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A ComGA-dependent checkpoint limits growth during the escape from competence

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Summary

In *Bacillus subtilis*, competence for transformation develops in 5–10% of the cells in a stationary phase culture. These cells exhibit a prolonged lag in the resumption of growth and cell division during the escape from competence. To better understand the basis of this lag, we have characterized competent cultures microscopically. To distinguish the minority of competent cells, a translational fusion between ComK, the competence transcription factor, and the green fluorescent protein (GFP) was used as a marker. Only 5–10% of the cells in a competent culture were fluorescent, indicating that ComK synthesis is an all or nothing event. To validate the identification of competent cells, we demonstrated the coincident expression of comEA, a late competence gene, and comK-gfp. Competent cells resemble stationary phase cells; the majority are single (not in chains), contain single nucleoids, and rarely contain FtsZ rings. Upon dilution into fresh medium, competent cells maintain this appearance for about 2 h. In contrast, the majority of non-competent cells rapidly resume growth, exhibiting chaining, nuclear division and FtsZ-ring formation. The late competence protein ComGA is required for the competence-related block in chromosome replication and cell division. In the competent cells of a comGA mutant culture, chromosomal replication and FtsZ-ring formation were no longer blocked, although competent comGA mutant cells were abnormal in appearance. It is likely that one role for ComGA is to prevent growth, chromosome replication and cell division until ComK can be eliminated by degradation. A mutation in the ATP-binding site of comGA inactivated the protein for transformation but did not prevent it from inhibiting DNA replication and cell division. The buoyant density difference between competent and non-competent cells depends on the competence-specific growth arrest.

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Introduction

Competent *Bacillus subtilis* cells are able to bind and internalize exogenous transforming DNA (Dubnau, 1999). The development of competence occurs at the onset of late exponential growth in certain media. Remarkably, competence is expressed in only a subpopulation of the cells (Nester and Stocker, 1963; Hadden and Nester, 1968; Haseltine-Cahn and Fox, 1968). Although the control of competence development is complex, the regulatory mechanisms converge at a crucial cell-fate determining step: the synthesis and stabilization of the competence transcription factor, ComK (Hahn *et al*., 1994; van Sinderen and Venema, 1994; van Sinderen *et al*., 1994, 1995; Turgay *et al*., 1998). ComK is needed for the expression of the late competence genes, encoding a set of proteins responsible for the binding, processing and internalization of transforming DNA. ComK also activates the transcription of genes needed for DNA repair and recombination (Haijema *et al*., 1995, 1996). However, the development of the transformable state, marked by the synthesis of ComK, involves more than the synthesis of the DNA uptake machinery. The precompetent phase, starting as early as 90–180 min before the appearance of transformability, is accompanied by a decreased rate of DNA and stable RNA synthesis (Nester and Stocker, 1963; Dooley *et al*., 1971). Additionally, the buoyant densities of the competent and non-competent cells in a given culture differ, permitting separation of these two subpopulations by equilibrium centrifugation (Hadden and Nester, 1968; Haseltine-Cahn and Fox, 1968). Using this separation method, it has been demonstrated that *comK* and the late competence genes are expressed preferentially in the competent subpopulation (Albano *et al*., 1987; Hahn *et al*., 1994).

Development of the low buoyant density characteristic of competent cells requires the first gene of the *comG* operon (Albano *et al*., 1987). Insertion of the transposon Tn*917* in *comGA* eliminated the low buoyant density fraction, whereas a polar insertion in *comGB*, the second gene of the operon, did not. These insertions did not affect the expression of the other known competence genes, but

Table 1. Frequency of Z rings[a].

	T_2	T_2 + 90 min
Wild-type non-competent cells	10%	85%
Wild-type competetent cells	12%	16%
Competent *comGA12* cellls	9%	90%

a. Frequency of Z rings at T_2 or 90 min after dilution of cultures with fresh competence medium.

completely abolished transformability. A polar insertion in *comGA* could be complemented for buoyant density separation (but not for transformation) by an intact copy of *comGA*. ComGA encodes a peripheral membrane protein with Walker A and B nucleotide-binding consensus motifs and is involved in permitting access of transforming DNA to the DNA-binding protein ComEA (Albano *et al.*, 1987; Chung *et al.*, 1998; Provvedi and Dubnau, 1999).

When a competent culture of *B. subtilis* is diluted into fresh medium, the number of transformants remains constant for 3–5 h after the non-competent cells have resumed growth (Nester and Stocker, 1963). During this growth lag, the synthesis of an enzyme specified by the donor DNA is also delayed, although integration of a donor gene occurs minutes after the addition of donor DNA. Clearly competent cells are physiologically distinct from non-competent cells. To improve our understanding of the cell biology of competence, we have studied competent cultures microscopically, both prior to and during the escape from competence. We have confirmed that competence-expressing cells are arrested in cell division and DNA replication and have demonstrated that ComGA plays a major role in this arrest, preventing DNA replication and FtsZ (Z)-ring formation. Our data also provides a partial explanation for the buoyant density difference between competent and non-competent cells.

Results

Expression of a ComK–GFP fusion as a gratuitous marker for competent cells

To distinguish competent from non-competent cells, we constructed a translational fusion between the first 10 codons of ComK and the N-terminus of the green fluorescent protein (GFP). This construct was introduced by a single reciprocal recombination event, so that the resulting strain (BD2711) carried a wild-type copy of *comK* in addition to *comK-gfp*. Comparison of fluorescent and differential interference contrast (DIC) images of BD2711, grown to competence, demonstrated that between 5% and 10% of the cells were fluorescent. Representative images are shown in Fig. 1A and B. This result is in accord with data obtained using other methods, indicating that about 10% of the cells in a competence culture express competence genes (Nester and Stocker, 1963; Hadden and Nester, 1968; Haseltine-Cahn and Fox, 1968; Albano *et al.*, 1987; Hahn *et al.*, 1994). As expected, no GFP signal was observed when loss of function mutations in *comK* or *comS* were placed in this background, or when BD2711 was grown in Luria–Bertani (LB) broth medium (not shown). These mutations and growth in LB broth are known to prevent the expression of competence (D′Souza *et al.*, 1994; van Sinderen *et al.*, 1994; Hamoen *et al.*, 1995). As a further proof that the *comK-gfp* construct permitted the accurate identification of competence-expressing cells, we used immunofluorescence detection of ComEA to demonstrate co-expression of ComK–GFP and ComEA. The ComEA signal (Fig. 1C) was observed throughout the cell. ComEA is an integral membrane protein (Inamine and Dubnau, 1995) and the brighter staining bands evident in Fig. 1C probably correspond to the double thickness of cell membrane between adjoining cells. The ComEA immunofluorescence signal was observed in 85% of the cells that had GFP fluorescence. No cells were observed containing a ComEA signal in the absence of GFP fluorescence. In spite of this bias, our results show that the presence of a ComK–GFP signal can be used to distinguish competent from non-competent cells. Because the majority of cells exhibited no ComK–GFP or ComEA-associated fluorescence, these results indicate that competence expression is an all or nothing event.

Three further observations (not shown) indicate that ComK–GFP provides a gratuitous marker for competence expression. First, the *comK-gfp* strain is transformable,

Table 2. Strains.

Strain	Genotype	Source
IS75	his leu met	–
BD1248	*his leu met comGA12* (*Tn*917*-lacZ*)	Albano *et al.* (1989)
BD1254	*his leu met comGB39* (*Tn*917*-lacZ*)	*Albano et al.* (1989)
BD2210	*his leu met comK mecA::spc comGA12* (*Tn*917*-lacZ*)	This work
BD2112	*his leu met mecA::spc*	This work
BD2685	*his leu met comGA*Δ (in frame)	Chung and Dubnau (1998)
BD2711	*his leu met comK-gfp*	This work
BD2713	*his leu met comK-gfp comGA12* (*Tn*917*-lacZ*)	This work
BD2717	*his leu met comK-gfp comGB39* (*Tn*917*-lacZ*)	This work
BD3018	*his leu met comK-gfp comGA-atp*	This work

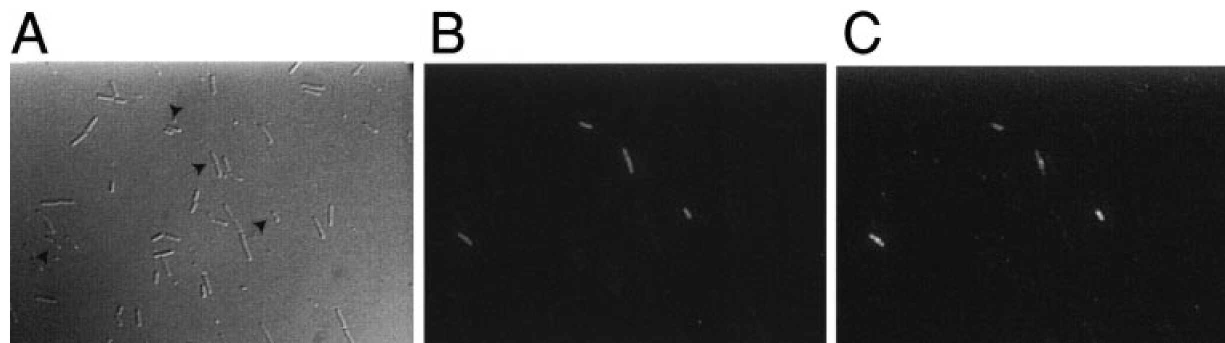

Fig. 1. Microscopic identification of competence-expressing cells.
A. DIC image of competent culture at T_2 (2 h after the transition from the exponential to the stationary growth phase).
B. Visualization of the field shown in A using GFP fluorescence.
C. Visualization of the field shown in A using immunofluorescence staining of ComEA. The competence-expressing cells are indicated by arrows in A.

since it also carries a wild-type copy of *comK*. Second, the ComK–GFP protein probably does not bind DNA. This was inferred from the observation that the GFP signal was distributed throughout the cell, whereas detection of wild-type ComK by immunofluorescence revealed a location coincident with the 4,6-diamidino-2-phenylindole (DAPI)-stained nuclear body. Third, Western blot analysis with anti-ComK and anti-GFP antisera revealed that the ComK–GFP signal was stable for at least several hours during outgrowth from competence, whereas that of ComK gradually disappeared, as previously reported (Turgay *et al*., 1998). These observations demonstrated that ComK–GFP was a useful marker for competence-expressing cells and that the marker persists throughout the escape from competence.

Competent and non-competent cells are indistinguishable at T_2

Strain BD2711 was grown to T_2, 2 h after the transition to stationary phase, at which time maximal competence was achieved, and samples were stained with DAPI for visualization of the chromosomes, and with tetramethylrhodamine-conjugated wheat germ agglutinin (WGA-TMR) for visualization of cell walls to determine cell length. Both the competent and non-competent cells occur predominantly as single cells and the distribution of cell lengths was similar for the two populations. Approximately 80% of the cells contained a single centrally located nuclear body. Immunofluorescence, using anti-FtsZ antibody, was used to visualize Z rings (Lutkenhaus and Addinall, 1997) as an indicator of cell division. These structures were absent in the great majority of the cells, both competent and non-competent (Table 1). By these criteria, the competent and non-competent cells were not distinguishable microscopically, and both populations had an appearance typical of stationary phase cells.

Competent cells retain the appearance of stationary phase cells during outgrowth

Nester and Stocker (1963) reported that the number of transformants for a given marker did not increase during incubation in fresh liquid media until 3–5 h after transformation. From this they inferred that competent cells exhibit a prolonged lag before resumption of growth, although part of the delay they observed must have been due to the segregation of the donor marker from the DNA heteroduplex which is the initial product of transformation. To further characterize this growth delay, BD2711 was grown to T_2, followed by 20-fold dilution into fresh competence medium. Samples were taken at 60 and 90 min after dilution and examined as described above, except that cell lengths were measured in separate samples using propidium iodide (PI) staining as well as with WGA-TMR. PI stains both RNA and DNA under the conditions used, leaving a gap where there is a septum. Similar results were obtained using the two methods. Results from the 90 min point are presented in Figs 2 and 3. Similar results were obtained with the 60 min samples. As expected for growing cells, most of the non-competent cells contained nucleoids in various stages of replication and many cells contained more than one nucleoid (Figs 2 and 3). In contrast, most of the wild-type competent cells contained solitary nucleoids that rarely displayed the elongated shape indicative of replicating nuclear bodies. Most ($> 80\%$) of the non-competent cells occurred in chains, indicating the prior completion of cell division, whereas more than 60% of the competent cells occurred as single cells just as they had at T_2 (Fig. 3). Cell-length measurements revealed no difference between the competent and non-competent cells of BD2711 when either PI (Fig. 3) or WGA-TMR (not shown) were used to stain the protoplasts or walls; nearly 80% of both populations were 1–2 microns in length (Fig. 3). The

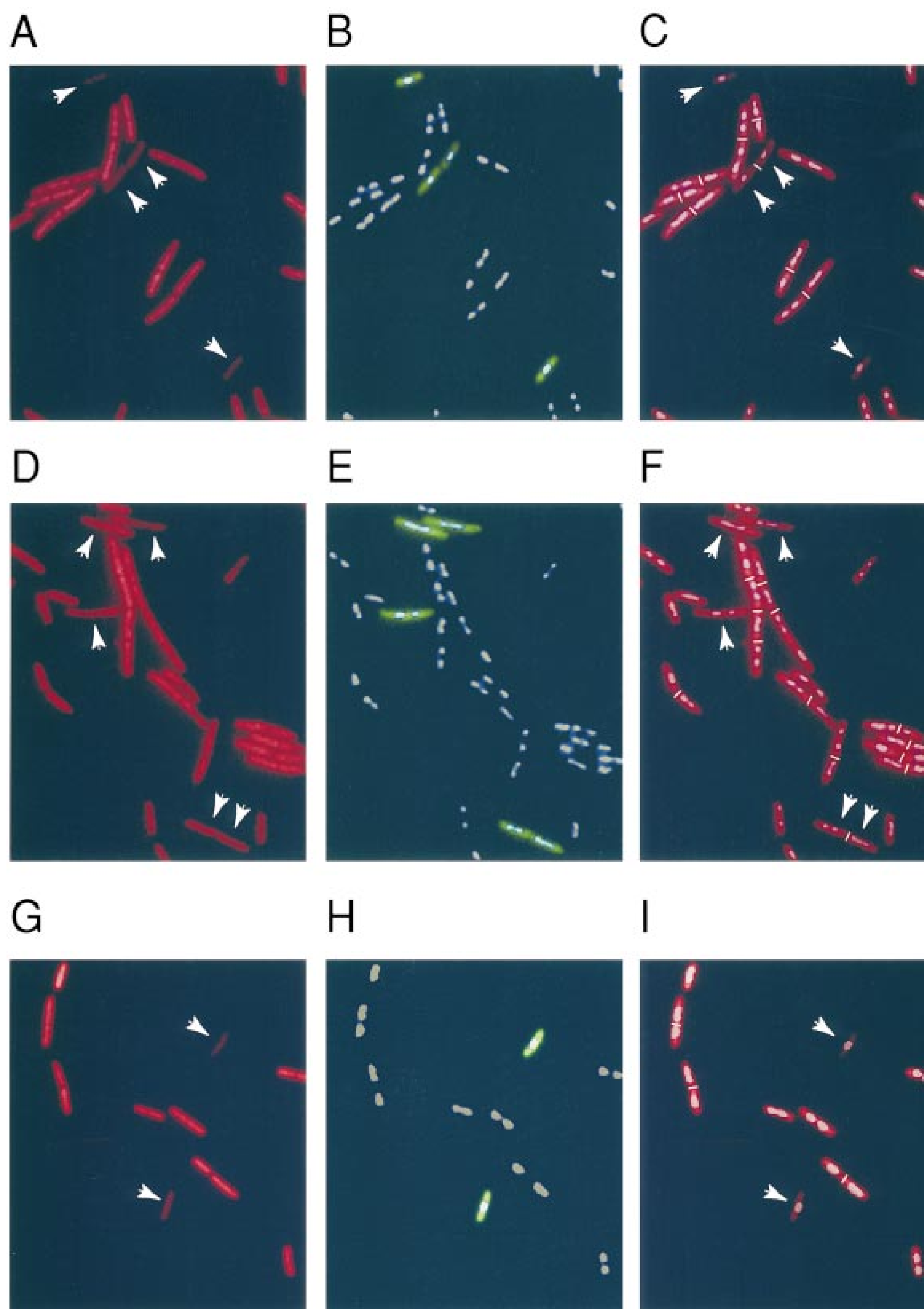

Fig. 2. Microscopic appearance of competence-expressing and non-competent cells at 90 min after dilution in fresh medium.
A–C, wild type (BD2711); D–F, *comGA12* (BD2713); G–I, *comGA-atp* (BD3018). A, D and G show PI fluorescence images. B, E and H show GFP fluorescence (pseudocoloured green) from the same fields, overlayed on DAPI fluorescence images. Panels C, F and I show overlays of the PI and DAPI fluorescence. The competence-expressing cells are indicated by arrows and the positions of cross-walls by white bars. The cross-walls were located based on the PI staining.

non-competent cells exhibited a marked increase in the frequency of cells with Z rings compared with the samples taken at T_2, whereas the competent cells did not (Table 1, Fig. 4A–C). In conclusion, by the criteria of Z-ring formation, chaining and the appearance and number of nuclear bodies, the non-competent cells appeared to be growing at 60 and 90 min after dilution in fresh medium, and it was evident that chromosome replication was underway. In contrast, the competent cells were still not distinguishable from cells in stationary phase. Additional experiments of the same kind revealed that the competent cells did not begin cell division and DNA replication until 2–3 h after dilution. One additional difference between the competent and non-competent cells is evident in the

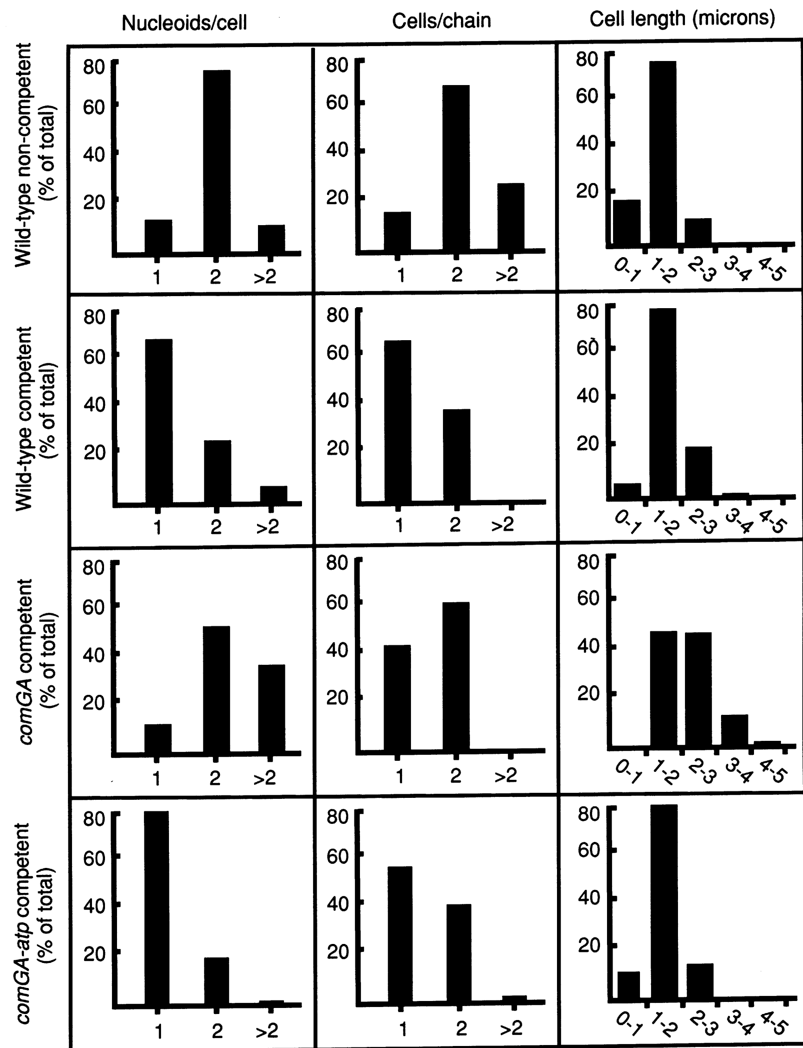

Fig. 3. Quantification of microscopic appearance of the wild-type (BD2711) competence-expressing and non-competence cells and of competence-expressing cells of the *comGA12* mutant (BD2713) and of the *comGA-atp* mutant (BD3018).

PI-stained images (Fig. 2A). The competent cells were consistently less fluorescent than the non-competent cells. This difference disappeared after about 2 h as the competent cells emerged from the growth arrest (not shown).

Buoyant density separation of competent and non-competent cells

Competent cells of *B. subtilis* may be prepared by a simple procedure that involves growth of a culture to stationary phase in a suitable medium (one-step method) (Albano *et al.*, 1987). Alternatively, they may be prepared by a two-step method in which a stationary phase culture is diluted 10-fold into fresh medium and incubated for about 90 min (Anagnostopoulos and Spizizen, 1961). When prepared by the two-step procedure, the competent and non-competent subpopulations have distinct buoyant densities, permitting their separation by isopycnic density gradient centrifugation (Hadden and Nester, 1968; Haseltine-Cahn and Fox, 1968). When competent cultures were subjected to density gradient centrifugation after preparation by the one-step method, nearly all of the cells banded at the lower density (not shown). In contrast, exponentially growing cells in competence medium, banded at the higher density position, characteristic of non-competent cells. These observations led to the hypothesis that during the 90 min incubation in fresh medium, the non-competent cells had resumed growth and, therefore, exhibited an elevated buoyant density, whereas the competent cells which had failed to resume growth, banded at a lower density. Competent and non-competent cells prepared using the one-step method do not resolve in density gradients, because the non-competent cells have not resumed growth. Support for this hypothesis was provided by a density fractionation experiment with BD2711 90 min after dilution (not shown). The majority of cells in the less dense fraction were single and

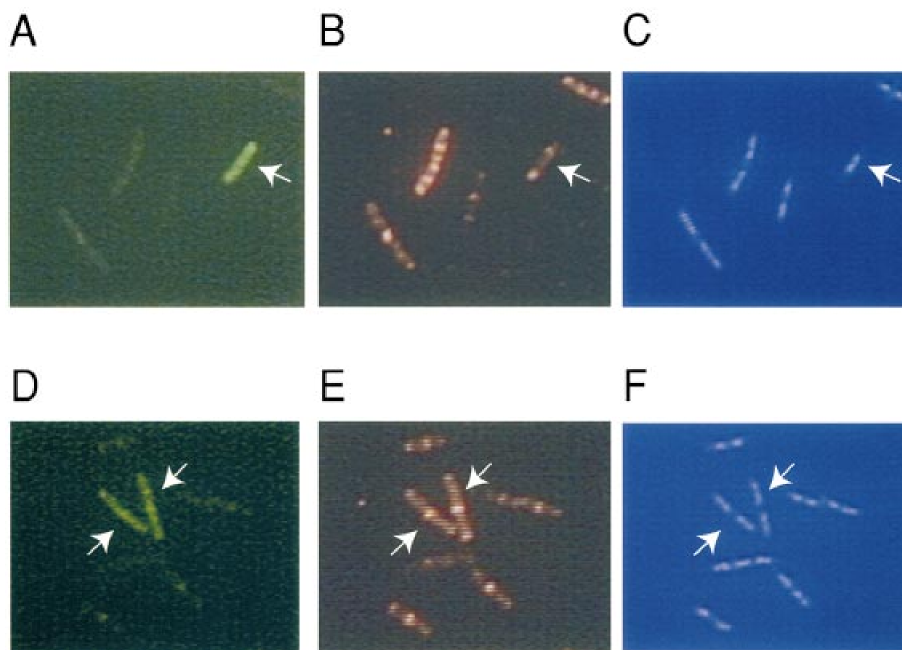

Fig. 4. Appearance of Z rings in the wild-type (BD2711) and *comGA12* mutant (BD2713) strains after 90 min of outgrowth. A–C, wild type; D–F, *comGA12* mutant. A and D, ComK-GFP fluorescence; B and E, FtsZ immunofluorescence; C and F, DAPI fluorescence. The competence-expressing cells are indicated by arrows.

mono-nucleated and about half exhibited a GFP signal, whereas the majority of the cells in the denser fraction were in chains, were multinucleated and only 2% exhibited GFP fluorescence. Many of the non-fluorescent cells in the less dense fraction were presumably non-competent cells that had not yet resumed growth, represented as mononucleated, single cells in Fig. 3.

Null mutations in *comGA* prevent buoyant density separation, because all of the cells, including those that express the wild-type competence genes, exhibit the higher density characteristic of non-competent cells (Albano *et al*., 1989). As *comGA* mutant cells exhibit high density, we predict that ComGA must be required for the replication and division arrest of competent cells. This prediction was tested microscopically.

The late competence protein ComGA arrests DNA replication, cell division and growth

*comGA12 (*a Tn*917* insertion in *comGA*) was introduced into BD2711 to create BD2713. This strain was grown in competence medium to T_2. Samples were taken at that time and 90 min after dilution in fresh medium. At T_2 the competent and non-competent cells were microscopically identical to those of the wild-type strain (BD2711) (not shown). However, after growth in fresh medium, the competence-expressing cells of BD2713 exhibited a distinct appearance. First, the competence-expressing mutant cells were slightly longer and contained more nucleoids than either the wild-type competent or non-competent cells (Figs 2D–F and 3). Second, they exhibited an increased tendency to occur in chains compared with the wild-type competent cells, suggesting that some cell division events had occurred, although perhaps slightly fewer than in the wild type non-competent cells (Figs 2 and 3). Third, in marked contrast to the wild-type competent cells, Z rings were as frequent as in the non-competent cells (Table 1). Figure 4 shows typical wild-type and *comGA12* competence-expressing and non-competent cells. Z rings are visible at mid-cell in the non-competent cells and in the two competence-expressing *comGA12* cells displayed as well as in all of the non-competent cells. In addition, FtsZ immunofluorescence is visible near the poles of almost all of the cells, including the wild-type competent ones, indicating that the failure to form Z rings was not due to the absence of FtsZ. In growing *B. subtilis*, FtsZ is normally detected near the poles as well as in medial Z rings (Levin and Losick, 1996).

Our results suggest that the replication and cell division arrest of competent cells requires ComGA and that ComGA also limits cellular elongation during the escape from competence. Because the competence-expressing *comGA* cells were slightly elongated and contained more nucleoids than the growing non-competent cells, it appears that the control of cell division was aberrant in these cells. This defect was manifested after Z-ring formation (Table 1) and must be competence dependent. It is also apparent in Fig. 2 that the weak PI-staining

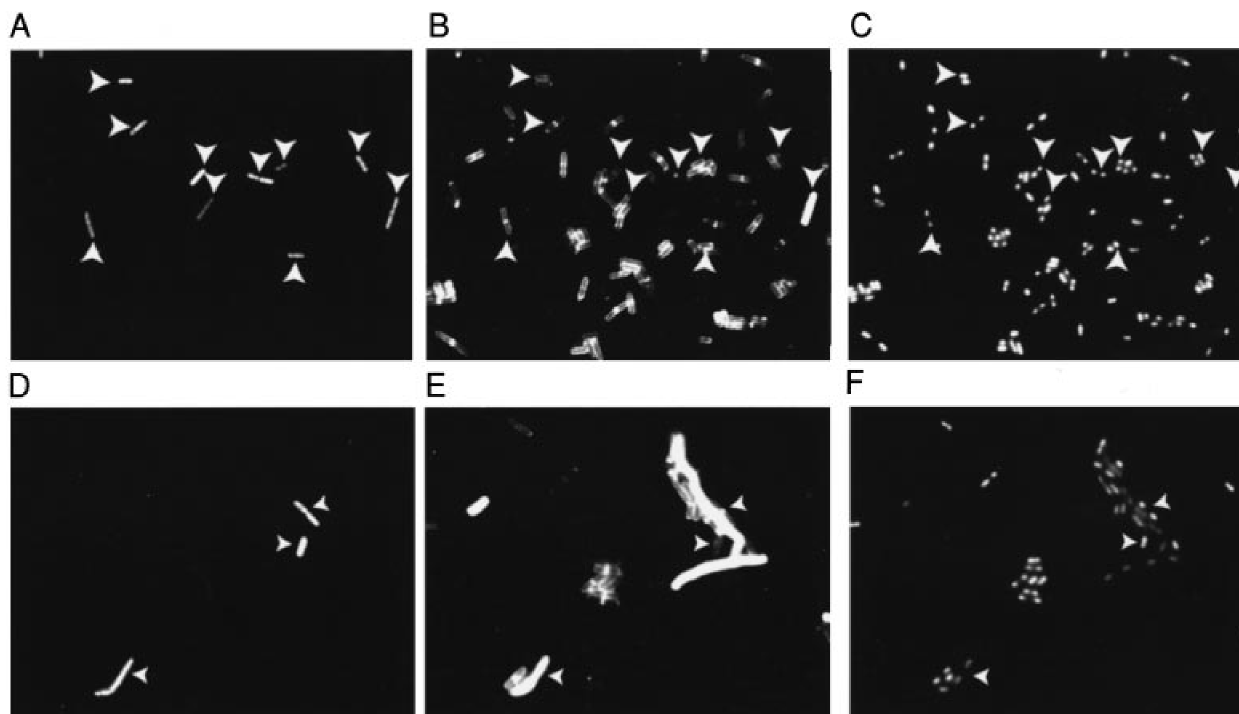

Fig. 5. Appearance of wild-type and *comGA12* cultures after 4 h of outgrowth. A–C and D–F represent the wild-type (BD2711) and *comGA12* (BD2713) cultures respectively. A and D exhibit ComK-GFP fluorescence, B and E display WGA-TMR staining and D and F show DAPI staining. Competence-expressing cells are indicated by arrow heads. B and E have been adjusted so that the 'normally staining' cells exhibit comparable brightness, in order to illustrate the enhanced staining intensity of the aberrant cells.

characteristic of wild-type competent cells was not evident in the *comGA12* mutant background.

The *comGA12* mutation is polar on the downstream six open reading frames (ORFs) of the *comG* operon. To determine whether the phenotype just described is due only to inactivation of *comGA*, a polar *comGB* mutant was constructed in the *comK*-gfp background (BD2717). The competence-expressing cells of this strain were indistinguishable from the wild type with respect to length, nucleoid number and chaining (not shown). We conclude that ComGA is the only ComG protein required for the block in replication and cell division. This result is consistent with the unique dependence on ComGA for the buoyant density fractionation of competent cultures (Albano *et al*., 1989).

We have attempted to determine whether the competence-expressing *comGA12* mutant cells ever manage to escape from competence and initiate normal growth. The wild-type (BD2711) and *comGA12* (BD2713) strains were grown to stationary phase in competence medium, diluted in fresh medium and then maintained in exponential growth by periodic dilution. After 4 h, many cells in each of the cultures still exhibited GFP fluorescence, consistent with the stability of the ComK–GFP fusion protein. Nearly all the wild-type fluorescent cells had the appearance of normally dividing cells (Fig. 5A–C). In contrast, three cells types were visible in the *comGA12* culture at 4 h, and Fig. 5D–F presents examples of each of them. Two types of competence-expressing cells were visible. The first type had a normal appearance. These cells were often present as doublets, had compact, centrally located nuclear bodies and stained normally with WGA-TMR. A second type stained heavily with WGA-TMR, were filamented (no evidence of cross-walls) and contained multiple nucleoids that stained more faintly than normal with DAPI (Fig. 5D–F). Finally, the *comGA12* culture contained additional cells that did not exhibit GFP fluorescence but were otherwise identical to the second class of GFP-fluorescent cells (filamented and stained heavily with WGA-TMR). Both types of heavily stained cells tended to be bent in shape, and such cells were nearly absent from the wild-type culture. A plausible interpretation of these various types of cells is as follows. The normal appearing competence-expressing *comGA12* cells may have successfully escaped from the competent state. If so, it appears that the penetrance of the *comGA12* mutation is not absolute. The cells that stained heavily with WGA-TMR may have been in the process of autolysis. Autolysis may permit greater access to WGA-TMR through the petidoglycan matrix, and may also expose *N*-acetyl glucosamine residues for WGA-TMR binding. The heavily stained cells that did not exhibit GFP fluorescence may have been competence-expressing cells that had partially lysed, releasing their cytoplasmic

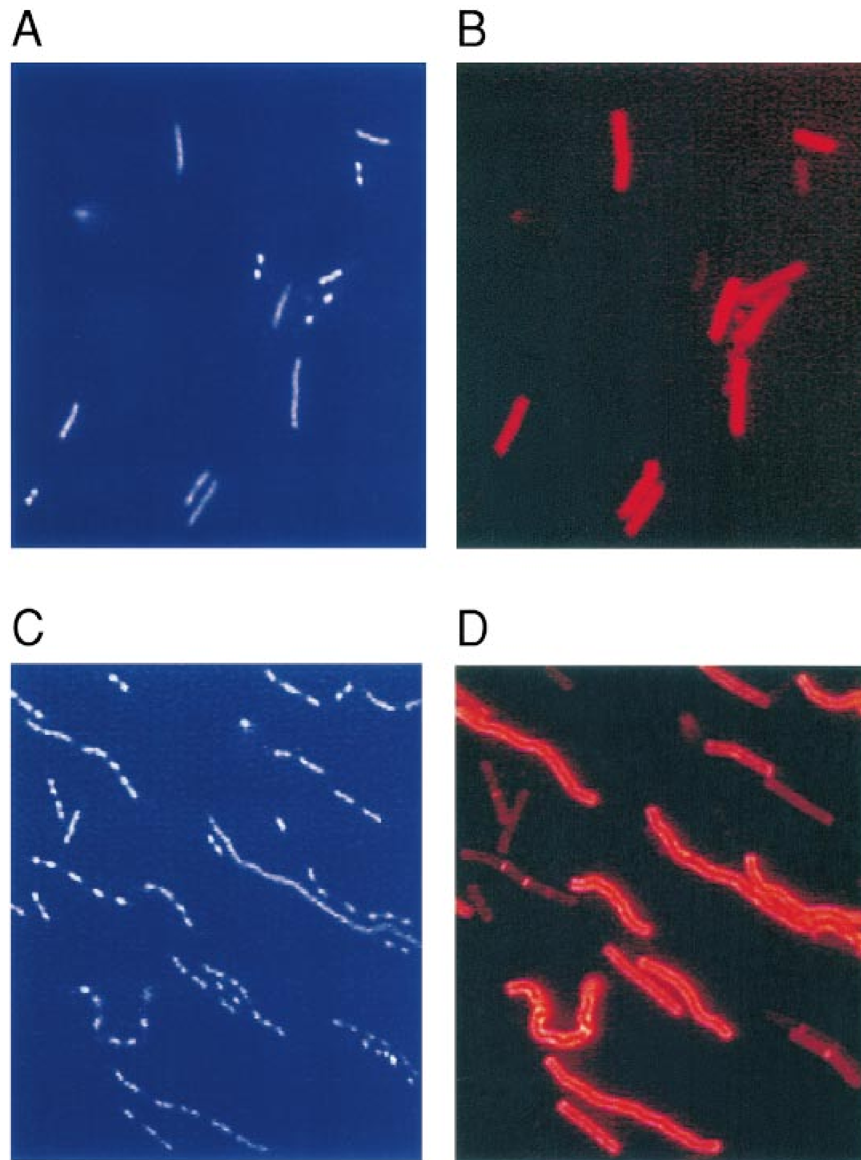

Fig. 6. Appearance of the *mecA* (A and B) and *mecA comGA12* (C and D) strains (BD2112 and BD2210 respectively). A and C, DAPI fluorescence. B and D, WGA-TMR fluorescence. The samples were taken at T_2.

contents. Whatever the precise interpretation of the various types of cells, it is clear that many of the ComGA-deficient cells retain an aberrant appearance even after 4 h, suggesting that they have failed to resume normal growth.

Phenotype of a mutation in the Walker A box of ComGA

ComGA is a member of a widespread family of proteins that have been implicated in pilus formation, protein secretion and twitching motility, in addition to competence (reviewed in Dubnau, 1999). These proteins possess Walker A and B nucleotide-binding motifs. To investigate the role of nucleotide binding in ComGA function, we have introduced a K to Q change at position 150 of *comGA*. This mutation was introduced, as described in *Experimental procedures*, into a background carrying the *comK-gfp* construct, in which it replaced the wild-type copy of *comGA.* The resulting strain (BD3018) was completely deficient in transformability and was unable to bind radiolabelled-transforming DNA (R. Provvedi and D. D., unpublished results), a phenotype identical to that of a *comGA* null mutant (Chung and Dubnau, 1998). Nevertheless, after 90 min incubation in fresh growth medium, the *comGA-atp* mutation had no detectable effect on cell length, chaining and the number of nucleoids per cell (Figs 2G–I and 3). This result distinguishes two independent roles for ComGA: in the

binding of transforming DNA and in mediating the competence-specific arrest in DNA replication, cell elongation and division. It also demonstrates that the binding of nucleotide may not be required for the second role. Additionally, the weak PI-staining characteristic of wild-type competence-expressing cells, but not of the *comGA12* mutant, is evident in the *comGA-atp* mutant cells (Fig. 2G).

Phenotype of a mecA comGA *double mutant*

It was noted above that although Z rings can form in competence-expressing cells of the *comGA12* mutant, these cells are not completely normal as they are slightly filamented. These defects are presumably dependent on the competence transcription factor ComK, as all known competence-related phenotypes require ComK for their expression. It is likely that the presence of ComK, or of some product dependent on ComK, prevents or delays cell division, acting at a step after Z-ring formation. This is supported by the marked filamentation of many *comGA12* mutant cells after 4 h of outgrowth. During the escape from competence, ComK is normally degraded by a MecA-dependent mechanism (Turgay *et al*., 1998). For this reason, in a *mecA* mutant ComK persists. These facts suggested that a *comGA mecA* double mutant might exhibit a pronounced filamentation phenotype. As a result of the absence of ComGA, cellular elongation would no longer be inhibited, while the persistence of ComK might prevent or delay cell division. Additionally, this filamentation phenotype should affect most cells, as in *mecA* strains nearly 100% of the population expresses competence (Hahn *et al*., 1995) and should be evident even at T_0, because in these strains ComK is expressed prior to stationary phase. These predictions were confirmed. In the double mutant strain (BD2210), dramatic filamentation was observed (Fig. 6). Approximately 50% of the double mutant cells were >8 microns in length, compared with only 5% in the *mecA* strain (BD2112). In both the *mecA* and *mecA comGA* strains, the nuclear bodies often presented an abnormal appearance. In many cases what appear to be single nucleoids extended over the entire length of the cell. This phenotype was described previously for *mecA* (Hahn *et al*., 1995). We have also observed that the *mecA* and *mecA comGA12* strains stain intensely with WGA-TMR and that the double mutant also exhibits an irregular and bent appearance as noted above for the *comGA12* mutant after 4 h in fresh growth medium. In the few *mecA* and *mecA comGA12* cells in Fig. 6 that exhibit less intense WGA-TMR staining, the nucleoids often appear more normal and the crooked shapes are not present. These normal-appearing cells are probably *comK* mutants, which tend to accumulate in *mecA* cultures (Hahn *et al*., 1995).

Discussion

The present results provide direct cytological confirmation of the classical conclusion that competent cells are blocked in growth (Nester and Stocker, 1963). These older reports were based on measurements of colony-forming units after transformation and on the penicillin susceptibility of transformant cells during outgrowth. Even without a growth block, a lag of at least one generation in the increase of transformant colony-forming units would be expected, as the initial product of transformation is a heteroduplex. The present results demonstrate that in addition to this effect, which clearly contributed to the older data, a block in cell division accompanies the competent state. This block lasts for about 2 h, while the non-competent cells are dividing. Because no transforming DNA was added to our cultures, and as the growth arrest occurs in the *comGA-atp* mutant that cannot take up DNA, the arrest is independent of transformation. Not only are cell division and elongation arrested, but DNA replication also appears to be blocked, as the nuclear bodies of competent cells retain the appearance of resting nucleoids. It has been reported that DNA replication and stable RNA synthesis are blocked in the presumptive competent cells during the development of competence (Dooley *et al*., 1971) and in newly transformed cells (McCarthy and Nester, 1967). The present results confirm that the inhibition of DNA replication continues during outgrowth. The weaker PI staining of competent cells may reflect a decreased content of RNA and DNA resulting from the inhibition of stable RNA and DNA synthesis.

Our results also provide a partial explanation for the density difference between competent and non-competent cells in a given population. Although the physical basis of this difference is unknown, it reflects a distinction between growing cells, which are more dense, and non-growing cells, which are less dense.

ComGA is required for the inhibition of elongation, Z-ring formation and DNA replication. Several lines of evidence suggest that in the absence of this ComGA-mediated arrest, the competence-expressing cells suffer adverse consequences. After 90 min of outgrowth, *comGA* mutant cells are slightly filamented and contain more nuclear bodies then dividing non-competent cells, suggesting the presence of a ComK-dependent defect that is exhibited when elongation and replication resume prematurely due to the absence of ComGA. This conclusion is further supported by the dramatic filamentation phenotype exhibited by the *mecA comGA* strain (Fig. 6). Additional preliminary data suggests that the timing of chromosome replication may be aberrant in the absence of ComGA (not shown). After 90 min of outgrowth, 22% of the competence-expressing *comGA12* cells (22 out of 102 cells examined) had nucleoids in

different stages of replication within a single cell, in contrast to only 5% of such cells (10 out of 201 cells examined) of the wild-type strain (not shown). In the wild type, after 4 h of outgrowth, only in 6 out of 70 cases (8.5%) were there nuclear bodies in different stages of replication within a single cell, similar to the 5% noted above at 90 min. However, in the 4 h culture from the *comGA12* mutant, 16 out of 61 (26.2%) of the fluorescent cells showed evidence of asynchonous replication, again similar to the 22% observed at 90 min. Among the non-fluorescent cells from the wild-type and mutant cultures, 9 of 118 (7.6%) and 6 out of 110 (5.5%) exhibited this phenotype. These numbers are similar to those reported above for the competence-expressing wild-type cells at 90 min (5%) and 4 h (8.5%). Finally, the intense WGA-TMR-staining characteristic of competence-expressing *comGA* mutant cells after 4 h of outgrowth (as well as of *mecA* and *comGA mecA* cells) may reflect autolysis, as noted above. Taken together, the filamentation, the possible defect in the timing of replication and the intense WGA-TMR-staining of ComGA-deficient competence-expressing cells suggest that the premature resumption of growth in the presence of ComK, due to the absence of ComGA, is deleterious. The abnormal appearance of *mecA* cells (Hahn *et al*., 1995) shows that an excess of ComK is deleterious even when ComGA is present. Either the ratio of ComGA to ComK must be above a critical value to permit an orderly escape from the competent state, or excess ComK exerts a toxic effect independent of ComGA.

The ComGA-mediated arrest provides a checkpoint, preventing the resumption of growth during the early stages of competence escape. This checkpoint may provide time for the elimination of ComK by MecA/ClpC/ClpP-mediated degradation. This is entirely consistent with the fact that the degradation of ComK during the escape from competence is complete after 2 h (Turgay *et al*., 1998), just when the competent cells resume growth and the ComGA-mediated arrest must, therefore, be reversed. A second possible role for the arrest, which is mutually compatible with the first proposed role, may be to provide time for the repair of nicks and gaps in the DNA introduced by transformation or by the competent state (Harris and Barr, 1971) before chromosome replication is allowed to resume. This is similar to the role of the SulA-mediated growth arrest that follows DNA damage in *Escherichia coli* (Gottesman *et al*., 1981). It is interesting that the SOS-induced protein SulA, such as ComGA, is a nucleotide-binding protein that blocks Z-ring formation (Bi and Lutkenhaus, 1993). SulA accomplishes this by the direct inhibition of FtsZ polymerization (Mukherjee *et al*., 1998).

ComGA-mediated growth inhibition operates only in the context of competence or perhaps during the emergence from stationary phase. This is clear from our observation that expression of ComGA from the P*spac* promoter during exponential growth in the absence of any other competence gene product does not result in a detectable defect in growth or cell division (not shown). In these experiments, Western blots were used to verify that the synthesis of ComGA was similar to that achieved in the competent state. This constraint on inhibition by ComGA is reasonable if ComGA is only needed to prevent the resumption of growth while DNA repair is underway and ComK is undergoing degradation. The primary target or targets for ComGA inhibition are unknown, but it is likely that the direct or indirect ComGA-mediated replication block occurs at the initiation step, as it has been reported that competent cells have completed a round of replication (Dooley *et al*., 1971). In *B. subtilis*, Z-ring formation can occur even if the initiation of DNA replication is blocked during spore germination, although the Z rings formed under these conditions are often located acentrically (Wu *et al*., 1995; Harry *et al*., 1999). This result suggests that the target for ComGA action is not simply the initiation of DNA replication. Similarly, inhibition of Z-ring formation does not necessarily inhibit cellular elongation or DNA replication; conditions that inhibit Z-ring formation and cell division often permit the formation of filaments with multiple nucleoids (Donachie, 1993). However, inhibition of cell elongation might be expected to halt both cytokinesis and replication. ComGA may, therefore, exert its primary effect by blocking elongation, perhaps interfering with murein synthesis. Alternatively, ComGA may have several targets or may act at an unknown step that governs all these processes.

ComGA plays an independent role in transformation, as in its absence no DNA binding to the cell surface occurs (Chung and Dubnau, 1998). ComGA is localized at the interior face of the membrane (Chung *et al*., 1998) and probably plays a role in assembling a structure that traverses the wall (Provvedi and Dubnau, 1999). Although the *comGA-atp* mutant cannot support the binding of transforming DNA, it does mediate the competence-specific inhibition of cell division and DNA-replication blocks. We conclude that there are different nucleotide binding requirements for the growth arrest and for the assembly of the wall structure that permits DNA binding to ComEA, the DNA receptor. Because the nucleotide-binding site is needed for construction of the putative wall-traversing structure, ComGA may play a chaperone-like role in this process. This suggestion is supported by the fact that orthologues of ComGA are required for the assembly of type 4 pili, but are not themselves part of these organelles (Nunn *et al*., 1990). In contrast, we have shown that nucleotide binding may not be required for the ComGA-mediated growth arrest. However, this simple conclusion must be regarded with caution. Because

alteration of the conserved lysine residue may decrease the affinity of ComGA for nucleotide (Fry *et al.*, 1986; Hung *et al.*, 1998), it is possible that the growth arrest simply requires a lower degree of binding site occupancy than the transformation role of ComGA. Orthologues of ComGA have been reported to assemble into hexameric rings (Krause *et al.* 2000), and it is possible that for the growth arrest, fewer of the monomer subunits need to bind nucleotide.

Experimental procedures

Bacterial strains and general methods

The bacterial strains used in this study are derivatives of *B. subtilis* 168 and are listed in Table 2. Competent cultures were grown as described previously (Albano *et al.*, 1987). Transformants were selected on tryptose blood agar base (TBAB) agar plates supplemented with chloramphenicol or erythromycin, both at 5 μg ml^{-1}, as required. DNA manipulations, cloning and standard molecular biological methods were as described (Sambrook *et al.*, 1989).

Construction of the comK*–gfp fusion*

To construct the translational *gfp–comK* fusion, the *comK* promoter region was amplified by PCR using the oligonucleotides K1 (GATC**CTGCAG**GTCTGTTTTCTGACTCAT) and K2 (GATC**GTCGAC**CGAGCACCATTTGAATG). The boldface sequences correspond to *Pst*I and *Sal*I cleavage sites respectively. The PCR products were digested with *Pst*I and *Sal*I and ligated to pSG1137 (Lewis and Errington, 1996), digested with the same enzymes. The resulting plasmid (p*comK-gfp*) contained an in frame fusion of *gfp* with the first 10 codons of *comK*. *pcomK-gfp*, was integrated into the chromosome of *B. subtilis* IS75 by Campbell-type recombination, with selection for chloramphenicol resistance. The resulting strain (BD2711) has an intact copy of *comK* as well as the *comK–gfp* fusion, both under control of the *comK* promoter. BD2713 was constructed by transformation of BD2711 using DNA from a strain (BD1248) with a Tn*917* insertion in *comGA* (*comGA12*) with selection for erythromycin resistance. BD2717 was constructed similarly, using DNA from a strain with a Tn*917* insertion in *comGB* (BD1254).

Construction of the comGA*-atp mutant*

The K150Q mutation in *comGA* was constructed using a QuikChange site-directed mutagenesis Kit (Stratagene). The mutagenic primers were 5′-CCGGGCCGACTGG**A**TC**C**G GG**C**AGACTACCACATTATACTCTCTCG and its complement. The boldface letters represent mutational changes. The underlined GGATCC sequence represents a *Bam*HI site that was created from the wild-type GGTTCA sequence. This did not alter the encoded amino acid residues. The underlined CAG codon encodes a Q residue in place of the original K residue of the Walker A box of ComGA. The template plasmid for mutagenesis was *pDR66-comGA*, which carries an intact copy of *comGA,* including the *comG* promoter and regulatory region, in the vector *pDR66* (Ireton *et al.*, 1993). To create *pDR66-comGA,* the plasmid pED19 (Albano *et al.*, 1989), which carries the entire *comG* operon, was cut with *Bgl*II and *Hin*dIII. The resulting *comGA* fragment was ligated to *pDR66* cut with *Bam*HI and *Hin*dIII to create *pDR66-comGA*. *pDR66-comGA* also carries a chloramphenicol resistance marker for selection in *B. subtilis* and the front and back sequences of the *amyE* gene. Both the mutant *comGA-atp* plasmid and the parent plasmid carrying the wild-type *comGA* were integrated at *amyE* and these constructs were transduced into BD2685, which carries a non-polar in frame deletion in *comGA* (Chung and Dubnau, 1998). To replace the wild-type chromosomal *comGA* with the *comGA*-atp allele, the mutant plasmid was integrated at the *comGA* locus by Campbell-like (single reciprocal) recombination, with screening for a chloramphenicol-resistant, Amy$^+$ transformant. Subsequent growth of this transformant in the absence of chloramphenicol, followed by replica plating onto chloramphenicol selective media permitted the isolation of clones in which the integrated plasmid had been lost by reverse recombination. These were screened for the presence of the *comGA-atp* mutation by PCR followed by restriction analysis using *Bam*HI.

Preparation of samples for microscopy

Samples were grown to competence (T_2) and then diluted 20-fold into fresh competence medium and incubated with shaking at 37°C. Samples were taken at T_2 and at intervals thereafter. Cells were prepared for fluorescence microscopy by fixation with 2.7% formaldehyde and 0.004% glutaraldehyde (Harry *et al.*, 1995) and permitted to attach to polylysine-coated slides. Cross-walls were visualized using PI without lysozyme treatment (Levin and Losick, 1996) or alternatively, cross-walls were visualized using WGA-TMR at a final concentration of 1 μg ml^{-1} after brief treatment with lysozyme (2 mg ml^{-1}). To visualize nucleoids, DAPI was added at a final concentration of 10 μg ml^{-1} together with the PI or WGA-TMR. Samples were mounted in Slow Fade (Molecular Probes).

FtsZ immunofluorescence

Anti-FtsZ antiserum was a kind gift from P. Levin. Antibodies were affinity purified on polyvinylidene dipluoride membranes. The membranes were prepared by blotting from polyacrylamide gels on which crude extracts of an *E. coli* strain overexpressing *B. subtilis* FtsZ (Wang and Lutkenhaus, 1993) had been resolved by electrophoresis. Antibodies were stripped from the membrane by incubation with 5 mM glycine-HCl buffer, pH 2.5. Immunofluorescence was carried out as described (Pogliano *et al.*, 1995) but without the methanol–acetone treatment. The secondary antibody was alexa 546 conjugated goat anti-rabbit (Molecular Probes).

Microscopy

Images were collected and analysed using the Openlab software package (Improvision) and then exported to Adobe

Photoshop where they were prepared for publication. We used a Zeiss Axiovert 135 M microscope equipped with an Orca digital camera (Hammamatsu), and a Zeiss 1.3 NA Plan Neo-Fluar 100× oil immersion objective. For detecting fluorescent images the following Omega Optical filter sets were used: DAPI, XF06; GFP, XF23; WGA-TMR and PI, XF34.

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