterium*

995 *johnsoniae* were grown at 30 °C in CYE medium. *Hirschia rosenbergii* was grown at 30 °C in marine

996 broth medium (Difco, Fisher Scientific). *Cellulophaga algicola* was grown at 30 °C in DSMZ

997 Medium 172. *Cytophaga hutchinsonii* was grown at 25 °C in CYE medium supplemented with 1%

998 glucose. *Bacillus subtilis*, *Bacillus megaterium*, *Lysinibacillus sphaericus,* and *Paenibacillus*

999 *polymyxa* were grown at 30 °C in nutrient broth medium. *Bacteroides ovatus*, *Bacteroides*

1000 *thetaiotaomicron*, *Bacteroides xylanisolvens*, *Parabacteroides distasonis*, *Chromobacterium*

1001 *violaceum*, *Providencia alcalifaciens*, *Roseburia intesinalis*, *Anaerostipes sp.*, *Clostridium boltae*,

1002 *Clostridium hathewayi*, *Lactoacillus reuteri,* and *Collinsella aerofaciens* were grown at 37 °C in

1003 GMM (Goodman et al., 2011). *B. theta* was also grown at 37 °C in TYG medium (Bacic and Smith,

1004 2008). Fixed *B. theta* cells isolated from the cecum and fecal matter of monocultured mice were a

1005 kind gift of the Andrew Goodman laboratory (Yale University). All cells that were grown in GMM

1006 or TYG medium were cultured anaerobically. The exact composition of all growth media is

1007 detailed in Table S1.

1008

1009 Cephalexin treatment of *E. coli* cells was performed by first growing the cells in the indicated

1010 growth medium as described above. Steady-state cultures were subsequently exposed to

1011 cephalexin (50 μg/ml) for a period of time corresponding to about two doublings of an unexposed

1012 population (1 to 6 h, depending on the growth medium) and then imaged.

1013

1014 For FtsZ depletion in *C. crescentus*, CJW3821 cells carrying *ftsZ* under the xylose-inducible

1015 promoter were grown to an OD_{660} of ~0.1 at 30 °C in PYE medium containing 0.3% xylose for

1016 proper FtsZ synthesis. Cells were then spun down (5000 x g for 5 min) and washed with fresh

1017 PYE containing no xylose. FtsZ depletion was then performed by growing cells in PYE at 30 °C for

1018 3-6 h.

1019

1020 **METHOD DETAILS**

1021

1022 ***Microscopy***

1023 Unless otherwise indicated, cells were imaged on agarose (1%) pads supplemented with the

1024 appropriate growth medium. For most experiments live cells were used, except for Figures 4 and

1025 5 for which cells were first fixed with 4% formaldehyde and for Figure 7C-F for which cells were

1026 fixed and permeabilized for FISH microscopy (see below).

1027

1028 Phase contrast and epifluorescence imaging was performed on a Nikon Ti-E microscope equipped

1029 with a 100X Plan Apo 1.45 NA phase contrast oil objective (Carl Zeiss), an Orca-Flash4.0 V2 142

1030 CMOS camera (Hamamatsu), and a Spectra X light engine (Lumencor). The microscope was

1031   controlled by the Nikon Elements software. The following Chroma filter sets were used to acquire

1032   fluorescence images: DAPI (excitation ET350/50x, dichroic T400lp, emission ET460/50m), CFP

1033   (excitation ET436/20x, dichroic T455lp, emission ET480/40m), GFP (excitation ET470/40x,

1034   dichroic T495lpxr, emission ET525/50m), YFP (excitation ET500/20x, dichroic T515lp, emission

1035   ET535/30m), mCherry/TexasRed (excitation ET560/40x, dichroic T585lpxr, emission

1036   ET630/75m) and Cy5.5 (excitation ET650/45x, dichroic T685lpxr, emission ET720/60m).

1037   Specialized microscopy setups used for FRAP experiments and single-molecule or single-particle

1038   tracking are detailed below.

1039

1040   ***GFP-µNS experiments***

1041   For GFP-µNS experiments in *E. coli*, we used a published protocol (Parry et al., 2014). Briefly, *E.*

1042   *coli* strain CJW6723 was grown at 30 °C in M9 medium supplemented with 0.2% glycerol to an

1043 OD_{600} = 0.05-0.1. The synthesis of GFP-µNS was induced by the addition of 200-500 µM IPTG for

1044   60-120 min. After induction, cells were spun down (5000 x g for 5 min) and washed with fresh

1045   M9 glycerol medium and grown for at least 60 min to allow for GFP maturation. For experiments

1046   in *C. crescentus*, strain CJW6723 was grown at 30 °C in M2 medium supplemented with 0.2%

1047   glucose to an OD_{660} = 0.05-0.1. GFP-µNS synthesis was induced by the addition of 0.3% xylose to

1048   the medium for 30-120 min. After induction, cells were spun down and washed with fresh M2G

1049   medium and grown for at least 60 min to allow for GFP maturation. Cells were then spotted on

1050   1.5% agarose pads containing M9 glycerol (*E. coli*) or M2G (*C. crescentus*) and imaged every 2 s at

1051   30 °C.

1052

1053   ***Photoactivated localization and single-molecule tracking experiments***

1054   For photoactivated localization microscopy and single-molecule (ribosome) tracking, cover slips

1055   and glass slides were washed in the following manner: sonication in 1 M KOH (15 min), sonication

1056   in milliQ H_2O (15 min) and sonication in 70% ethanol (15 min) with 3-5 milliQ H_2O rinses between

1057   wash solution changes. Cleaned glass slides and cover slips were then dried with pressured air.

1058   Cells were spotted on a 1.5% agarose pad made with M9 medium supplemented with 0.2%

1059   glycerol, 0.1% casamino acids, and 1 µg/ml thiamine for *E. coli* or M2 medium supplemented with

1060   0.2% glucose for *C. crescentus*. Imaging was performed with an objective heat ring set at 30 °C. All

1061   images were acquired on an N-STORM microscope (Nikon) equipped with a CFI Apo TIRF 100×

1062   oil immersion objective (NA 1.49), lasers (Agilent Technologies) emitting at 405 nm (0.1-10%)

1063   and 561 nm (15-100%), and a built-in Perfect Focus system. Raw single-molecule data were taken

1064   at a frame rate of 200 to 10 frames per second in a field of view of 36 × 36, 120 x 120, or 200 x

1065   200 pixels (depending on the frame rate) with an Andor iXon X3 DU 897 EM-CCD camera (Andor

1066   Technology). Rifampicin treatment was performed by exposing cells to 200 μg/ml (*E. coli*) or 50

1067   μg/ml (*C. crescentus*) rifampicin for 2 h in liquid culture before sampling and imaging.

1068

1069   ***Fluorescence recovery after photobleaching experiments***

1070   For the FRAP experiments, filamentous cells (generated either by a 2 h treatment with 50 μg/ml

1071   cephalexin for *E. coli* or a 3-6 h FtsZ depletion in *C. crescentus*) were spotted on 1.5% agarose pads

1072   with M9 medium supplemented with 0.2% glycerol, 0.1% casamino acids, and 1 μg/ml thiamine

1073   or PYE. Cells were imaged at room temperature (~22 °C) with a Nikon E80i microscope equipped

1074   with 100X Plan Apo 1.45 NA phase contrast objective and an Andor iXonEM+ DU-897 camera

1075   controlled by the Metamorph software. Fluorescence photobleaching was performed using a

1076   Photonic Instrument Micropoint laser system at 488 nm. Cells were imaged once before

1077   photobleaching, then bleached (for ~0.5 s), and subsequently imaged at equal intervals (3-6 s for

1078   450 s depending on whether *E. coli* or *C. crescentus* was imaged).

1079

1080   ***Fluorescence in situ hybridization experiments***

1081   For FISH experiments, *E. coli* cells were grown in LB medium at 30 °C, *C. crescentus* cells were

1082   grown in PYE medium at 30 °C, and the other bacterial species were grown as described above.

1083   FISH was performed similarly to previous methods described by our laboratory (Kim and Jacobs-

1084   Wagner, 2018; Montero Llopis et al., 2010). Briefly, exponentially growing cells (OD$_{600}$ < 0.3) were

1085   fixed in a 4% formaldehyde solution (4% formaldehyde, 30 mM NaHPO$_3$ pH 7.5) for 15 min at

1086   room temperature followed by 30 min on ice. The samples were spun down (5000 x g for 3 min)

1087   and washed in phosphate-buffered saline (PBS) treated with diethyl pyrocarbonate (DEPC) 3

1088   times. The cell pellets were resuspended in DEPC-treated PBS (8.0 g/l NaCl, 0.2 g/l KCl, 1.44 g/l

1089   Na$_2$HPO$_4$ and 0.24 g/l KH$_2$PO$_4$) and adhered to poly-L-lysine-coated coverslips. Cells were then

1090   lysed with 70% ethanol for 5 min at room temperature. Pre-hybridization was then performed

1091   with a 40% formamide, 2x saline-sodium citrate solution (SSC, 300 mM NaCl, 30 mM sodium

1092   citrate, pH 7.0) containing 0.2 mM vanadyl ribonucleoside complex (VRC) for 2 h at 37 °C.

1093   Immediately   afterwards,   hybridization   was   performed   with   EUB338   (5'-

1094   GCTGCCTCCCGTAGGAGT-3', 5'-monolabeled with Cy5) in a solution containing 4 nM EUB338,

1095   40% formamide, 2x SSC, 0.2 mM VRC, 10% dextran sulfate, 0.1% bovine serum albumin, and 0.4

1096   mg/ml *E. coli* tRNA. Hybridization proceeded for 16 h at 37 °C and was then washed 5 x with wash

1097   solution (50 % formamide, 2x SSC) and 10x with DEPC-treated PBS. Finally, 1 μg/ml DAPI was

1098   added to the coverslip, which was then mounted on a glass slide for imaging.

1099

1100

1101

1102 ***DNA dye labeling***

1103 For live cells, the nucleoid was visualized by incubating 1 µg/ml DAPI with cells in their growth

1104 medium for 10 min. Due to a lack of labeling efficiency with DAPI in live cells of some of the species

1105 that were studied, all species for Figures 4 and 5 (with the exception of *E. coli* in different

1106 conditions) were fixed with 4% formaldehyde for 15 min at room temperature and 30 min on ice.

1107 They were then washed three times with 1 x PBS and spun down at 7000 rpm. Fixed *A. excentricus,*

1108 *A. tumefaciens, B. subvibrioides, C. algicola, C. hutchinsonii, H. rosenbergii, B. bacteroides, B.*

1109 *diminuta, C. indologenes, F. johnsoniae, M. xanthus, P. syringae, R. leguminosarum, S. meliloti* and *C.*

1110 *crescentus* were stained with 1 x SYBR Green. Fixed *B. theta, B. ovatus, V. harveyi* and *P. distasonis*

1111 were stained with 1 µg/mL DAPI. Fixed *B. megaterium, C. violaceum, J. lividum, V. fischeri, B.*

1112 *subtilis, B. thailandensis, E. coli, Anaerostipes sp., B. xylanisolvens, C. aerofaciens, C. hathewayi, L.*

1113 *reuteri, P. alcalifaciens, R. intestinalis, C. boltae, L. sphaericus* and *P. polymyxa* were stained with 1

1114 µg/ml Hoechst 33342.

1115

1116 ***Image processing***

1117 Cell outlines were generated using the open-source image analysis software Oufti (Paintdakhi et

1118 al., 2016). Nucleoids were detected using Oufti's objectDetection module. For comparison

1119 purposes, we used the same nucleoid detection parameters for all image datasets (see

1120 Supplemental Information for parameters), with a single exception (see below). The resulting cell

1121 lists were further processed and analyzed in MATLAB (Mathworks) using custom-built

1122 algorithms (see below).

1123

1124 One experiment required optimizing our nucleoid detection pipeline. Elongation of *dnaC2* cells

1125 under restrictive conditions (related to Figure 2C-D) led to a decrease in the fraction of nucleoid-

1126 bound HU-mCherry signal. To overcome this, we used an adjusted nucleoid detection function:

1127 Nucleoid_Detection_High_Background.m that uses MATLAB built-in functions to threshold and

1128 identify nucleoids within Oufti cell meshes.

1129

1130 SeqA-mCherry signal information was added to *E. coli* cell lists using the MATLAB function

1131 Add_SeqA_Area.m (see Supplemental Information for the code). DnaN-CFP information was

1132 added to *C. crescentus* cell lists using the MATLAB function Add_DnaN_Area.m.

1133

1134 ***Support Vector Machine model for curation of cell contours***

1135 In similar fashion as before (Campos et al., 2018), we used an automated approach to identify

1136 poor and incorrect cell detections across our datasets. We trained a support vector machine

1137 (SVM) model based on 11 normalized phase-contrast features: cell length, cell volume, integrated

29

1138   phase signal, mean cell contour intensity, minimum cell contour intensity, maximum curvature of

1139   cell contour, minimum inflated cell contour intensity, mean intensity gradient across the cell

1140   contour, maximum variability in contour intensity, mean variability in contour intensity and

1141   maximum cell pixel intensity. We visually scored 20,265 cells and used 30% of them (6,080 cells)

1142   to train the SVM model. The model was evaluated using a k-fold cross-validation approach,

1143   leading to a generalized misclassification rate of 9.9%. We used the remaining 70% of the dataset

1144   (14,185 cells) to validate the model. The SVM classifier achieves a balanced classification rate of

1145   90.9% and features an AUROC of 0.9640.

1146

1147   The SVM model underperformed for species (e.g., *C. crescentus*) and mutants (e.g., *dnaC2* at the

1148   restrictive temperature) with morphologies that deviated significantly from *E. coli*'s typical rod

1149   shape. Therefore, in these instances, we resorted to visual inspection and curation of the obtained

1150   cell contours. For time-lapse experiments, visual inspection and manual curation of the cell

1151   contours was also required.

1152

1153   ***Growth rate measurements***

1154   Growth rates were measured in 96-well plates in a Synergy2 microplate reader (BioTek). Cultures

1155   were first grown to stationary phase and re-inoculated into 150 µl fresh medium (1/300).

1156   Cultures were subsequently grown for 60 h at 37 °C with OD_{600} measurements every 4 min. The

1157   maximal growth rate was extracted from the obtained growth curves by fitting the Gompertz

1158   function (Zwietering et al., 1990).

1159

1160   ***Osmolality measurements***

1161   Osmolality of growth media was measured using a Precision Systems 6002 Touch Micro

1162   OSMETTE™ osmometer, which uses the freezing point method for osmolality measurements. All

1163   measurements were conducted in duplicate.

1164

1165   **QUANTIFICATION AND STATISTICAL ANALYSIS**

1166   ***Cellular characteristics***

1167   Properties of individual cells (cell and nucleoid dimensions, DAPI fluorescence intensity,

1168   fluorescent marker behavior, etc.) were extracted from cell lists obtained from Oufti using the

1169   MATLAB function Extract_Cell_Properties.m. Morphological features (e.g., cell length, width, area,

1170   and volume) were determined by summing the dimensions of each individual segment of the cell

1171   mesh identified by Oufti. See https://oufti.org/ for more details.

1172

1173

30

1174   ***Correlation coefficients***

1175   Kendall correlation coefficients between variables were calculated using MATLAB's built-in corr

1176   function.

1177

1178   ***Unconstrained linear fits***

1179   Unconstrained linear fits were performed using MATLAB's built-in polyfit function.

1180

1181   ***Nucleoid exclusion of ribosomes***

1182   The extent of ribosome exclusion was determined by calculating the signal correlation factor

1183   (SCF) between the DNA and ribosome signals. For each individual cell, the SCF was calculated by

1184   examining a specific "correlation area", corresponding to an intracellular region determined by

1185   two user-specified parameters. The restriction of the calculation to this area was required to

1186   ensure optimal correlation calculations for cells with different shapes and sizes as the smaller

1187   cytoplasmic volume at the cell poles and the cell periphery leads to a general decrease in

1188   fluorescent signal which, in turn, artificially generates positive biases in the calculation of the SCF.

1189   The first parameter was the number of pixels, starting from the cell poles, to exclude from the

1190   calculation. The second parameter was the number of pixels, starting from the cell centerline, to

1191   include in the calculation. Together, these parameters defined the correlation region for which

1192   the correlation between pixel values was determined. Different combinations of these two

1193   parameters were scanned for each growth condition and species, and parameters were chosen

1194   by finding the minimal average SCF. The minimal average SCF was selected to avoid the positive

1195   SCF biases introduced by the cell poles and periphery. The following MATLAB functions were

1196   used for this analysis: Pixel_Correlation_Multiple_Experiments_Scan.m,

1197   Pixel_Correlation_Parallel.m, Cell_Pixel_Correlation.m, Extract_Cell_Pixels.m, Cell_Projection.m

1198   and Taylor_Smooth.m.

1199

1200   ***Mean squared displacements of single ribosomal particles***

1201   Particle locations determined using the *uTrack* package (Jaqaman et al., 2008) were linked into

1202   trajectories based on a previously described algorithm (Crocker and Grier, 1996). Briefly, the

1203   most likely trajectories were constructed by minimizing the sum of squared particle

1204   displacements between two consecutive frames. Trajectories of lengths smaller than five

1205   displacements were removed. Mean squared displacements (MSD) at various time delays were

1206   then calculated from individual trajectories. For each frame interval, an ensemble-averaged MSD

1207   was obtained by averaging individual MSD curves weighted by the corresponding trajectory

1208   lengths. For each MSD curve, the slope was determined by fitting the three smallest time delays

1209   using least squares regression and by further dividing by a factor of 4 to obtain the apparent

1210   diffusion coefficient D_a. Similarly, the slope of the log-log MSD curve was determined by fitting

1211   the three smallest time delays to obtain the anomalous exponent α. Due to the short average

1212   trajectory length, only the three smallest time delays were used to ensure reliable determinations

1213   of these values.

1214

1215   ***Mean squared displacements of GFP-μNS particles***

1216   Cell meshes obtained from Oufti were used to limit particle localization to the region within cells

1217   and prevent spurious trajectory linking between cells. Particle localization was performed using

1218   the function uNS_Particle_Tracking.m to fit a 2D Gaussian to filtered images.

1219

1220   **DATA AND SOFTWARE AVAILABILITY**

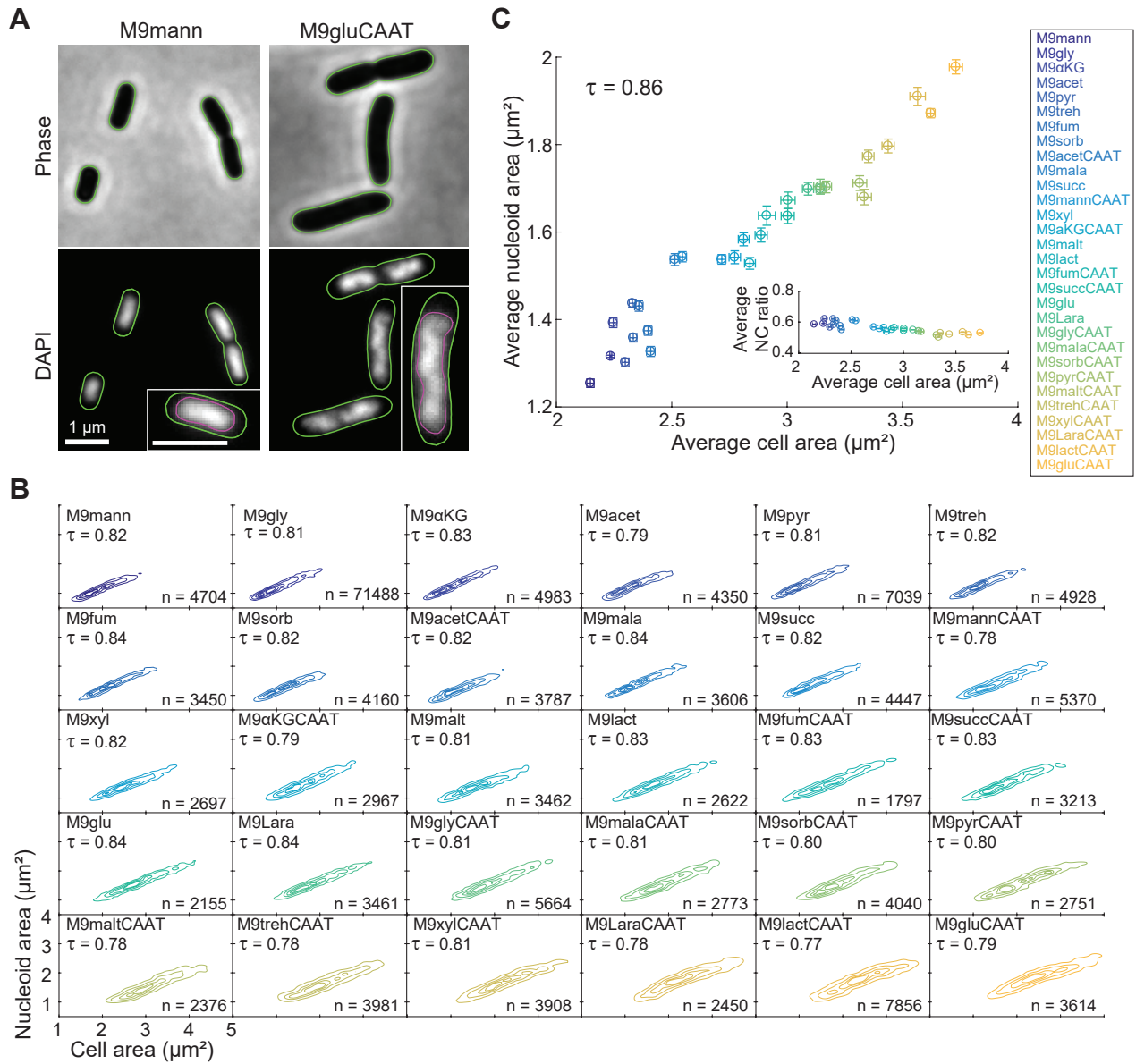1221   All computer code is provided in the Supplemental Information and can also be found at

1222   https://github.com/JacobsWagnerLab/published.

1223

Figure 1

A

Relative density 0 — 1

Relative SeqA-mCherry area

Cell area (µm²)

B

C

D

1 µm

B

τ = 0.45
τ = 0.74
τ = 0.55

Nucleoid area (µm²)

D

C

B

n = 11610
n = 42454
n = 16840

Cell area (µm²)

C

2 µm

Time at 37 ºC

D

Mean ± SD
at 37 ºC
Fit ± 99% CI
at 30 ºC

Nucleoid area (µm²)

Cell area (µm²)
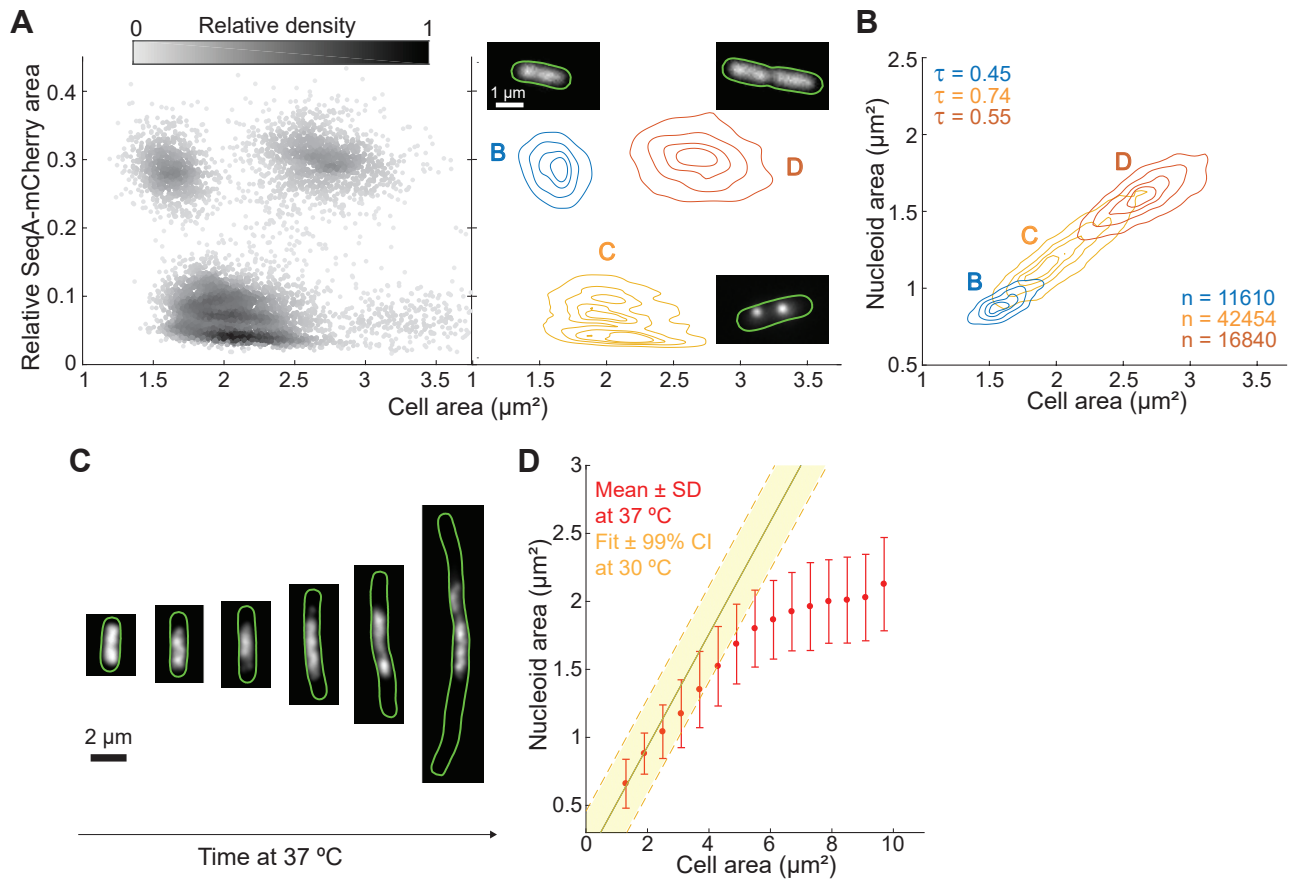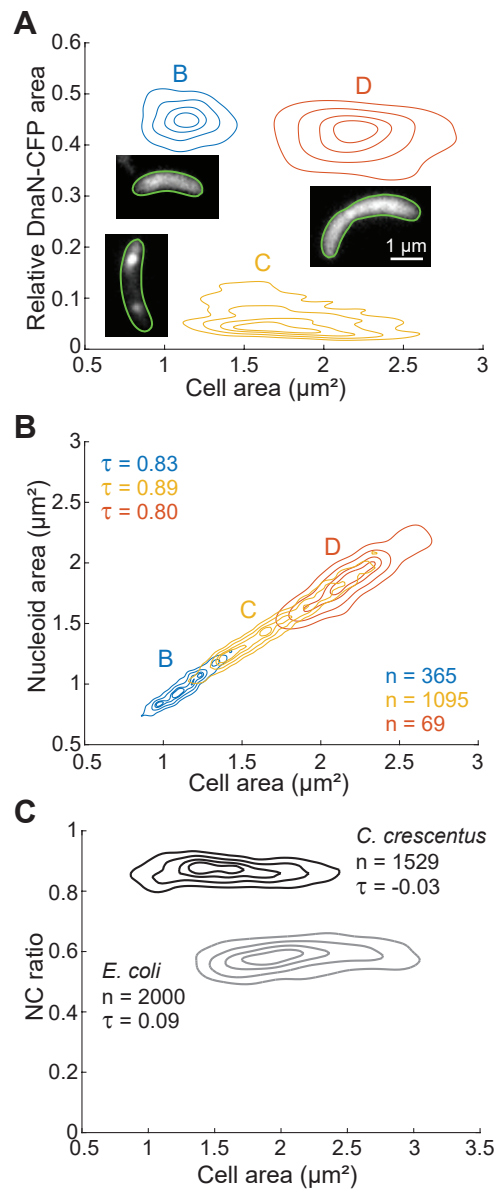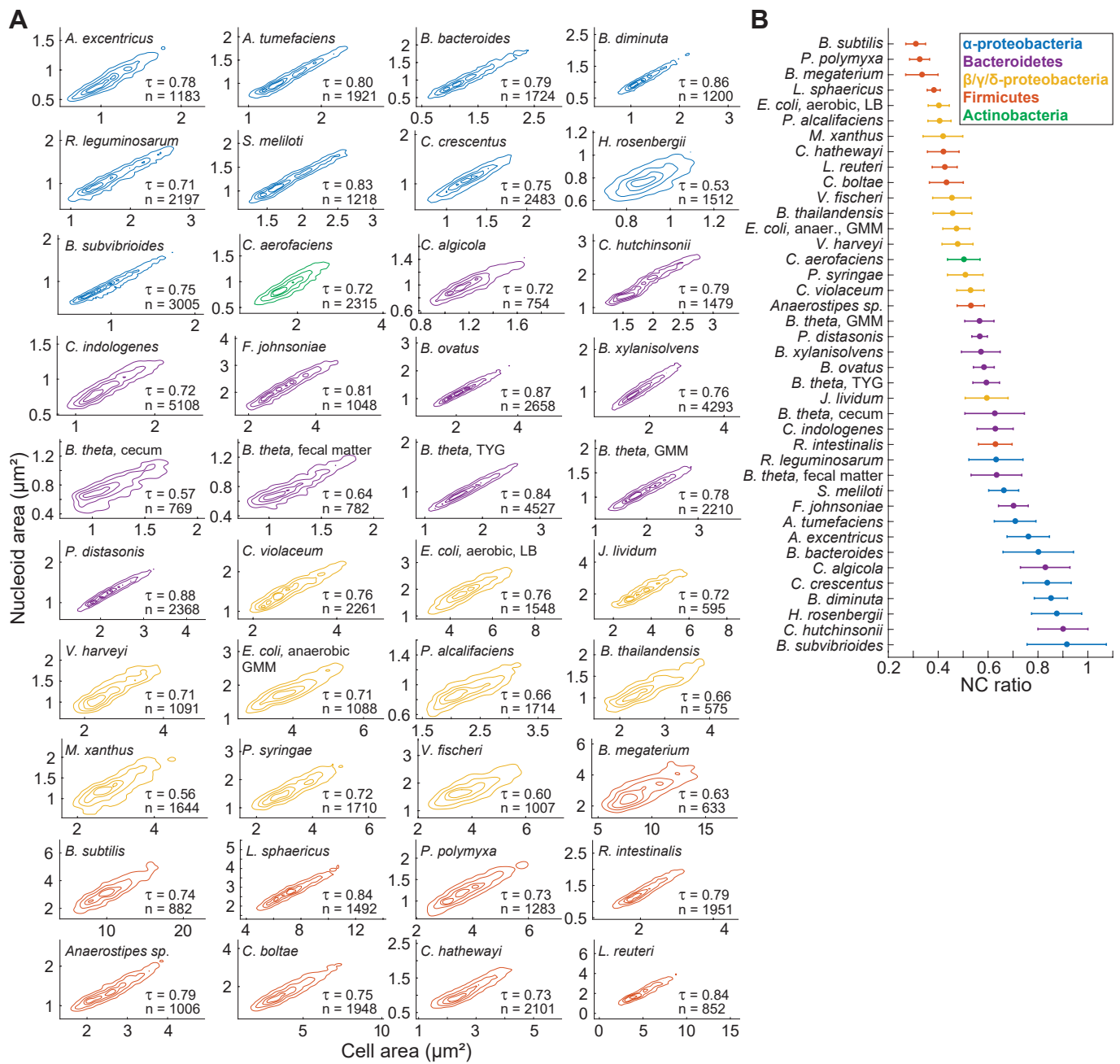
Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

DAPI — RpsB-mEos2

M9gly   M9gluCAAT

RpsB-mEos2

DAPI

1 µm

Fluorescence intensity (AU)

0   0.5   1
Relative cell length

B

τ = 0.62

Average SCF

-0.2
-0.4
-0.6
-0.8

0.52   0.56   0.6
Average NC ratio

M9mann
M9gly
M9treh
M9sorb
M9mala
M9succ
M9mannCAAT
M9glyCAAT
M9malaCAAT
M9sorbCAAT
M9trehCAAT
M9gluCAAT

C

Phase   DAPI   RplA-GFP   EUB338-Cy5

1   2

1 µm

D

DAPI — RplA-GFP ---- Cy5-EUB338

Cell 1   Cell 2

Fluorescence intensity (AU)

1
0.8
0.6
0.4
0.2
0

0 0.2 0.4 0.6 0.8 1   0 0.2 0.4 0.6 0.8 1
Relative cell length   Relative cell length

E

---- RplA-GFP/DAPI
—— Cy5-EUB338/DAPI
—— Cy5-EUB338/RplA-GFP

Frequency

4
3
2
1
0

-1   -0.5   0   0.5   1
SCF

F

τ = 0.75

Average SCF

0.5
0
-0.5
-1

0.3 0.4 0.5 0.6 0.7 0.8 0.9
Average NC ratio

τ = -0.82

Average SCF

0.5
0
-0.5
-1

1   2   3   4   5
Average cell area (µm²)

A. excentricus
C. crescentus
B. diminuta
C. indologenes
A. tumefaciens
S. meliloti
V. harveyi
P. syringae
J. lividum
V. fischeri
E. coli

Figure 7