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PHENOTYPIC HETEROGENEITY IN BACTERIAL POPULATIONS

Darja ŽGUR-BERTOK [a)]

[a)] Univ. of Ljubljana, Biotechnical Fac., Dept. of Biology, Večna pot 111, 1000 Ljubljana, Slovenia, Prof., Ph.D., e-mail: darja.zgur.bertok@bf.uni-lj.si.

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ABSTRACT

Genetically uniform bacterial cells exhibit heterogeneity such as intrapopulation differences in metabolism as well as variation in growth rate. Additionally, phenotypic heterogeneity in more complex developmental processes where a portion of a population performs specialized functions has been described. Heterogeneity within populations of bacterial cells ensures that a small fraction of the population is prepared to survive adverse environmental conditions. Phenotypic heterogeneity is mediated by two mechanisms: (i) genotypic alterations such as, mutations and rearrangements of specific DNA fragments or (ii) epigenetic phenomenon. Here examples of genotypic as well as epigenetically regulated phenotypic heterogeneity from several bacterial species are presented.

Key words: microbiology / bacteria / phenotypic heterogeneity / genotype / epigenetic regulation

FENOTIPSKA HETEROGENOST V BAKTERIJSKIH POPULACIJAH

IZVLEČEK

Genetsko enake bakterijske celice izkazujejo heterogenost kot sta npr., znotraj populacijske razlike v metabolizmu in hitrosti rasti. Poleg tega so opisani primeri fenotipske heterogenosti zapletenih procesov razvoja pri katerih del populacije vrši posebne naloge. Heterogenost znotraj populacij bakterijskih celic zagotavlja, da je majhen del populacije pripravljen na morebitne neugodne pogoje okolja. Fenotipsko heterogenost posredujeta dva mehanizma: (i) genotipske spremembe kot so mutacije in prerazporeditve določenih fragmentov DNA ali (ii) epigenetski pojavi. V pričujočem prispevku so opisani primeri genotipske kakor tudi epigenetsko uravnane fenotipske heterogenosti nekaterih vrst bakterij.

Ključne besede: mikrobiologija / bakterije / fenotipska heterogenost / genotip / epigenetsko uravnavanje

INTRODUCTION

Genetic differences and environmental influences are the basis of variation among individuals. However, variation is also observed in genetically identical organisms and cells under the same environmental conditions. The advent and increased use of flow cytometry and fluorescence microscopy to analyse individual cells has revealed a number of examples of heterogenous gene expression in genetically uniform populations of bacteria. Microorganisms have to survive and multiply in often rapidly changing and hazardous environments. Alterations in availability of nutrients, temperature, salinity, osmolarity and pH are frequent. Additionally, microorganisms are repeatedly confronted by adverse conditions such as, antibiotics, toxins, mutagens, bacteriophage and radiation. It is presumed that phenotypic heterogeneity ensures that some bacterial cells are better prepared if a sudden change in environmental conditions occurs.

Phenotypic heterogeneity can arise due to genotypic alterations such as genome rearrangements or mutations. Additionally, phenotypic heterogeneity can also be mediated by heterogeneity in expression of individual genes due to epigenetic phenomenon where no genotypic alterations are involved and growth conditions are homogenous. Such heterogeneity arises due to "noise", random fluctuations in rates of protein synthesis and degradation. Two types of noise are distinguished: intrinsic and extrinsic (Swain *et al*., 2002). Intrinsic noise is a reflection of random bursts of activity of a promoter while extrinsic noise reflects cell to cell variation in activity of proteins regulating expression of a given gene. Noise can sometimes lead to bistability when a population of genetically identical cells forms two subpopulations. Individual cells in such populations follow or do not follow a specific developmental pathway.

In the presented review examples of phenotypic heterogeneity within bacterial populations are presented and some of the pathways responsible for heterogeneity are discussed. Cases of phenotypic heterogeneity based on genotypic modifications as well as on epigenetic phenomenon are presented. Some are well documented while others have received only recent attention.

GENOTYPIC MODIFICATIONS AND PHENOTYPIC HETEROGENEITY

Phase and antigenic variation

Phase variation involves reversible alterations in specific genomic loci. The best characterized are those engaged in the synthesis of antigenic surface structures such as outer lipopolysaccharides (LPS), pili and flagella (Henderson *et al*., 1999; Hallet 2001). Phase variation assists bacteria in evading host immune defences and colonisation of new ecological niches. To meet this end bacteria have evolved a number of molecular mechanisms. Generally, some act by turning individual genes "on" or "off", while others enable expression of multiple phenotypes via rearrangements of DNA sequences. Phase variation has been described in a number of bacterial pathogens namely, *Salmonella thyphimurium*, *Neisseria gonorrhea, Neisseria meningitidis*, *N. gonorroehae*, *Haemophilus influenzae* and *Escherichia coli* (Dybvig *et al*., 1993). Phase variation can be mediated by reversible changes in the length of short DNA sequence repeats associated with genes for surface structures. Nucleotide sequences are gained or lost by slipped-strand mispairing (SSM) (van Belkum *et al*., 1998), which can take place during chromosomal replication as well as DNA repair or recombination. Changes in DNA repeat length can result in translation frameshift mutants.

Phase variation can also be mediated by inversion of a DNA fragment belonging to a specific locus via recombination (Hallet and Sheratt, 1997). For example *Salmonella typhimurium*, by inverting a promoter with respect to the structural gene switches expression of alternative alleles for flagellin, the flagellum protein. Alternatively, some pathogens, such as *N. gonorroehae* exhibit antigenic variation of pilin, the structural protein of pili, which is mediated by intramolecular recombination between variable silent DNA casettes and expre ssed loci (Howell-Adams and Seifert, 2000). Recombination between silent and expressed genes has also been demonstrated for variable surface lipoproteins and proteins in *Borrelia burdorferi* and *B. hermsii*, respectively (Casjens *et al*., 2000; Barbour and Restrepo, 2000). Additionally, in species exhibiting natural competence for transformation, such as *Neisseria meningitidis,* intergenomic recombination to yield phase variation occurs between cells (Swartley *et al*., 1997).

Mutators

Another example of genotypic heterogeneity is the appearance of bacterial cells designated mutators. Under constant environmental conditions mutation rates are low as most are deleterious. However, when bacterial populations are faced with variable environmental conditions higher mutation rates are detected (Ishii *et al*., 1989; Travis and Travis 2002). Mutators, bacterial cells with high mutation rates relative to the wild type have been described within different bacterial species and have been detected at high frequencies among pathogenic bacteria (LeClerc *et al*., 1996; Oliver *et al*., 2000; Richardson *et al*., 2002).

In laboratory strains of *E. coli* and *Salmonella typhimurium,* mutators arise at frequencies of 10^{-5} to 10^{-6} (LeClerc *et al*., 1998, Boe *et al*., 2000,) while in natural isolates mutators occur at much higher frequencies of approximately 1–5% (LeClerc *et al*., 1996). Commonly, mutators in natural strains have defects in genes involved in DNA repair, such as the methyl-directed mismatch repair pathway (MMR) genes, *mutS*, *mutL*, *mutH* and *uvrD* (LeClerc *et al*., 1996, Matic *et al*., 1997). The MMR pathway is a DNA repair system that corrects base mismatches in newly replicated DNA and also represents the main barrier preventing recombination of mismatched heteroduplexes. Defects in MMR thus allow a broader range of interspecies DNA exchange which implicates that horizontal gene transfer influences survival and growth. Additionally, evidence also suggests that mutators could play an important role in the evolution of resistance to antibiotics.

EPIGENETIC REGULATION AND PHENOTYPIC HETEROGENEITY

The *lac* network

A classical example of epigenetic heterogeneity is the utilization of lactose as a source of carbon and energy (reviewed in Miller and Reznikoff, 1978). The protein products of the *lac* structural genes *lacZYA,* enable uptake and utilization of β-galactosides. *lacZ* encodes the enzyme β-galactosidase which breaks a β-galactoside, such as lactose, into glucose and galactose; *lacY*, encodes a permease responsible for the transport of lactose into the cell and *lacA* encodes a β-galactoside transacetylase that transfers an acetyl group from acetyl-CoA to β-galactosides (Figure 1). A separate operon encodes the repressor LacI which prevents expression of the three structural genes. LacI is in turn inhibited by allolactose. As early as 1957, Novick and Weiner showed that an *E. coli* population with a low level of *lac* operon induction yields two populations of cells, one with high *lac* expression and the other non *lac* expressing. The *lac* system has subsequently been very well characterized (Ozbudak *et al*., 2004). In the absence of lactose cells harbor only very few molecules of β-galactosidase. Immediately following the addition of lactose only a few cells will be able to utilize lactose. In these lactose utilizing cells random, noninduced transcription of the *lac* operon occurs as the repressor is present in only approximately 10 molecules. It follows that enzyme levels within the bacterial population are heterogeneous. Cells with high levels of permease accumulate lactose more rapidly and further in these cells, due to higher levels of β-galactosidase, transglycosylation proceeds to form the inducer, allolactose. This in turn leads to increased transport of lactose, transglycosylation, induction and production of galactose and glucose as sources of carbon and energy. Subsequently, variability decreases as the lactose metabolizing cells become predominant.

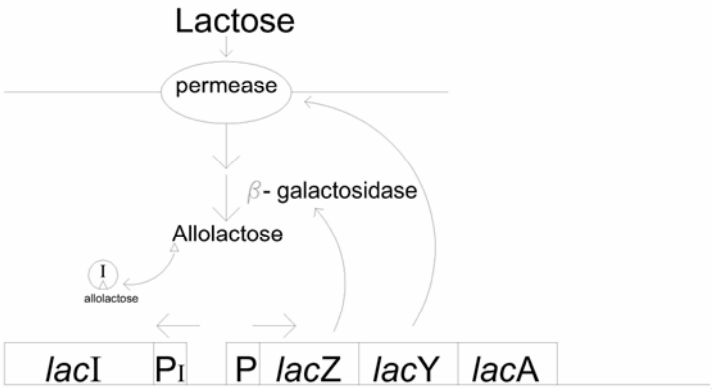

Figure 1. Induction of the *lac* operon.
Slika 1. Indukcija operona *lac*.

Lysogeny versus the lytic cycle

Another example of heterogeneity is the outcome of infection of *Eschericiha coli* cells by bacteriophage λ (Campbell 1996). Infection can result in either lysis or lysogeny (integration of bacteriophage DNA into the bacterial chromosome) due to competition between the cI repressor and Cro protein for the operators of the two divergent early promoters P_L and P_R. The operators consist of three binding sites which have different orders of binding for cI and Cro. In a lysogenic cell, cI has gained occupancy of the operators however, if it is replaced by Cro, the lytic cycle follows (Folkmanis *et al*., 1977) (Figure 2). The first genes transcribed from the two early promoters P_L and P_R are leftward *N* and rightward *cro*. *N* is an antiterminator of transcription which allows expression of the downstream *cII*. The cII protein is labile due to protease degradation however, if sufficient concentrations are available, it will together with the cIII protein activate the *cI* repressor gene, switching off expression of *cro* which is crucial for the lytic cycle. Again a key regulator the cI repressor, is present in only a few copies per cell. Thus, lysogeny is maintained by the continuous production of the repressor as well as by its adequate partitioning to the two daughter cells at cell division (Dodd *et al*., 2005).

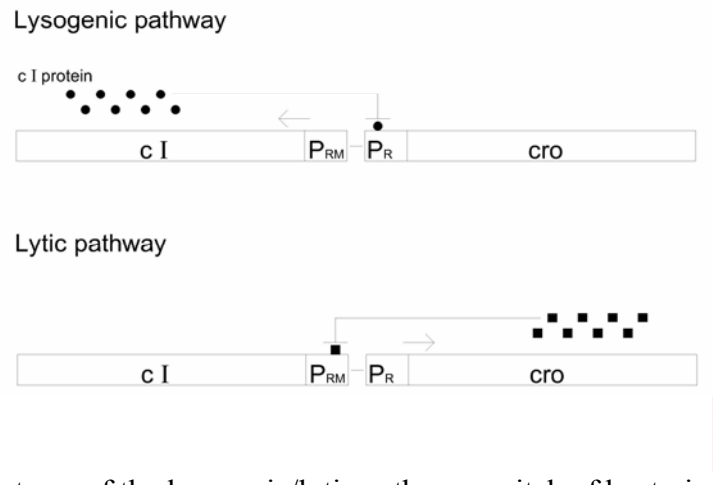

Figure 2. The key features of the lysogenic/lytic pathway switch of bacteriophage λ.
Slika 2. Poglavitne značilnosti preklopa lizogene/litične poti bakteriofaga λ.

Persisters

In 1944, Bigger discovered that even though the addition of penicillin to staphylococci induced lysis, a small fraction of the cells remained viable even after prolonged incubation and resumed growth when the antibiotic was removed. The surviving cells were named persisters. Subsequently, persisters were described in various bacterial species following treatment with antimicrobials (Brooun *et al*., 2000; Keren *et al*., 2004). Recently, biofilm resistance to antimicrobials has been shown to be based on the presence of persisters (Spoering *et al*., 2001). The proportion of persister cells has been shown to be higher in a stationary phase population, with a pronounced increase at mid to late-exponential phase in *E. coli*, *P. aeruginosa* and *Staphylococcus aureus* (Keren *et al*., 2004). Persisters exhibit a state of reduced growth which is entered into spontaneously and is a pre-existing characteristic and not produced in response to antibiotic treatment (Balaban *et al*., 2004). As yet only the *hipAB* operon, encoding a toxin-antitoxin module, has been identified as affecting development of a persister state. HipA is a toxin inhibiting macromolecular synthesis and promoting persistence by protecting the cells from the damaging effects of antibiotics that act in a growth-dependent manner (Keren *et al*., 2004a).

Regulation of competence in Bacillus subtilis

A well characterized example of bistability is the development of competence for transformation in B. subtilis. Transformation comprises the uptake of naked DNA from the environment and subsequent recombination which incorporates the DNA into the bacterial genome.

Competence development in *B. subtilis* is expressed as a response to nutrient limitation and quorum sensing. Even under optimal conditions only a small fraction of the cells in a culture will develop competence (reviewed in Dubnau 1999; Hamoen *et al*., 2003). ComK is the key transcription factor regulating transformation which includes DNA-binding, uptake and recombination. Regulation of *comK* expression is complex and involves autoregulation. However, even under conditions optimal for competence development only up to 10% of the cells will synthesize ComK and develop competence. Expression of ComK is tightly regulated and is induced in response to nutrient limitation and quorum sensing, the ability of bacteria to communicate and coordinate behaviour via signaling molecules. Binding of ComK as a tetramer composed of two dimers induces *comK* transcription. An autostimulatory loop of *comK* expression is the only factor required for bistability of *comK* expression (Figure 3). In some cells, due to noise in expression, the concentration of *comK* exceeds a threshold, activating the positive loop and subsequently competence development.

Figure 3. Positive autoregulation of *comK* is central for competence development in *B. subtilis*.
Slika 3. Pozitivno samo uravnavanje je ključno za razvoj kompetence pri *B. subtilis.*

On the other hand in *Streptococcus pneumoniae,* natural competence is presumed to be developed by the large majority of the population under inducing conditions. However, it has recently been shown that DNA is actively released by competence-induced lysis of a subfraction of the cells (Steinmoen *et al.*, 2002). Cell lysis and DNA release is brought about by lysins such as LytA synthesized by competent cells and which are most probably attached to the cell surface (Steinmoen *et al*., 2003).

Sporulation

Sporulation, the formation of a dormant spore which exhibits extreme resistance to environmental conditions is another example of bistability in *B. subtilis*. The key transcriptional regulator of sporulation is Spo0A and its activity is controlled by phosphorylation mediated by a phosphorelay system (Burbulys *et al*., 1991). Under nutrient limiting conditions only some cells activate Spo0A. Sporulation is energy consuming and is irreversible following an early stage. Similarly as for ComK, a positive feedback loop involving transcription of *spo0A* and its phosphorylation controls bistability of sporulation gene expression. A threshold level of active Spo0A has to be reached in order to initiate sporulation (Chung *et al*., 1994). Additionally, sporulating cells can produce a killing factor which destroys nonsporulating cells (Gonzalez-Pastor *et al*., 2003). Nutrients that are released by the non sporulating cells can be used by the sporulating cells. Further, bistability of entry into sporulation allows remaining cells which had not sporulated to resume growth upon sudden nutrient availability.

Cell lysis in Pseudomonas aeruginosa biofilm development

Bacteria often grow in biofilms where they undergo complex differentiation (Stoodley *et al.*, 2002). Microcolony formation within a biofilm is a coordinated, adaptive response that facilitates biofilm development and dispersal. Quorum sensing has been shown to be involved in microcolony development in some organisms for example: P. aeruginosa (Davies *et al.*, 1998), Burkholderia cepacia (Huber *et al.*, 2001) and Aeromonas hydrophila (Lynch *et al.*, 2002).

Recently, cell death – killing inside microcolonies has been demonstrated in wild-type *P. aeruginosa* biofilms (Webb *et al*., 2003). Prophage-mediated cell death and lysis inside microcolonies have been linked with the accumulation of reactive oxgygen species (ROS). Prophage - mediated cell death benefits a subpopulation of surviving cells and has an important role in subsequent biofilm differentiation and the dispersal of surviving biofilm cells. The dispersing cells have been shown to be more similar to planktonic cells than to mature biofilm cells, indicating that dispersing biofilm cells revert to the planktonic mode of growth (Sauer *et al*., 2002).

Colicin production

Colicins are plasmid-encoded bacteriocins, synthesized by and active against cells of Escherichia coli and sometimes related species such as Shigella and Salmonella spp. Colicin-producing strains are found with high frequency among natural isolates and have been demonstrated to play a role in intraspecies population dynamics (Kirkup and Riley, 2004). Production of colicins is characteristically encoded by a cluster of three genes: a gene encoding the colicin activity protein; the immunity gene encoding the immunity protein which potects the producing strain and a lysis gene, encoding the lysis protein. Regulation of colicin K is induced primarily by an increase in ppGpp due to nutrient depletion (Kuhar and Žgur-Bertok, 1999; Kuhar *et al.*, 2001). Furthermore, colicins are released semispecifically, by cell lysis and the colicin K activity gene cka is expressed in only 3% of the bacterial population upon induction by nutrient starvation. On the other hand the immunity gene is expressed in the large majority of

cells. The LexA protein has been shown to play a key role in establishing differential expression of colicin synthesis at the level of transcription (Mulec *et al.*, 2003).

CONCLUSIONS

Bacteria have evolved molecular mechanisms which allow divergence into populations of phenotypically separate subpopulations. Heterogeneity within bacterial populations can be mediated by specific genotypic modifications or alternatively, by epigenetic mechanisms. In this review several examples have been presented however, it is quite likely that such phenomenon are very widespread. As bacteria evolved these mechanisms to survive adverse environmental conditions understanding the molecular basis of bacterial heterogeneity has important implications for the food preservation industry and for antimicrobial chemotherapy.

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