Genome economization and a new approach to the species concept in bacteria

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The direct experimental evidence presented here shows that *Escherichia coli* cells can lose a part of their DNA during prolonged starvation. Under stringent conditions cells with a reduced DNA content achieve reproductive advantage over those that maintain their original genome size. Thus, the majority or nearly all of the cells of a long-starved bacterial population undergo genome size reduction. The loss of DNA seems to occur at random in different cells of a population and, thus, their DNA content may vary significantly from one another. The heterogeneity at the DNA level seems to be reflected in conspicuous morphological variability as well. We suggest that, in evolutionary terms, the general dynamics of bacterial genome organization involve two contrasting mechanisms: genome economization (size reduction by DNA loss) and genome loading (acquisition of exogenous DNA and its maintenance in the genome). The former, strengthening the so-called *r* strategy, might have resulted in the limited genome size of prokaryotes ranging up to 9.5 Mb. The latter explains the widespread horizontal, interspecific gene transfer (general genetic mixing) in bacteria. In the light of the above findings we propose a species concept in bacteria which is comparable to the biological species concept based on reproductive incompatibility.

Keywords: genome organization; genome economization; lateral gene transfer; microbial diversity; species concept, bacteria

1. INTRODUCTION

Prokaryotes are known as organisms which are well adapted to extremely exhausted conditions (Morita 1992). In such cases they can use metabolites released by lysed cells (Stewart & Carlson 1986; Redfield 1993) and propagate at a decreased growth rate (Marr 1991). When nutrients become limiting, bacteria can differentiate through rare beneficial mutations (Zambrano *et al.* 1993) that occur at an increased rate (hypermutations) during extended starvation (Bridges 1997). These well-characterized mutants (Zambrano *et al.* 1993; Huisman & Kolter 1994; Fischer *et al.* 1998) become resistant to nutritional stresses explaining why their clones can take over stationary-phase cultures.

However, the genome analysis of long-cultured starving *Escherichia coli* populations presented here suggests the significance of other factors as well. A genome reducing mechanism that we call genome economization also seems to play an important role in the adaptation of bacteria to prolonged starvation. We present direct evidence that prokaryotic cells propagated in exhausted media can lose a part of their genetic information in response to starvation. As suggested earlier (Stouthamer & Kooijman 1993; Vellai *et al.* 1998),

bacterial cells may achieve a relatively faster reproduction rate (*r* selection) under stringent conditions by DNA reduction if deletion occurs within disused sequences coding for functions that are non-essential in reproduction. A cell with a smaller genome size has a selective advantage over a state with a larger genome size under certain conditions. It has also been demonstrated that two cells belonging to a single bacterial species may differ in some 20% of their chromosome size (Bergthorsson & Ochman 1998). Furthermore, it is well known that the size of prokaryotic genomes is limited, ranging up to 9.5 Mb (Fonstein & Haselkorn 1995). In agreement with the limitation of genome size, genome economization may be one of the most important factors in the organization and evolution of prokaryotic genomes. The limited genetic potential due to genome streamlining might have been the barrier against a further increase in cellular complexity (Vellai *et al.* 1998; Vellai & Vida 1999), explaining why present-day bacteria are presumably no more complex than their ancestors were two billion years ago (Maynard Smith & Szathmáry 1995). The view of genome economization is also supported by some recent studies on completely sequenced bacterial genomes (Blattner *et al.* 1997; Andersson & Kurland 1998; Andersson *et al.* 1998; Lawrence & Ochman 1998) which have demonstrated the loss of a substantial amount of genomic DNA in the speciation dynamics of prokaryotes.

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Figure 1. The growth curve of a 'natural' population of the *E. coli* XL1-Blue strain which was cultured in a non-selective and gradually exhausting LB medium. Cells of the starting culture carried a plasmid-encoded ampicillin resistance gene. In the late decline phase of the growth cycle the number of cells retaining the plasmid DNA decreases (dashed curve and black triangles), whereas the proportion of cells losing their plasmid DNA becomes larger. The solid curve illustrates the total number of cells (circle). The reproduction rate of cells losing their 'unnecessary' DNA content increases relative to those maintaining their original genome size.

2. MATERIAL AND METHODS

(a) Long-term culturing of *E. coli* cells

Escherichia coli XL1-Blue strain carrying a plasmid-encoded ampicillin resistance gene was grown in a non-selective Luria broth (LB) medium (Sambrook *et al.* 1989) for 55 days under optimal conditions (incubated at 37 °C and shaken at 300 r.p.m.). For cell counting, some of the culture was spread onto both LB and LB^{Amp} plates every day in an appropriate dilution and single colonies were counted (figure 1).

(b) Genome size determination by pulsed-field gel electrophoresis

Escherichia coli CSH strain was cultured in gradually exhausted LB medium for more than one month (shaken at 300 r.p.m. and incubated at 37 °C). After the 50th day of propagation, some of the starved culture was spread onto fresh LB plate. After incubation, single colonies were separately transferred into fresh LB media and grown to OD₆₀₀=1. Cells from different cell clones were embedded into low melting agarose (Sigma) and their total genomic DNA was prepared by lysozyme-proteinase K treatment. Having a relatively rare cutting site, the unbroken DNA was fragmented into large pieces by *in situ* digestion with *NotI* restriction enzyme and screened by pulsed-field gel electrophoresis (PFGE). This technique makes the analysis of the DNA structure of entire bacterial genomes possible (Smith *et al.* 1987). A 1% agarose gel (15 cm) was used in a 33 cm × 33 cm Pharmacia LKB apparatus. The running conditions were 170 V for 26 h with a 10–30 s pulse time at 14 °C. The gel was stained with ethidium bromide and photographed with ultraviolet light in the conventional manner (figure 2a).

(c) Electron microscopy

Escherichia coli CSH cells were propagated in LB medium for more than one month with vigorous shaking (300 r.p.m.) at 37 °C and a part of the starving culture was subsequently transferred onto a fresh LB plate. The cells were grown (the proportion of dead cells was negligible), then 1% glutaraldehyde solution buffered with sodium cacodylate (0.1 M and pH = 7.2)

was poured onto the culture and a mixed suspension was made in the fixative with the help of pipetting. Thereafter, the cells were examined either *in toto* on a coated grid after staining with 0.5% phosphotungstic acid (figure 3a) or the bulk of the suspension of the cells was embedded in 2% agar. Post-fixation was carried out in cacodylate-buffered 0.5% OsO₄ solution. After staining with uranyl acetate, the samples were dehydrated and embedded in Durcupan (Fluka) cut with an ultramicrotome, stained with lead citrate and examined in an electron microscope (Robards & Wilson 1993) (figure 3b–f). The great majority of the cells shown by the pictures of the transmission electron microscopic sections (figure 3b–f) were alive at the time of fixation as revealed by the quality of their intracellular material.

3. RESULTS

(a) Experimental evidence for genome economization

An *E. coli* population bearing copies of a plasmid with a gene encoding ampicillin resistance was cultured in a gradually exhausted non-selective medium simulating the natural conditions that are generally characterized by a limitation of food resources. The growth rate of this population shows that, in the late decline phase of the growth cycle, the proportion of cells losing their plasmid DNA becomes increasingly larger relative to those retaining their plasmid (figure 1). The gene encoding ampicillin resistance was not lost when nutrients were plentiful (two to five day culture), not even in antibiotic-free medium, despite the fact that in this case it possesses no actual function. The loss of plasmid DNA, i.e. the reduction in DNA content, occurred in the late decline phase, implying that disused ('unnecessary') sequences reduce the fitness of bacteria under starvation. This experiment indicates that (i) the cells of a prokaryotic population tend to possess a decreasing DNA content during prolonged starvation, and (ii) the non-essential DNA may be best located on plasmids, as was proposed by Stouthamer & Kooijman (1993).

Our molecular data of entire genomes obtained by PFGE clearly show that the DNA content of clones of seven individual *E. coli* cells from nine randomly chosen ones deriving from a starved population differs (loss or rearrangement of bands, as indicated by the arrows in lanes 4–12 of figure 2a) from that of the initial cells (lane 2 of figure 2a). In cells derived from the exponential- or stationary-phase culture (two to three day culture), there was no detectable genome reduction or heterogeneity by PFGE (lane 3 of figure 2a). However, after one month of propagation in exhausted medium the majority of the starved cells underwent genome size reduction, implying that, in the long term, nearly all cells of the starving population accumulate deletions. For instance, in lane 9 of figure 2a a ca. 30 kb deletion allows B and C bands to be distinguished. Most cells lack the 'Q' band the size of which (ca. 100 kb) corresponds to the 'F' plasmid (Smith *et al.* 1987). This observation, in accordance with our first experiment, strongly supports the suggestion that non-essential DNA may be best located on plasmids (Stouthamer & Kooijman 1993). Based on the physical map of *E. coli* K12 (Smith *et al.* 1987), with the exception of the band indicated by asterisks in figure 2a (see the order of *NotI* bands in figure 2b), the shifted and lacking

Figure 2. (a) Pulsed-field gel separation of *NotI* restriction fragments of the genome of long-starved *E. coli* CSH cells. Lane 1 is the lambda ladder PFGE marker, lane 2 is the genomic DNA of a clone of an individual *E. coli* cell cultured in rich media (see the physical map of the *E. coli* K12 genome in Smith *et al.* (1987)), lane 3 is the *NotI*-digested genome of a clone of a cell deriving from the stationary-phase (two-day) culture and lanes 4–12 are genomic DNAs of clones of individual cells from a long-starved population. The arrows indicate lacking or shifted *NotI* bands due to large deletions which occurred in the genome (with the exception of the band indicated by asterisks; see the text) as implied by the analysis of the physical map of *E. coli* K12. For example, the series of arrows at the bottom indicate *ca.* 100 kb DNA loss. This size corresponds to the band of the 'F' plasmid (see Smith *et al.* 1987) that has been lost in most starving cells. (b) The order of *NotI* bands of the genome of *E. coli*. The physical map of the genome of *E. coli* was determined in Smith *et al.* (1987).

bands (arrows) resulted from large deletions that had occurred in the genomic DNA and not from single-base mutations within *NotI* recognition sites. In the latter case, if the position of a fragment alternates, the position of another *NotI* fragment should also be changed due to either the loss of a former or appearance of a new *NotI* restriction site (see the arrows indicated by asterisks). We think it reasonable to assume that starved cells also possess several microdeletions (loss of a few base pairs) due to mutations which, however, are not detectable by PFGE. Similar genome decay has been previously reported in the starved marine bacterium ANT-300 (Moyer & Morita 1989; Morita 1992) as well as in starved *Mycoplasma capricolum* cells (Seto & Miyata 1998). However, these observations were based on indirect measurement of the DNA content of cells by microscopy. In many cases, prokaryotic cells from the exponential growth phase contain their chromosome in more than one copy. This may imply the possibility of a reduction in the copy number of chromosomes. To avoid the effect of such factors, we analysed the genome by PFGE which provided compelling evidence for genome size reduction.

Because the loss of DNA occurs at random in different cells (or more precisely it may be random within sequences encoding non-essential functions), their DNA contents may differ significantly from one another (figure 2). As has been suggested (Matic *et al.* 1995), adverse environmental conditions induce the SOS response and repress the mismatch repair system of bacteria, increasing the genetic variation and, thus, the fitness of stressed bacterial populations. In agreement with the studies of Milkman & Bridges (1990) and Milkman (1997) on chromosome evolution in *E. coli*, the

population dynamics of the starved *E. coli* culture presented here reveal (figure 2b) that the clonal frame (the initial DNA sequence) of a population loses its uniformity in nucleotide sequence as mutations (or recombination introducing new DNA from other clones; Milkman 1997) occur within different cells and remain in their (sub)clones. It seems that bacteria are genetically diverse not only within species (for example, the genome size in natural isolates of *E. coli* can vary by as much as 1 Mb, which was shown by Berghthorsson & Ochman (1998)), but within natural populations as well.

(b) Morphological heterogeneity in starving *E. coli* populations

The heterogeneity at the DNA level seems to be reflected at the cell morphology level as well. The originally rod-shaped and uniform-sized cells from normal growth conditions become morphologically diverse as a result of starvation. Both the size and the appearance of starved, but viable, cells may differ greatly from one another as shown by electron microscopy. Many of them have a stretched rod, coccoid or even branched shape (figure 3a). These observations imply a higher microbial diversity than previously thought.

4. DISCUSSION

(a) The size restriction and dynamics of the prokaryotic genomes

Under long-term starvation, when the availability of energy is limited, the nucleotide synthetic apparatus of the non-compartmentalized bacterial cell is subsaturated with substrates and catalytic components (Jensen & Pedersen 1990). As a consequence of the limitation in

Figure 3. Conspicuous and frequent morphological heterogeneity from a starving *E. coli* population as revealed by electron microscopy. (a) A randomly selected sample of total cells stained by phosphotungstic acid shows different forms with various shapes and sizes (magnification $\times 5,600$). Note that even dividing cells may differ considerably in length and width. (b-f) Morphological heterogeneity of cells in section at higher magnification ($\times 7,100$) demonstrating among others stretched rod and branched cells (arrows).

DNA synthesis, an inverse correlation can be seen between the growth rate and DNA content of starving cells belonging to a single microbial population (Vellai *et al.* 1998). Mutations such as deletions occurring randomly in different cells arise at an unexpectedly increased rate (hypermutation) during nutritional stress (Zambrano *et al.* 1993; Bridges 1997). Mutator genotypes with increased mutation rates produce rare beneficial mutations more often than wild-type genotypes, allowing a faster response to selection. Deletion may cause cell death or may result in (within the disused DNA; Stouthamer & Kooijman 1993) the loss of a part of the genetic information that is not expressed under conditions of extended starvation (Vellai *et al.* 1998), obligate parasitism or an intracellular life style (Andersson & Kurland 1998). This might explain why the non-coding DNA content of bacterial genomes is relatively low (ca. 9% on average; Andersson *et al.* 1998), as compared to that of eukaryotes. Cells with a reduced DNA content can achieve a faster reproduction rate relative to those maintaining their original genome size, which strengthens the so-called *r* strategy (Carlile 1982). On the basis of the experimental evidence presented here, we propose that, under prolonged starvation, when cells compete for survival and propagation, one of the most important driving forces in bacterial genome organization is the genome size reduction through deletion. Since cells retaining their original DNA content are selected out by those with a reduced genome by overgrowth, presumably the majority or nearly all of the cells of the long-starved population undergo substantial genome reduction.

Contrary to the loss of genetic material, more is known about the DNA acquiring mechanisms that occur when cells are transferred into favourable conditions. Bacterial cells are able to take up exogenous DNA, mostly actively, by genetically regulated natural transformation, conjugation or transduction (Solomon & Grossman 1996) and recombine it into their genome by homologous recombination. The double-strand DNA gaps (deletions) can be

repaired through gene conversion copying template sequences derived from DNA uptake (Kusano *et al.* 1994). This mechanism, which is capable of restoring lost genetic information, provides an advantage for cells in constantly changing environments. Moreover, the DNA uptake frequently takes place over large phylogenetic distances (Munoz *et al.* 1997; Aravind *et al.* 1998; Chistoserdova *et al.* 1998), leading to a widespread horizontal, interspecific gene transfer in bacteria.

These dual dynamics of the bacterial genome organization, genome reduction due to selection for a faster growth rate and the acquisition of exogenous DNA for adaptation to changing conditions, have indeed been demonstrated in evolutionary terms (Martin 1999). In some recent studies on completely sequenced prokaryotic genomes, Blattner *et al.* (1997) and Lawrence & Ochman (1998), as well as Andersson *et al.* (1998), demonstrated the existence of extreme decreasing and increasing mechanisms of genome size in prokaryotic speciation dynamics. For instance, an unexpectedly dynamic evolution of the *E. coli* genome has been presented: the chromosome of *E. coli* acquired ca. 1600 kb of horizontally transferred DNA after diverging from *Salmonella enterica*. Most of the transferred DNA was deleted subsequently, as well as a significant part of the ancestral DNA, resulting in 547.8 kb of transferred genes maintained in the *E. coli* genome. On the basis of these data we state that the universal genome organization of prokaryotes consists of two contrasting mechanisms: the genome reducing and the genome reloading processes. It is the dynamics of these two processes that have resulted in the limited genome size of bacteria ranging up to 9.5 Mb (Fonstein & Haselkorn 1995) and their general genetic mixing (figure 4).

(b) *The species concept in bacteria*

Bacteriologists have long tried to develop a species concept that is comparable to the biological species concept based on reproductive incompatibility (Mayr 1942). This effort is seemingly futile because of the

Figure 4. Schematic representation of DNA flow through prokaryotic chromosomes over time. The general dynamics of genome organization of prokaryotes involve two opposing mechanisms: genome loading through the acquisition of exogenous DNA and its maintenance in the genome and DNA loss by deletion. The former explains the widespread interspecific gene transfer in bacteria. The latter, which strengthens a faster reproduction rate, may have resulted in the limited genome size of prokaryotes. The non-exchangeable genes (grey) are related to the most ancient biological processes such as protein synthesis and core energy conversion. They represent a group within the so-called essential, non-losable genes. These genes can evolve through mutations only, i.e. without interspecific gene exchange, providing the genetic basis for the separation of species. When the flow of such genes across populations is interrupted due to spatial isolation, the accumulation of sequence divergence leads to the starting of a speciation process.

widespread interspecies gene exchange in bacteria. However, our results on genome economization and recent data confirming dramatic changes in genome size during the evolution of prokaryotes (Blattner *et al.* 1997; Andersson & Kurland 1998; Andersson *et al.* 1998; Lawrence & Ochman 1998) led us to present a model for the universal dynamics of prokaryotic genome organization (figure 4), providing a possible solution to this outstanding problem of microbial taxonomy.

Bacteria are the only organisms that have been selected for their ability to take up exogenous DNA actively and recombine it into their genomes (Maynard-Smith *et al.* 1991). The uptake of exogenous DNA occurs frequently among distantly related taxa of prokaryotes, resulting in the lateral gene transfer which appears to be one of the most important processes in their speciation dynamics (Lawrence & Ochman 1998; Martin 1999). In principle, any bacterial gene can be transferred and expressed; however, only certain sequences can be maintained in evolutionary terms. A newly acquired gene or an ancestral one which possesses no actual function over a long period of time is likely to be lost from the genome through deletion (Blattner *et al.* 1997; Andersson & Kurland 1998; Andersson *et al.* 1998; Lawrence & Ochman 1998; Vellai *et al.* 1998).

However, despite the general genetic mixing, partial reproductive isolating mechanisms may exist in prokaryotes (Maynard Smith *et al.* 1991; Woese 1998) due to barriers against lateral transfer. Genetic barriers that separate prokaryotic species by preventing interspecies genetic exchange of certain genes are primarily recombinational (Matic *et al.* 1995). For example, the comparative sequence analysis of the *E. coli* genome with other representative genomes sequenced so far has revealed genes for proteins that are either highly conserved across various species (they are mainly translation proteins such as aminoacyl synthetases and ribosomal proteins) or unique, having no match in any other genome (Blattner *et al.*

1997). Thus, there are certain subsystems in prokaryotic genomes which are integrated through encoded proteins into a specialized, complex cellular function and, thus, cannot change through lateral transfer (Woese 1998). Concerning sequence transferability and maintainability, we state that prokaryotic genomes can be divided into two parts: exchangeable and non-exchangeable (non-compatible) sequences. The latter ones cannot be transferred functionally between species, i.e. they evolve without interspecies exchange, because their products function in complex structural interactions. If such genes change (become exchanged by another one) or are deleted, then the cell either dies immediately or its clone, which has a smaller reproduction rate, is selected out over time. For instance, rRNAs with a specific, highly conserved structure and ribosomal proteins together form the functional site of ribosomes. A component of such complexes can only be exchanged if the other components are modified simultaneously, maintaining the structure-dependent original function. Furthermore, these genes are often present in more than one copy in the genome as cases of genetic redundancy. At present, we are still in the initial stages of gathering the data provided by comparative genomics which will lead to an exact determination of the non-exchangeable sequences. As additional bacterial genomes are sequenced, exchangeable and non-exchangeable patterns may begin to be identified.

In contrast to eukaryotic species, which are usually separated by reproductively isolated genomes, prokaryotic species can be characterized primarily by reproductively isolated sequences. In bacteria, divergence from an ancestral species occurs when a part of the originally entirely exchangeable sequences becomes non-exchangeable (partial reproductive isolation) due to constantly changing niches. As a result of the spatial isolation, the gene flow (within the so-called non-exchangeable sequences) between two or more populations is interrupted, initiating the process of speciation. During the long isolation process, the

exchangeable genes, the acquisition of which generally presents an immediate adaptive advantage, can be frequently transferred between species. This lateral transfer may cause a considerable variation in DNA sequences within a species. Such transferable genes may strengthen the so-called sequence isolation process through the acquisition of new properties (ecotypes) and contribute to a rapid adaptation to novel environments.

Presumably, at an early stage in the evolution of living systems, the transfer of genetic information was pervasive and non-specific (Woese 1998). Later, however, parallel to the increase in genetic and cellular complexity, certain sequences became connected to each other through the interacting cellular functions that they encoded and evolved together to accommodate various conditions through mutations only. This has led to the existence of genetic barriers, implying that the definition of reproductive isolation (at least partial isolation) can be extended to bacteria.

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