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minireview

Two Tales of Prokaryotic Genomic Diversity: *Escherichia coli* and Halophiles

*Lejla Pašić, Jerneja Ambrožič Avguštin, Marjanca Starčič Erjavec, Blagajana Herzog-Velikonja, Zdravko Podlesek and Darja Žgur-Bertok**

Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia

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Summary

Prokaryotes are generally characterized by vast genomic diversity that has been shaped by mutations, horizontal gene transfer, bacteriocins and phage predation. Enormous genetic diversity has developed as a result of stresses imposed in harsh environments and the ability of microorganisms to adapt. Two examples of prokaryotic diversity are presented: on intraspecies level, exemplified by *Escherichia coli,* and the diversity of the hypersaline environment, with the discussion of food-related health issues and biotechnological potential.

Key words: Escherichia coli, genomic diversity, virulence, antibiotic resistance, SOS response, bacteriocins, halophiles, metagenomics

Introduction

Prokaryotes may exhibit vast intraspecies as well as intra-habitat genomic diversity. The basis of diversity among bacteria are mutations in concert with short generation times, horizontal gene transfer, production of bacteriocins and phage predation. Man-induced influences, namely indiscriminative antibiotic use, various food additives, intensive animal husbandry and global travel have in the last decades promoted rapid evolution and dissemination of antibiotic resistance and virulence factors.

In addition, prokaryotes inhabit extremely diverse ecological niches including habitats that are considered harsh with respect to temperature, salt concentration, pH pressure or nutrient availability. The particular stresses imposed by such environments have been strong selective forces in evolution. The ability of microbes to adapt regardless of environmental conditions has yielded enormous genetic diversity, which vastly surpasses that of their eukaryotic counterparts.

Until recently investigation of prokaryotic diversity had been inaccessible. To be studied, microbes had to be obtained in pure culture, limiting prokaryote recovery to less than one percent of cells present in a given population (*1*). In the past decade, the development of high throughput sequencing technology had permitted the generation of large datasets of environmental DNA sequences without the need for prior cultivation. A new field, metagenomics, has emerged unveiling the extent of microbial genetic potential and diversity in the environment.

Here we highlight two examples of prokaryotic genomic diversity: on an intraspecies level, exemplified by the highly versatile *Escherichia coli*, and the diversity in the hypersaline environment. Health issues, particularly related to the food chain, and biotechnological potential are discussed.

Intraspecies Diversity among *Escherichia coli*

Escherichia coli is a Gram-negative rod-shaped enterobacteria found in the intestine of humans and many animals. *E. coli* is the prototypic commensal species of the facultative anaerobic microbiota in the lower intes-

*Corresponding author: E mail: darja.zgur.bertok@bf.uni-lj.si

tine of healthy warm-blooded organisms. It is also the most abundant facultative anaerobe of the human gut microbiota. Commensal *E. coli* inhabits the mucous layer of the colon and colonizes the gastrointestinal tract of infants within a few hours after birth (*2*). Nevertheless, *E. coli* is a highly versatile species, hence, strains are also isolated from the environment, namely plants, soil, water and food.

In healthy humans, different *E. coli* clones often coexist, with some being more abundant or predominant than others (*3*). A complete turnover in the *E. coli* gut population is established within a period of two weeks to a month (*4*). In 2013, Smati *et al.* (*5*) identified a small reservoir of *E. coli* clones that are present in the human gut, the so-called minor clones. Their presence could explain fluctuations in the composition of the *E. coli* microbiota observed within individuals. It is also well established that the composition of the gut microbiota, including *E. coli* strains, is affected by host species, diet and physiology (*6*).

E. coli strains can be assigned to one of four main phylogenetic groups: A, B1, B2 and D, on the basis of allelic variation at enzyme-encoding genes detected by multilocus enzyme electrophoresis (*7*,*8*). The individual phylogenetic groups are associated with particular ecological niches, thus strains belonging to B2 and D groups are generally considered as extraintestinal and virulence associated in humans, while A and B1 groups are generally commensal. Clermont *et al.* (*9*) established a rapid and simple method to determine *E. coli* phylogenetic groups employing a triplex PCR based on the amplification of three fragments (a fragment of the *chuA* gene, a fragment of the *yjaA* gene, and a fragment of the TSPE4.C2 noncoding sequence). The presence or absence of combinations of these three amplicons is used to assign *E. coli* to one of the four phylogenetic groups. In order to increase the discriminative power of phylogenetic group analysis, Escobar-Páramo *et al*. (*10*) proposed to further define the combinations of the three amplicons using phylogenetic subgroups (A_0, A_1, $B2_2$, $B2_3$, D_1 and D_2). In addition, most enterohaemorrhagic *E. coli* (EHEC), including O157:H7, are assigned to the minor phylogenetic group E (*11*). Several roles have been assigned to commensal *E. coli*: (*i*) protection of the host against colonization by pathogenic bacteria, (*ii*) breakdown of nutrients/waste products, and (*iii*) synthesis of vitamin K.

Pathogenic E. coli

Normally commensal *E. coli* and its human host can coexist with mutual benefit for decades. Nevertheless, certain *E. coli* strains can cause an extensive variety of intestinal and extraintestinal infections, and several pathovars have been defined (*2*). Enteropathogenic *E. coli* (EPEC) are characterized by attaching and effacing lesions in the ileum; EHEC provoke haemorrhagic colitis (HC) with attaching and effacing (A/E) lesions similar to those of EPEC, and due to the production of Shiga toxin (also designated verotoxin) may cause haemolytic-uraemic syndrome (HUS) with severe renal damage. Enterotoxigenic *E. coli* (ETEC) produce heat-stable (ST) and heat-labile (LT) enterotoxins to provoke infantile diarrhoea and traveller's diarrhoea. Two additional intestinal pathogenic pathovars are the enteroaggregative and enteroinvasive *E. coli* (EAEC and EIEC, respectively). Extraintestinal pathogenic *E. coli* (ExPEC) strains have the ability to cause disease at multiple anatomical sites, *e.g.* urinary tract infections (UTI), neonatal meningitis (NMEC), pneumonia, skin and soft tissue infections, and sepsis (*12*). A number of virulence factor genes of the *E. coli* pathovars are located on pathogenicity islands, which are characterized by a G+C content different from that of the rest of the chromosome and are generally acquired *via* horizontal gene transfer.

Pathogenic *E. coli* strains that provoke infections encode characteristic virulence factor genes (*2*). Any component of a microbe that is required for, or potentiates its ability to cause disease is designated as a virulence factor. Characteristic groups of virulence factors are: adhesins, toxins, iron uptake systems and host immunity-evading systems. Virulence factors facilitate colonization and invasion of the host, avoidance or disruption of host defence mechanisms, injury to host tissue, and/or stimulation of excessive fluid and electrolyte secretion or stimulation of noxious host inflammatory response (*13*). Adhesins are among the first virulence factors that come into play during establishment of an infection. Besides their primary role as adhesin molecules, they can also function as invasins, promoters of biofilm formation and transmitors of signals to epithelial cells resulting in inflammation. Various adhesins have been identified and studied (*14*). The most common, encoded in many pathogenic and commensal *E. coli*, are type-1 fimbriae, which are generally not regarded as virulence factors (*15*). Toxins are important virulence factors in a variety of *E. coli*-mediated intestinal and extraintestinal diseases that affect an astonishing variety of fundamental eukaryotic processes and thereby harm the host; they can also act as invasins (*2*). Bacteria have developed specialized uptake systems to capture iron, an essential cofactor for many basic bacterial metabolic pathways. The most prominent iron uptake systems are the siderophores, iron-binding molecules that are taken up by special siderophore receptors and ATP-consuming porin-like transporters in the bacterial outer membrane (*16*). In addition to siderophore synthesis, strains can use siderophores produced and released into the extracellular medium by other bacteria and even fungi (*17*). Apart from the siderophores and their receptors, autotransporters, virulence-associated proteins in Gram-negative bacteria, can also play a role in obtaining iron, for example the haemoglobin protease Hbp (*18*). A wide array of host immunity-evading systems, ranging from polysaccharide capsules to serum-resistant proteins, are known in pathogenic *E. coli* (*2*). Capsules are discrete structural layers of extracellular polysaccharides that protect pathogens from assaults such as opsonophagocytosis and complement-mediated killing (*19*); and in the case of acidic polysaccharide capsules (K antigens), they can act as 'sponges' to sequester and neutralize antimicrobial peptides (*20*). Another virulence factor that *E. coli* uses to avoid host defence is TcpC, a Toll/interleukin-1 receptor (TIR) domain-containing protein that inhibits Toll-like receptor (TLR) and MyD88-specific signalling, thus impairing the innate immune response (*21*). Notably, *E. coli* strains causing intestinal and extraintestinal infections have different arrays of specific virulence factors. Assignment of pathogenic *E. coli* strains

into pathovars is based on the carriage of a common set of virulence factor genes (*2*) (Table 1).

The gut microbiota represents a reservoir of virulence determinants for intestinal and extraintestinal pathogenic *E. coli* strains and thus, the genetic armory of the faecal *E. coli* population is important in the pathogenetic potential of its flora (*3*). This has been indicated by studies directed to commensal *E. coli* strains that possess virulence factors typical for extraintestinal pathogenic *E. coli* (Table 2; *22–27*).

Table 1. *Escherichia coli* pathotypes and characteristic virulence factors

Pathotype	Adhesin	Toxin
Intestinal pathogenic *E. coli*		
enterotoxigenic *E. coli* (ETEC)	fimbriae CF, fimbriae K88, fimbriae K99, fimbriae 987P, fimbriae F17	heat-stable toxin (ST), heat-labile toxin (LT)
enteropathogenic *E. coli* (EPEC)	fimbriae Bfp, intimin	
enteroinvasive *E. coli* (EIEC)	similar to *Shigella* spp.	similar to *Shigella* spp.
enterohaemorrhagic *E. coli* (EHEC)	intimin	Shiga-like toxin (Stx), enterohaemolysin (E-Hly)
enteroaggregative *E. coli* (EAEC)	fimbriae GVVPQ	enteroaggregative heat-stable (EAST), enterotoxin
necrotoxic *E. coli* (NTEC)	fimbriae P	cytotoxic necrotizing factor (CNF)
Extraintestinal pathogenic *E. coli*		
uropathogenic *E. coli* (UPEC)	fimbriae P fimbriae S Afa/Dr family of adhesins	haemolysin (Hly) cytotoxic necrotizing factor (CNF) uropathogenic specific protein (Usp)
sepsis/meningitis-associated *E. coli* (MNEC)	fimbriae S, K1-capsule	invasin (Ibe)

Table 2. Prevalence of ExPEC virulence factors among faecal or rectal *Escherichia coli* isolates in different studies

	N(isolates)/%					
	A	B	C	D	E	F
Virulence factor (VF)						
Toxin						
cnf1	5 (6)	6 (4)	n.a.	6 (7)	9 (13)	3 (5)
hlyA/hlyD	7 (8)	7 (4)	15 (20)	10 (11)	10 (14)	6 (9)
usp	22 (24)	n.a.	n.a.	n.a.	n.a.	n.a.
ibeA	12 (13)	3 (2)	7 (9)	n. a.	11 (15)	4 (6)
Adhesin						
fimH	79 (88)	n.a.	70 (92)	87 (99)	65 (92)	64 (96)
papGII + GIII	10 (11)	n.a.	3 (4)	n.a.	19 (27)	7 (10)
sfa/foc	15 (17)	6 (4)	4 (5)	11 (13)	11 (15)	6 (9)
afa/draBC (*drb*)	4 (4)	2 (1)	4 (5)	2 (2)	4 (6)	0 (0)
Iron uptake system						
aer (*iucD/iutA*)	35 (39)	27 (16)	20 (26)	35 (40)	14 (20)	20 (30)
fyuA	59 (66)	n.a.	51 (67)	n.a.	39 (55)	23 (34)
ireA	18 (20)	n.a.	n. a.	n.a.	5 (7)	n.a.
iha	35 (39)	n.a.	13 (17)	n.a.	10 (14)	n.a.
hbp	14 (16)	n.a.	n.a.	n.a.	n.a.	n.a.
Host immunity evading systems						
kpsMTII	52 (58)	n.a.	53 (70)	62 (70)	34 (48)	16 (24)
tcpC	7 (8)	n.a	n.a.	n.a.	n.a.	n.a.

A=90 isolates from Slovenia (*22*); B=168 isolates, including 56 isolates from Paris, France, 57 isolates from Olib and Silba, Croatia, and 55 isolates from Mali (*23*); C=76 isolates from Minneapolis, USA (*24*); D=88 isolates from Michigan, USA (*25*); E=71 isolates from Minneapolis, USA (*26*); F=67 isolates from Barcelona, Spain (*27*). n.a.=not applicable

Foodborne E. coli

Foodborne infections are of great health and economic significance. Therefore, analyzing foods using standard phenotypical methods for the presence of foodborne pathogenic bacteria is a routine procedure in Slovenian food producing and processing plants. Some *E. coli* strains are significant foodborne patogens causing diverse diarrhoeal diseases. The most important and the only recognized zoonotic *E. coli* strains in Slovenia so far have been classified as enterohaemorrhagic *Escherichia coli* (EHEC). The hallmark of these strains is the production of Shiga-like toxins/verotoxins; therefore, they are often denoted as Shiga toxin-producing *E. coli* (STEC)/verotoxin-producing *E. coli* (VTEC). STEC cause a spectrum of human diseases, including bloody diarrhoea, haemorrahagic colitis (HC) and haemolytic-uraemic syndrome (HUS) usually following consumption of contaminated food (*2*,*28*, *29*). A large variety of VTEC serotypes have been associated with disease, the most prevalent being O157:H7. However, enterohaemorrhagic *E. coli* O26:H11 has recently emerged as the most important non-O157:H7 pathogen (*30*,*31*). In accordance, data for Slovenian VTEC isolates indicate that the number of O26:H11 belonging to phylogenetic group B1 is steadily increasing (Trkov, unpublished results). Recently, a study performed at the Department of Biology, Biotechnical Faculty, University of Ljubljana, and the Institute of Public Health of the Republic of Slovenia, assessed food supply as a possible source of antimicrobial-resistant and VTEC strains. Sixty *E. coli* isolates from different food items (vegetables, raw meat products, sausages, *etc.*) were screened with PCR for the presence of enterovirulence genes *vtx1*, *vtx2*, *eae*, *ehxA*, *ipaH*, *eltA*, and *estA* and assigned to a phylogenetic group according to Clermont *et al.* (*9*). While one isolate belonged to either the extraintestinal virulence-associated phylogenetic group B2 or D, forty-three isolates were assigned to the low-virulence, predominantly commensal phylogenetic group A and sixteen isolates to group B1. Although strains of phylogenetic group B1 are reported to be predominantly commensal, five out of 16 B1 isolates carried at least one of the virulence genes associated with VTEC strains. Thus, genes for intimin (*eae*) and EHEC-associated enterohaemolysin (*ehxA*) were found in one isolate from minced meat with spices and different *vtx* alleles alone or in combination with *ehxA* in other five isolates from raw beef and tartar beefsteak (*32*). In a second study, sixty-six *E. coli* isolates from patients' stool samples from the Ljubljana region, with diarrhoea of unknown origin were analysed. Twelve isolates were determined as *E. coli* O26 and all belonged to phylogenetic group B1. They carried an *eae* gene, nine were *ehxA* positive and two carried an additional *vtx1* or *vtx2* gene. Further, six isolates were determined as O157. All carried the *eae* gene and additionally either the *ehxA* and/or *vtx2* gene (*32*). The treatment of EHEC infections with bactericidal antibiotics, particularly with β-lactams, is generally not recommended due to possible induction of Shiga toxin production (*33*).

Other non-EHEC enteropathogenic strains of *E. coli* cause generally less severe infections with few complications in developed countries. These infections are rarely treated with antimicrobial agents. It is likely, therefore, that less attention has been dedicated to less pathogenic and nonpathogenic *E. coli* strains originating from food, animals used for food, animal food production systems and other environments, although they are often a permanent or transient part of the microbial community of the human gastrointestinal tract. As certain *E. coli* strains have been studied solely in terms of human and avian pathogens, horizontal gene transfer of virulence and resistance genes between strains of different origin, aided by the abundant use of antibiotics in human/veterinary medicine and food production systems, has been completely overlooked. Consequently, new pathogenic and/or antimicrobial-resistant strains emerge 'unexpectedly', as for example the EHEC O104:H4 outbreak in Germany in 2011 (*34*,*35*).

Foodborne *E. coli* as a source of antibiotic resistance genes

The emergence of antibiotic-resistant bacteria has become one of the major challenges of global health care systems, both from the point of view of economic and human costs (*36*). Hospitals were traditionally considered to be the major selective environment of antibiotic-resistant bacteria and genes. However, the increasing frequency of some, previously almost exclusively hospital-associated infections, *e.g.* infections caused by extended-spectrum β-lactamase (ESBL)-producing *E. coli*, among community-dwelling patients has prompted researchers to consider other reservoirs of resistant strains and/or genes. Recently, it has been proposed that the dissemination of resistances to β-lactams and other antimicrobials in the community could also occur through the food chain from animal production environments, where antimicrobials have been administered for therapeutic and prophylactic use, as well as growth promoters since the mid 1940s (*37*). The major mechanism of resistance to β-lactam antibiotics in *E. coli* and other Gram-negative species is the production of β-lactamases. In the last two decades numerous ESBL enzymes that hydrolyse extended-spectrum cephalosporins evolved due to mutations in TEM-1, TEM-2, SHV-1 and CTX-M genes. The genes are located on chromosomes and plasmids. The latter are successfully disseminated through horizontal gene transfer among the *Enterobacteriaceae* and even broader. The presence of ESBL-producing *E. coli* in the intestinal tract of food-producing animals has recently been described by several authors (reviewed in *38–40*). Of particular concern is the steady increase in the prevalence of *E. coli* and other bacterial species producing ESBL and/or AmpC enzymes in food-producing animals such as poultry, cattle, swine, horses and rabbits, and food products in different European countries. Indeed, several authors have suggested a possible transmission of ESBL-producing *E. coli* from poultry to humans, most likely through the food chain. Leverstein-van Hall *et al.* (*41*) compared *E. coli* isolates from retail chicken meat, food-producing animals and humans and found that 19 % of the human isolates carried ESBL genes on plasmids that were genetically indistinguishable from those obtained in poultry isolates, and that 94 % of the retail chicken meat was contaminated with ESBL-producing *E. coli*, of which 39 % had the same genotypes as the ones found in human isolates. On the other hand, a comparative analysis showed a high diversity in virulence and antimicrobial gene con-

tents among ESBL-positive *E. coli* isolates from animals and humans from the UK, the Netherlands and Germany. The genotypes of the majority of the isolates from humans were different from those isolated from animals, while many human isolates from the compared countries were similar. However, the results should be interpreted in light of the fact that nearly half of the human isolates belonged to or were highly similar to the pandemic clonal group ST131 (*42*). Different prevalence of ESBL/AmpC-producing isolates in different countries could partly be the result of various amounts of antimicrobials used in food-producing animals. Dierikx *et al.* (*43*) detected ESBL/AmpC-producing isolates in cloacal swabs from broilers at all 26 tested broiler farms in the Netherlands, which, compared with other European countries, use more antimicrobials for the treatment of food-producing animals. However, they also found a high prevalence of ESBL/AmpC-positive birds at one farm that did not use any antimicrobials during the whole production period. In Slovenia, the prevalence of ESBL/AmpC-producing *E. coli* in food animals and slaughterhouses has been studied since 2009. One of the studies included 72 strains isolated from samples of slaughtered poultry in the period between October 2011 and May 2012. Fifty-five (76.3 %), one (1.3 %) and sixteen (22.2 %) isolates carried group CTX-M-1, CTX-M-2 and SHV genes, respectively. Twenty-one (29 %) belonged to phylogenetic group A_0, 23 (31 %) to group B1, and 29 (40 %) to group D (*39*,*44*). Virulence genes *iha*, *kpsMTII*, *fluA* and *usp*, which are associated with human pathogenic *E. Coli*, were also detected (unpublished results). These data are worrisome since foodborne bacteria that enter the human gastrointestinal tract may become a constant part of the human endogenous microbiota (*45*). Recently, some authors have suggested that food is a possible source of ExPEC strains associated with different (*e.g.* urinary tract) infections (*46*,*47*). This possibility should not be underestimated. It is also of concern that metallo-β-lactamase-producing *E. coli* was recently isolated from mastitic milk samples (*48*). New Delhi metallo-β-lactamases, NDM1, inactivate carbapenems, powerful β-lactam antibiotics with broad spectrum antibacterial activity, employed as antibiotics of last resort for multiresistant *Klebsiella pneumoniae* and *E. coli* strains. It would therefore be an extremely alarming scenario if carbapenemase-resistant strains and/or resistance genes could easily, *via* food, enter our gastrointestinal tract – particularly if we consider that enterobacteria carrying *e.g.* an bla_{NDM} gene are resistant to almost all antimicrobials except colistin and tigecycline (*49*).

Mechanisms shaping E. coli genomic diversity

The primary mechanisms shaping *E. coli* genomic diversity are mutations, horizontal gene transfer, and bacteriocin production. *E. coli* naturally transfers DNA by two mechanisms of horizontal gene transfer, conjugation and transduction. Conjugation proceeds between two bacterial cells and is driven by conjugative plasmids, while transduction is mediated by bacteriophage. Both conjugation and tranduction are known to promote the dissemination of virulence and antibiotic resistance genes. Mutations can be either spontaneous or induced. In addition, DNA damage in *E. coli* activates the SOS response, a coordinated DNA damage response, which induces error-prone DNA polymerases to promote an elevated mutation rate and horizontal gene transfer. The SOS response thus generates genetic diversity, adaptation, but also evolution of antibiotic resistance, control of virulence factor synthesis and dissemination of antibiotic resistance as well as virulence factors.

Two proteins control the SOS response, a repressor LexA and an activator RecA. In the absence of DNA damage, a LexA dimer represses transcription of more than 50 genes including *lexA* and *recA* by binding to SOS boxes (a 20-base-pair consensus palindromic DNA sequence). Upon DNA damage, RecA is activated (RecA*) after binding to a single-stranded DNA (ssDNA), forming a nucleoprotein filament. RecA* stimulates self-cleavage of LexA leading to derepression of SOS genes (*50*).

While food is a source of infection, it is also a source of mutagens, introduced either as preservatives, flavour enhancers or produced during food processing (*51*). Mutagenic substances damage DNA, but they are not necessarily carcinogenic to human cells, since they may be altered (*e.g.* detoxified in the liver) or do not have the abiliy to enter a human cell. Direct correlation between mutagenicity and carcinogenicity is thus broken, the risk of non-carcinogenic mutagens is downgraded.

Nevertheless, some food additives (*e.g.* sodium nitrate, effective *via* nitrous acid transformation), although regarded 'safe' for human consumption, provoke DNA damage in intestinal bacteria and trigger the SOS response. In an attempt to repair DNA damage, this stress response leads to increased rearrangements within the genome and opens a Pandora's box of genetic elements that are mobilized, including latent bacteriophages, transposons and plasmids, able to spread among related bacteria (*52*). In addition, an increase in mutation rate paves the path for pathogens to acquire a new or increase the existing antibiotic resistances, and to accumulate and spread pathogenicity factors.

Further potential factors that affect the bacterial SOS response are antibiotics, used for human therapy as well as in veterinary medicine either for disease treatment and prevention or as growth promoters in food animals. Although the risk to human health seems negligible (*53*), presumably innocuous concentrations of some antibiotics, particularly fluoroquinolones, increase the expression of SOS-controlled genes (*54*). Alarmingly, even fresh vegetables could accumulate the extensively used fluoroquinolone ciprofloxacin, when sewage sludge containing drug residues is used as a fertiliser (*55*).

As the development of new antibiotics, particularly for new bacterial targets, is at a standstill, different approaches should be explored. The life-span of the existing antibiotics could be prolonged by blocking bacterial adaptation, controlled at least in part by the SOS system. In recent years, a three-dimensional model of the complex between LexA and RecA proteins has emerged (*56*, *57*), revealing some potential sites for the design of selective inhibitors.

Bacteriocins

In addition to the diversity of virulence factor production and antibiotic susceptibility, *E. coli* strains also exhibit a high level of diversity of bacteriocin produc-

tion. Bacteriocins are produced by all major lineages of bacteria as well as some archea presumably for defense or invasion of ecological niches. Bacteriocin activity was initially described in *Escherichia coli* in 1925 by Gratia (*58*). The most extensively studied are bacteriocins of the Enterobacteriacae, particularly those of *E. coli* designated colicins (*59*). Approximately 40 % of *E. coli* strains produce colicins.

E. coli strains produce two types of bacteriocins, colicins and microcins. Colicins are plasmid-encoded, high molecular mass bacteriocins (>20 kDa). More than 20 colicin types have been described. Evolutionary investigations of colicin diversity have revealed two modes of evolution: recombination characteristic of pore formers and point mutations among nuclease colicins (*60*). Generally, colicin production and release are encoded by a cluster of three genes: (*i*) the activity gene for colicin synthesis, (*ii*) immunity gene for protection of the producing cell, and (*iii*) a lysis gene for semispecific colicin release by cell lysis (*59*,*61*).

Killing of a sensitive cell proceeds by colicin binding to a specific receptor in the outer membrane, followed by translocation through the outer membrane *via* the Tol or TonB translocation system to the target. Colicins exhibit a three-domain structure: the N-terminal domain for translocation of the colicin into the target cell, the central domain for binding of the colicin to a specific cell surface receptor, and the C-terminal catalytic domain with a short region for binding of the immunity protein.

Colicins destroy sensitive cells by one of three mechanisms: (*i*) pore formation, (*ii*) nuclease activity, DNA or RNA degradation, or (*iii*) inhibition of peptidoglycan and lipopolysaccharide O-antigen synthesis.

Colicins have been shown to mediate population and community level interactions (*62–64*) promoting microbial diversity within *E. coli* populations in the mammalian colon (*65*). In addition, investigation of the transcriptional response of *E. coli* to the nuclease colicins, E3 and E9 revealed that the former upregulated genes of mobile genetic elements (phage recombinases, insertion sequence origin) while the latter upregulated the damage-inducible SOS response including the error-prone DNA polymerase V, suggesting that colicins may also promote genetic diversity in *E. coli* populations (*66*). Colicin synthesis is controlled primarily by the DNA-damage-inducible SOS system, as the synthesis is repressed by its key regulator, the LexA protein (*59*). The large majority of colicin operons harbour two overlapping LexA boxes located downstream of the Pribnow box (*67*) to which two LexA dimers bind. The two LexA boxes effectively repress expression, protecting the producer from overt lysis (*68*). Upon DNA damage, most SOS genes are induced almost immediately while colicin-encoding operons are expressed following a pronounced lag (*69*). The lag in expression is presumed to assist surviving cells by killing competitors (*70*). Regulation by the transcriptional regulator CRP (cAMP receptor protein)-cAMP complex as well as the IscR (iron-sulphur cluster) transcriptional regulator have been shown to provoke a lag in expression of some colicins. Thus, in the absence of DNA damage in metabolically active cells, the pore-forming colicin K synthesis is repressed by the LexA and IscR repressors. Only after a prolonged SOS response and as metabolism slows down due to nutrient exhaustion, is colicin synthesis induced presumably to eliminate defective cells as well as to provide nutrients to related neighbours (*69*). A post-transcriptional regulatory mechanism involving the stringent response has been shown to regulate colicin K synthesis (*71*). Furthermore, a recent study has shown that some colicin-producing strains induce expression of other colicins (*70*).

Compared to colicins, microcins are smaller molecular mass bacteriocins (<10 kDa). They are generally hydrophobic, heat and pH stable and resistant to protease degradation. Gene clusters for microcin synthesis harbour genes for the microcin precursor, immunity, secretion proteins and modification enzymes. Microcins exhibit a narrow antimicrobial spectrum of activity against phylogenetically related species and are therefore presumed to be engaged in competition within the intestinal tract. Stress such as nutrient depletion has been shown to induce microcin synthesis (*72*).

To obtain insight into the roles that bacteriocins play among *E. coli* populations, various collections of strains have been examined for the presence of colicins and microcins. Thus, among a collection of strains from patients with bacteraemia of urinary tract origin, 66 % of the strains encoded at least one bacteriocin, with 43 % encoding one or more colicins and 54 % one or more microcins. Among the bacteriocin-encoding strains, 43 % encoded one type of bacteriocin, 45 % two, 8 % three and 4 % encoded four different bacteriocins (*73*). Furthermore, the analysis revealed that among strains of the B2 phylogenetic group, most strains encoded more than one bacteriocin, while bacteriocins were least frequent among strains of the B1 group. On the other hand, the majority of microcin-encoding strains belonged to the B2 phylogenetic group. Microcins were found to co-occur with toxins, siderophores, adhesins and the Toll/interleukin-1 receptor domain-containing protein TcpC. The results indicated that microcins contribute to virulence of *E. coli*, provoking bacteraemia of urinary tract origin.

Application of bacteriocins

The discovery of penicillin and subsequently other antibiotic classes represented milestones in the battle against infectious diseases. However, the misuse and overuse of antibiotics has prompted the emergence and spread of antibiotic resistances. Bacteriocins represent a much needed alternative approach to control infections. Of the bacteriocins produced by Gram-negative bacteria, a number of studies have demonstrated that colicins are effective against *E. coli* strains associated with diarrhoea including serotype O157:H7 (*74–80*). In addition, colicins have been shown to be effective against uropathogenic strains (*81*) and to prevent colonization of urinary catheters (*82*). Colicin E1 also exhibited inhibitory activity against *L. monocytogenes* in broth culture and in ready-to-eat (RTE) products (*83*), while colicins H and G exhibited inhibitory activity against *Salmonella* strains isolated from clinical cases (*84*).

Microbial Diversity in Hypersaline Enviroments

Dominance of a single genus or a species in a given niche is highly unusual in prokaryotic communities. Instead, prokaryotes most often live in highly complex

multispecies assemblages in which they interact with one another (*85*). This complexity impedes our attempts to understand some basic concepts in the evolution of prokaryotic species: the mechanisms that generate and maintain diversity and the mechanisms of interaction with other biotic compounds. In these terms, extreme environments can be considered as ideal model systems, since they often support microbial communities that are simple in structure: dominated by a single or a very small number of prokaryotic species, with a vast diversity of low-abundance species. Here we aim to summarize findings on the diversity of prokaryotes in the hypersaline environment as well as some implications of these findings in the evolution of prokaryotic species.

Metagenomics of solar saltern crystallizer

Early metagenomic studies on crystallizer brine reported that its microbial community is composed mostly of euryarchaeal Halobacteriaceae and halophilic members of Bacteroidetes (*86*). In different salterns, prokaryotic communities were fairly similar in terms of taxonomic census but differed in the relative proportions of the present taxa. These differences were attributed to local differences in salt production technologies imposed by prevailing weather conditions and geology (*87*,*88*). For example, in the Slovenian Sečovlje saltern, where salt production is limited to the arid part of the year, the crystallizer ponds are shallow and measure only a few centimetres in depth. As a consequence, the water retention time is reduced to less than 24 h. It does not allow large microbial blooms and supports up to 10^4 cells per mL (*88*). Not surprisingly, the prokaryotic community is dominated by fast-growing members of the archaeal genus *Halorubrum* (66 % of sequences retrieved) (*88*,*89*). Another such example are the salterns of the Tamil Nadu region in India, which are fed with hypersaline spring water mixed with seawater and led to the ponds from borewells. Although the salt production technology is very similar to that in Slovenia, the differences in water composition result in the dominance of members of the archaeal genus *Natrinema* (3 % of sequences) (*90*).

In contrast to the above salterns, salterns in arid areas allow for brine to be replenished on a yearly basis. An example is the crystallizer of the Bras del Port saltern, Spain, which is characterized by large numbers of prokaryotic cells and virus-like particles, up to 10^9 per mL (*91*). As shown recently in a large-scale metagenomic study, this brine is remarkably complex in terms of taxonomy (*92*). However, in terms of community structure, the brine community is fairly simple. As indicated in early studies (*e.g. 86*), a vast majority (up to 79.0 %) of environmental 16S rRNA sequences can be affiliated with a single species, *Haloquadratum walsbyi*. This species is followed in abundance by the hyperhalophilic bacterium *Salinibacter ruber* (9.0 %), while the remaining taxa represent only a minor portion of the encountered diversity (*91*).

Like many other prokaryotes that are either ecologically relevant or very abundant, *Haloquadratum walsbyi* is difficult to cultivate. Today, only two isolates of this species are available (*93*,*94*). The genomic sequence of the *H. walsbyi* strain HBSQ001, originally isolated from the Bras del Port saltern, Spain, was published in 2006 (*95*) and the sequence of strain C23, originally isolated from a saltern in Geelong (Australia) followed in 2011 (*96*). Both genomes shed a number of signatures typical for halophilic organisms: *e.g.* low GC content (in %) and the presence of halophilic proteins. Likewise, two genomic sequences of *Salinibacter ruber* are available, and correspond to strains M31 and M8 isolated from the Salinas de Levante in Majorca, Spain (*97*,*98*).

Another important aspect of a prokaryotic community is its temporal dynamics. The stability of the crystallizer communities at different time points was addressed in the Chula Vista saltern, California, USA (*99*). This metagenomic study followed the composition and metabolic potential of microbial and viral communities at various time points ranging from 24 h to more than one year. At the level of microbial species, both community composition and metabolic potential remained stable with time. However, at the level of microbial strain and viral genotype, the community experienced dramatic temporal changes. The study suggested that the observed cycling of the taxa likely reflects the interplay at the level of viral genotypes and virus-sensitive microbial strains (*99*).

The above proposed concept was not entirely new. Natural populations of bacterial species have long been considered to be composed of distinct clonal lineages (*100*). These are defined as asexual descendants of a given genotype that differ from the originator only *via* mutation and mitotic recombination. Judging by the predictions of competition of mutants in laboratory populations of *Escherichia coli*, the ecologically superior lineage would eventually overcome all the others. This, in turn, reduces genomic diversity (*100*). If such a population encounters an increase in predating virus population, then a massive lysis event is expected to collapse the population. Actually, these events are occasionally observed, particularly in man-made systems, for example in the lactic industry (*101*).

Diversity among halophiles

Viral pressure is the main factor that controls the relative abundance of crystallizer taxa (*91*). However, it causes less than 5 % of prokaryotic losses and no massive lysis events (*91*). This indicates that the clonal lineages of dominant species (*i.e. Haloquadratum walsbyi* and *Salinibacter ruber*) might differ in phage susceptibility. This intraspecies variability is reflected in genomic sequences either as the presence of variable genomic regions that are absent from one or more lineages, or are unique to certain lineages (*102*). One way to distinguish the variable genomic regions from the core genome is to tile metagenomic reads of the environment from which the organism was isolated to its reference genome, a method known as genomic recruitment. The regions that are common to all lineages will be well represented in the environment and will recruit a large number of highly similar fragments of metagenomic DNA. In contrast, highly variable regions will be underrepresented in the plot and will form 'metagenomic islands'.

A genomic recruitment experiment performed using genomic sequence of *Salinibacter ruber* M31T and the metagenome of the Chula Vista saltern identified three meta-

genomic islands (*103*). These presented a number of features typical of highly unstable genomic regions. They had atypically low GC content (56 *vs.* 66 %) and contained three out of four phage integrases found in the *S. ruber* genome. The average coding region densities of the three metagenomic islands were 54.0, 64.7 and 45.4 %, respectively, compared to 84.8 % for the whole *S. ruber* genome. The regions further had high numbers of pseudogenes and short hypothetical proteins. Compared to the entire genome, the islands were enriched with genes involved in carbohydrate transport and metabolism, cell wall/membrane/envelope biogenesis, recombination, replication and repair. Remarkably, while one island resembled a lysogenic phage, two *S. ruber* islands contained genes that code for cell-surface polysaccharides and are involved in biosynthesis of polysaccharide components of cell wall. This variability at the level of cellular envelope was suggested to play a role in defence against phage predation. In fact, phages have been shown to target lipopolysaccharides through their host recognition machineries (*104*) or strain-specific polysaccharases (*105*).

The observed variability of gene clusters coding for the polysaccharide component of the cell wall seems to be a general phenomenon in prokaryotes. A similar pattern of intraspecies genomic variability was observed in the genomes of *Haloquadratum walsbyi* HBSQ001 and C23 (*96*,*106*), but also in available genomes of abundant marine microbes *Candidatus Pelagibacter ubique*, *Prochlorococcus marinus*, *Burkholderia* sp. 383, *Shewanella* sp. MR-4, *Aeromonas hydrophyla* ATCC 7966 and *Synechococcus* sp. WH812 when compared to marine metagenome (*107*). All species had metagenomic islands which contained genes that encode extracellularly exposed products and that could act as potential phage recognition sites.

Based on these observations, Rodriguez-Valera *et al.* (*107*) proposed the constant diversity dynamics model which assumes that the appearance of a mutant or recombinant that is ecologically superior to its peers leads to only transient domination over ecologically less successful lineages. Under this model, the expansion of invasive lineage is met by an increase in numbers of viruses that target the lineage-specific cell-surface receptor. This way, the invasive lineage is selected against and replaced by original normal fitness lineages avoiding massive losses. Therefore, under this model of population dynamics, the high level of clonal lineage diversity is steadily guarded by predating phage. It results in a large pan-genome within the bacterial population as indicated by metagenomic data and mathematical modelling (*107*).

Biotechnological aspects of adaptations to hypersaline conditions

The solar saltern crystallizer brine is one of the harshest environments with respect to total salt concentration. It supports highly specialized halophilic archaea, bacteria, fungi and viruses (*e.g. 88*,*89*,*99*,*108*). To compensate for high extracellular sodium chloride concentration, these organisms use two strategies. The so-called 'salt-in' halophiles (most of archaea) accumulate molar concentrations of potassium and chloride, a strategy that requires extensive metabolic adaptation. In contrast, the 'salt-out' halophiles (most of bacteria and fungi) accumulate high intracellular concentrations of compatible solutes.

The majority of enzymes originating from 'salt-in' halophiles perform their functions *in vitro* and *in vivo* at 4.0–5.0 M NaCl (*109*). Halophilic enzymes offer the possibility to extend the repertoire of the already available biocatalysts and aid in industrial processes where high salt concentrations inhibit mesophilic enzymes. For instance, highly salt-tolerant proteases of halophilic archaea are often also thermo- and alkalitolerant. For example, a protease isolated from saltern dweller *Haloferax lucentensis* VKMM 007 remained stable in the temperature range from 20 to 70 °C, at NaCl concentrations from 0.85 to 5.13 M and in pH range of 5.0–9.0. Furthermore, it retained proteolytic activity in the presence of various polar and non-polar solvents, surfactants and reducing agents (*110*). Halophiles present other characteristics of considerable biotechnological interest, such as bacteriorhodopsins, light-driven proton pumps used in photoelectrochemical cells (*111*), carotenoid pigments and gas vesicles – potential pathogen peptide delivery vehicles (*112–114*). Halophiles have long been recognized in food industry. Halophilic archaea are involved in the production of various compounds that give the characteristic taste, flavour and aroma to salt fish products such as Thai fish sauce (nam pla) (*115*). In fact, the first halophilic microorganisms were discovered in spoiled foods that were salted for preservation.

The high-salt tolerance of haloarchaea enables their cultivation under non-sterile and cost-effective conditions. However, such biotechnological advances are often hampered by long generation times of halophilic archaea that range from 1.5 h for *Haloterrigena turkmenica* (*116*) to up to 1–2 days reported for *Haloquadratum walsbyi* (*91*). Recently, optimization of medium components using response surface methodology resulted in significant reduction of the time required for cell cultures of halophilic archaea to reach the stationary phase while providing a nearly 2.4-fold increase in biomass production (*117*). This pioneering study proved that statistical experimental design techniques are useful tools for screening for nutrients with significant impact on growth rate.

Conclusion

Prokaryotes display enormous intraspecies as well as intra-habitat diversity. Mutations, horizontal gene transfer, bacteriocin production and phage production are shaping prokaryotic evolution and genomic diversity. While *E. coli* isolates are part of the gut microbiota of man and warm blooded animals, a number of pathovars with distinct combinations of virulence factors have evolved and they provoke intestinal and extraintestinal infections. Of great concern are those associated with the food chain (*e.g.* EHEC and those carrying antibiotic resistance determinants). In the hypersaline environment microbial communities are dominated by a single or a very small number of prokaryotic species and a vast diversity of low-abundance species. Phage-host interactions have been shown to control the relative abundance of taxa in the crystallizer, while variable genomic regions indicate intraspecies variability.

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