chromosome or because of reversions on the parental chromosome, double auxotrophs are often used to reduce the probability of nonplasmid-containing cells outgrowing the desired construction.

One weakness in both of these strategies is that, even when the cell loses the plasmid, the plasmid-free cell will retain for several divisions enough gene product to provide antibiotic resistance or the production of an auxotrophic factor (see Fig. 14.5). Thus, cells that will not form viable colonies on selective plates (about 25 generations are required to form a colony) can still be present and dividing in a large-scale system. These plasmid-free cells consume resources without making product.

Another related problem, particularly in large-scale systems, is that plasmid-containing cells may protect plasmid-free cells from the selective agent. For example, auxotrophic cells with a plasmid may leak sufficient levels of the auxotrophic factor that plasmid-free cells can grow. With an antibiotic, the enzyme responsible for antibiotic degradation may leak into the medium. Also, the enzyme may be so effective, even when retained intracellularly, that all the antibiotic is destroyed quickly in a high-density culture, reducing the extracellular concentration to zero. Although genes allowing the placement of selective pressure on a culture are essential in vector development, the engineer should be aware of the limitations of selective pressure in commercial-scale systems.

The other useful addition to plasmid construction is the addition of elements that improve plasmid segregation. Examples are the so-called *par* and *cer* loci. These elements act positively to ensure more even distribution of plasmids. The mechanisms behind these elements are incompletely understood, although they may involve promoting plasmid-membrane complexes (the *par* locus) or decreasing the net level of multimerization (the *cer* locus). Recall from Example 14.1 that multimerization decreases the number of independent, inheritable units, thus increasing the probability of forming a plasmid-free cell.

Any choice of vector construction must consider host cell characteristics. Proteolytic degradation may not be critical if the host cell has been mutated to inactivate all

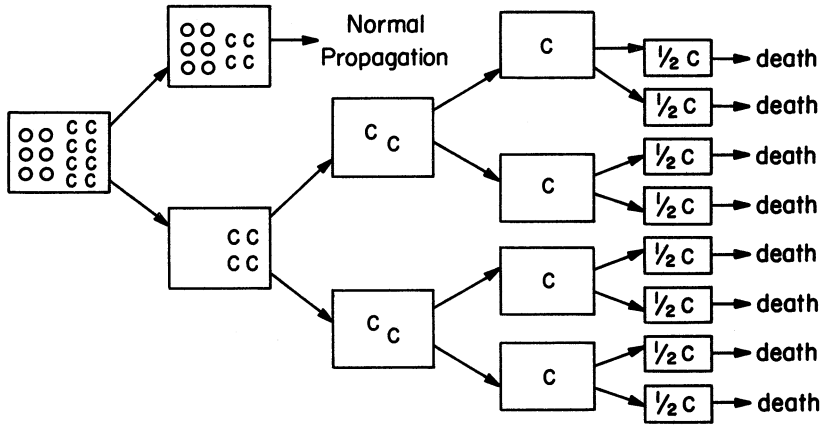

Figure 14.5. Newly born plasmid-free cells usually contain sufficient complementing factor (C) to withstand killing by a selective agent or starvation from the lack of a growth factor. In this case, the plasmid-free cell undergoes three divisions before the complementing factor is reduced to an ineffective level.