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Bacterial acid stress response: from cellular changes to antibiotic tolerance and phenotypic heterogeneity

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Most bacteria are neutralophiles but can survive fluctuations in pH in their environment. Herein, we provide an overview of the adaptation of several human, soil, and food bacteria to acid stress, mainly based on next-generation sequencing studies, highlighting common and specific strategies. We also discuss the interplay between acid stress response and antibiotic tolerance, as well as the response of individual cells.

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Introduction

Bacteria have evolved sophisticated acid resistance systems to survive and even multiply in acidic environments (Figure 1a). Acid stress sensing and adaptation enable neutralophilic bacteria to maintain constant intracellular pH under moderate acid stress [1]. As acidification of the cytoplasm has severe effects on all macromolecules of a cell that might lead to protein unfolding, decreased enzyme activity, membrane damage, and DNA damage [2–4], bacteria have evolved a variety of acid stress resistance systems to counteract and survive in mild and highly acidic environments [5]. Detailed information on the molecular mechanisms of acid resistance in bacteria can be found in reviews by Cotter & Hill 2003, Foster 2004, Kanjee & Houry 2013, Lund et al. 2014, Guan & Li 2020, Arcari et al. 2020, Schwarz et al. 2022, and Rai et al. 2022 [5–12].

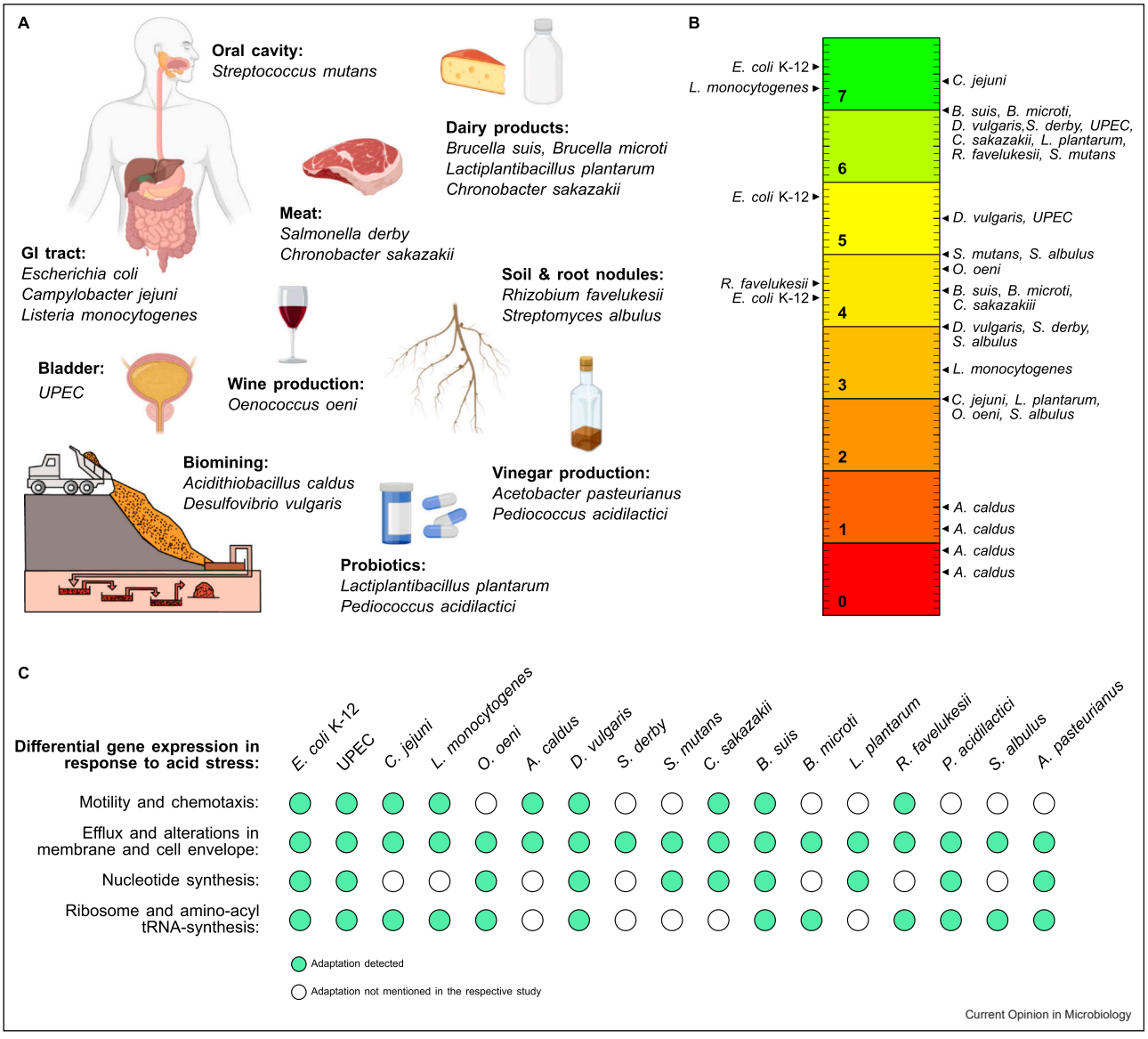
Here, we focus on systemic studies of the acid response of various bacteria and describe common and species-specific acid resistance mechanisms. We will also discuss how acid stress resistance facilitates development of antibiotic resistance [13]. Although most studies of stress response dynamics have focused on the population level and thus on the average response of millions of cells, recent work has also examined the acid stress response of individual cells, revealing phenotypic heterogeneity, which is also discussed.

Acid stress-dependent alterations of the transcriptome and proteome of various bacteria

Significant advances have been made in understanding how bacteria interact with their environment, particularly after the development of powerful next-generation sequencing techniques such as bacterial single-cell RNA-Seq, dual RNA-Seq, and triple RNA-Seq [14–17]. This trend of rapid large-scale data collection has now also impacted the field of acid stress research, as more and more next-generation sequencing techniques have become routine workflows in many laboratories. Since 2020, over a dozen studies, which are reviewed here, have been published with the aim of revealing genome-wide responses to acidity in a broad range of bacterial species (Figure 1), using a variety of techniques. These included mainly bulk RNA-Seq to analyze bacterial transcriptomes, with fewer studies using mass spectrometry to examine the translational level [18,19] (Table 1). Additionally, ribosome profiling (Ribo-Seq) has been employed to evaluate global protein synthesis rates under mild and severe acid stress in *E. coli* K-12 MG1655 (designated as *E. coli*) [20]. Ribo-Seq is a powerful tool that provides a global overview of protein synthesis rates and enables the detection of unidentified small open-reading frames [21,22]. Further insights into the microbial defense against low pH were provided by transposon-insertion-directed sequencing (TraDis), also called Tn-Seq. In the respective studies, transposon libraries were used for deep sequencing to provide information on the contribution of single genes to fitness in acidic habitats in uropathogenic *E. coli* (UPEC) and *Salmonella derby* [23,24].

Recent multi-omics studies focused on bacterial species from a broad range of phylogenetic classes, including *Acidithiobacillia*, *Actinomycetes*, *Alphaproteobacteria*, *Bacilli*, *Deltaproteobacteria*, *Epsilonproteobacteria*, and *Gammaproteobacteria* (Table 1). These organisms colonize a variety of environmental niches, including the

Figure 1



Overview of *omics* studies from 2020 to 2023, investigating global acid stress responses in various bacteria. **(a)** Habitats and industrial applications of bacterial species being studied using next-generation sequencing. **(b)** Visual representation of pH values selected as acid stress and controls for experiments listed in Table 1. **(c)** Schematic overview of acid stress responses. Common adaptions detected in several bacterial species are highlighted in green, adaptations that were not mentioned in the respective studies are marked in white. Figure **(a)** is created using BioRender.com and Affinity Designer 1.10.4.

gastrointestinal tract (GI tract), the human oral cavity and bladder, processed and unprocessed foods, acidic soils and root nodules, as well as biomining sites (Figure 1a). Among the strains listed in Table 1, *Escherichia coli*, *Campylobacter jejuni*, and *Listeria monocytogenes* colonize the human GI tract and can induce diseases. Further investigated bacteria include *Streptococcus mutans*, which is associated with dental caries and causes dysbiosis in the oral cavity [19], and UPEC, which is the primary cause of urinary tract infections. In addition, genome-wide acid responses of other food-borne pathogens, including *Salmonella derby*, *Chronobacter sakazakii*, *Brucella suis*, and *Brucella microti* have been characterized [24–26]. Other bacteria that have been the subject of recent multi-omics studies were *Rhizobium favelukesii* [27], an alfalfa-nodulating rhizobia frequently found in low-pH soils, and *Streptomyces albulus*, which is used for the industrial production of ε-poly-L-lysine [28]. Additionally, *Oenococcus oeni*, *Pediococcus acidilactici*, and *Acetobacter pasteurianus* are

Table 1

Bacterial species, methods, and growth media used to study genome-wide responses to acid stress.

Strain	Family	Class	Methods	Growth medium	Reference
Acetobacter pasteurianus Ab3	*Acetobacteraceae*	*Alphaproteobacteria*	RNA-Seq	Yeast extract–peptone–dextrose (YPD) medium + 20 g/l ethanol	[45]
Acidithiobacillus caldus CCTCC M 2018054	*Acidithiobacillaceae*	*Acidithiobacillia*	RNA-Seq	Modified Starkey medium	[37]
Brucella suis ATCC 23444, *Brucella microti* CCM4915	*Brucellaceae*	*Alphaproteobacteria*	RNA-Seq	Tryptic Soy broth (TSB) and Gerhardt's minimal medium (GMM)	[25]
Campylobacter jejuni NCTC11168	*Campylobacteraceae*	*Epsilonproteobacteria*	Microarray	Mueller Hinton (MH)	[38]
Cronobacter sakazakii CICC 21544	*Enterobacteriaceae*	*Gammaproteobacteria*	RNA-Seq	TSB	[26]
Desulfovibrio vulgaris ATCC 7757	*Desulfovibrionaceae*	*Deltaproteobacteria*	RNA-Seq	Self-created medium containing yeast extract	[36]
Escherichia coli K-12 MG1655	*Enterobacteriaceae*	*Gammaproteobacteria*	RNA-Seq, Ribo-Seq	Luria-Bertani (LB)	[20]
Escherichia coli serotype ST131 strain EO499 (UPEC)	*Enterobacteriaceae*	*Gammaproteobacteria*	RNA-Seq, TraDIS	M9 medium supplemented with glucose and cas-amino acids + 100 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) /100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)	[23]
Lactiplantibacillus plantarum ZDY2013	*Lactobacillaceae*	*Bacilli*	RNA-Seq	De Man, Rogosa, and Sharpe (MRS) broth	[39]
Listeria monocytogenes 6179 and R479a	*Listeriaceae*	*Bacilli*	RNA-Seq	TSB	[40]
Oenococcus oeni SD-2a	*Leuconostocaceae*	*Bacilli*	RNA-Seq, metabolomics	Fructose–malate–acid–tomato juice broth medium	[48]
Pediococcus acidilactici CGMCC 17856	*Lactobacillaceae*	*Bacilli*	Proteomics	MRS	[18]
Rhizobium favelukesii LPU83	*Rhizobiaceae*	*Alphaproteobacteria*	RNA-Seq	Sucrose–glutamate minimal medium + 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) /MES	[27]
Salmonella derby 14T	*Enterobacteriaceae*	*Gammaproteobacteria*	Tn-Seq	LB	[24]
Streptococcus mutans UA159	*Streptococcaceae*	*Bacilli*	Proteomics	Tryptone yeast medium + 1% (w/v) glucose	[19]
Streptomyces albulus M-Z18	*Streptomycetaceae*	*Actinomycetes*	RNA-Seq	Agar slant medium	[28]

See text for details.

exposed to acidity during industrial applications (Figure 1a). *Oenococcus oeni* is commonly used in wine production for malolactic fermentation, during which L-malate is converted to L-lactate and carbon dioxide under low-pH conditions [29]. *Pediococcus acidilactici* is utilized in the production of vinegar and as a probiotic food supplement [18,30]. Acid tolerance is crucial for these applications because acetic and lactic acids accumulate during vinegar production [31], and probiotic supplements are supposed to exhibit robustness toward acidity encountered in the stomach [32]. While most transcriptome and proteome analyses were conducted with neutralophilic bacteria, *Acidithiobacillus caldus* is acidophilic and commonly used for bioleaching to recover valuable metals from reduced sulfides [33]. During this process, metal ions are oxidized, and sulfuric acid is produced, resulting in an ambient pH of < 1.5 [34]. Despite being exposed to extreme acid stress, acidophilic organisms are capable of maintaining an internal pH of approximately 6.0 [35]. In this regard, the transcriptome of *A. caldus* was studied within the pH range of 0.6–1.5, whereas other bacteria were typically subjected to experiments performed within the range of pH 3.0–4.5, or 5.0–5.8 (Figure 1b). In addition to *A. caldus*, *Desulfovibrio vulgaris*, a sulfate-reducing bacterium, can be applied during biomining to remediate acid mine wastewater [36].

A comparison of these global systemic studies (Table 1) reveals both common changes in the expression of specific genes and species-specific adaptation strategies. Remarkably, despite substantial differences in sampling time points, stress treatment, stress duration, growth medium, pH (Figure 1b), oxygen availability, strain physiology, and habitats (Figure 1a), a number of common acid stress adaptions are detectable, even among distantly related bacteria. These adaptations include differential expression of genes associated with motility and chemotaxis, efflux and alterations in membrane and cell envelope, nucleotide synthesis, and ribosome and amino-acyl tRNA synthesis (Figure 1c). For example, differential expression of motility and chemotaxis-associated genes is found in *E. coli*, UPEC, *A. caldus*, *B. suis*, *C. jejuni*, *D. vulgaris*, *C. sakazakii*, *L. monocytogenes*, and *R. favelukesii* [20,23,25–27,36–40]. pH-dependent expression of genes involved in flagellar assembly and locomotion, as well as pH taxis, has previously been observed in both *E. coli* and *B. subtilis* and represents a strategy to migrate to territories with desired proton concentrations [41–43]. In response to low pH, modifications in inner and outer membrane composition are noted, which include differential synthesis of transporters, efflux pumps, and fatty acids. These adaptations restrict proton uptake and accelerate proton extrusion, and are detectable in all studied species (Figure 1c). A common adaptation of this type is the upregulation of genes encoding the subunits of F_oF_1-adenosine triphosphate (ATP) synthase, which exports protons in an ATP-dependent manner [44], in *E. coli*, *A. caldus*, *A. pasteurianus*, *B. microti*, and *S. mutans* [19,25,37,45]. Bacterial nucleotide metabolism is also undergoing significant changes (Figure 1c). Particularly in *B. suis*, *E. coli*, and *S. mutans*, genes involved in purine biosynthesis are upregulated under acid stress [19,20,25]. It is worth noting that DNA damage is accelerated by acid stress, and DNA depurination increases proportionally with lower pH [46]. Depurination refers to the loss of purine caused by hydrolysis of *N*-glycosyl bonds, which occurs more rapidly compared with depyrimidination [47]. Thus, the observed increase in purine biosynthesis in acid-stressed bacteria might provide additional purine nucleotides for DNA repair. This hypothesis is supported by metabolomics data from *O. oeni*, which suggest a decrease in the abundance of nucleotides upon acid treatment [48]. In the same study, increased expression of genes related to translation and post-translational modifications is suggested. Indeed, genes encoding ribosomal subunits are differentially expressed upon acid stress, for example, in *E. coli*, *C. jejuni*, *D. vulgaris*, *P. acidilactici*, *O. oeni*, *R. favelukesii*, and *S. albulus* (Figure 1c) [18,20,27,28,36,38,48]. Accordingly, amino-acyl tRNA synthesis in *E. coli*, UPEC, and *L. monocytogenes* is affected by low pH [20,23,40].

In addition to common adaptation strategies, there are also different species-specific mechanisms. The plasmid-encoded putative metal-ion sensing riboswitch is specific for the acid response of *L. monocytogenes* [40]. Acid stress in *B. microti* induces production of the cold-shock protein CspA. A deletion of the corresponding gene decreases the survival of these bacteria in acidified media [25]. Intriguingly, *cspA* is a pseudogene in *B. suis*, a feature that could explain the observed difference in acid tolerance between *B. suis* and *B. microti* [25]. Among other transcriptional changes in the acidophilic *A. caldus*, a decrease in external pH resulted in downregulation of several transcriptional regulators of the LysR family, which have been demonstrated to be essential for maintaining cytoplasmic pH [37]. In *E. coli* K-12, about 2% of all ribosome profiling reads at pH 4.4 mapped to the *asr* region, which encodes an acid-shock protein [20]. This highlights the crucial role of Asr, a periplasmic chaperone that contributes to membrane integrity by preventing aggregation of proteins with positive charges [49]. Moreover, altered pyruvate metabolism was observed in *P. acidilactici* and *O. oeni* [18,48]. UPEC in acidic habitats, is protected by serine deamination resulting in increased levels of pyruvate and ammonia. Presumably, the produced pyruvate is taken up by the putative pyruvate transporter YhjX [50].

In conclusion, while many mechanisms of acid resistance are conserved in a variety of bacterial genera, there are also species- and strain-specific adaptations, which is

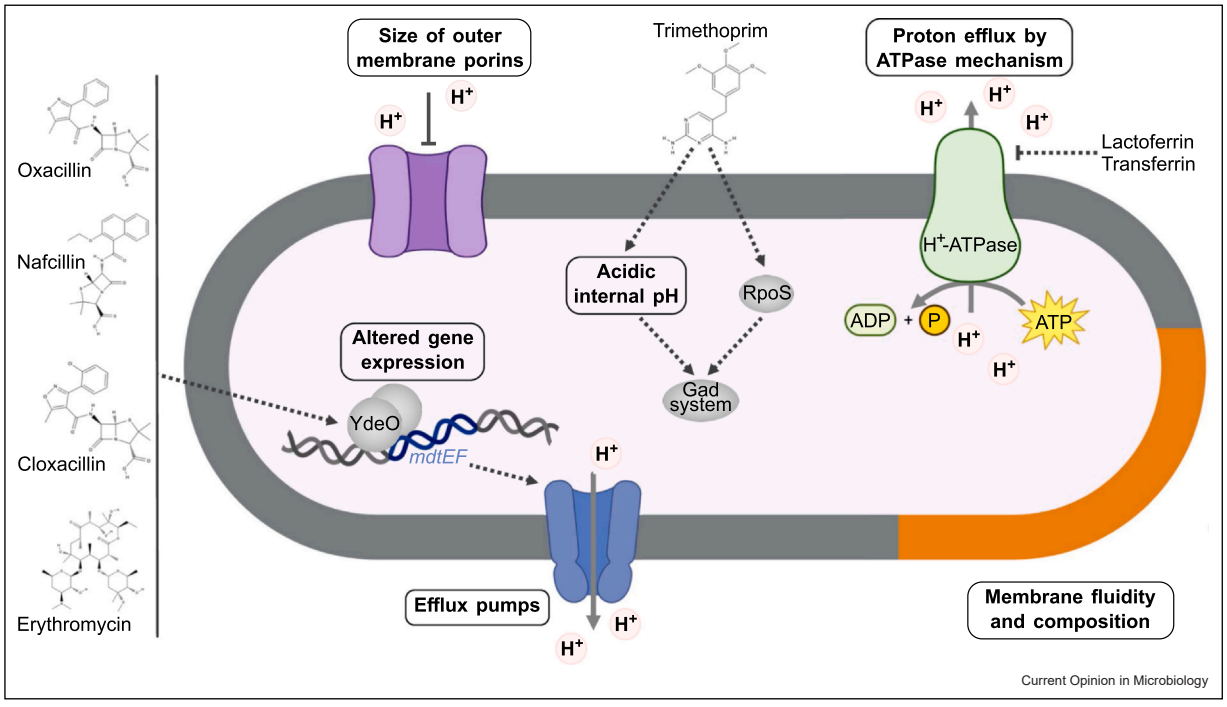
ultimately reflected in the different degrees of acid tolerance among bacteria [5].

Acid stress and antibiotic tolerance

The activation of stress response programs often protects bacteria from subsequent higher levels of the same stress, or other environmental stresses. Acid-induced changes in the size of outer membrane porins, and membrane fluidity and lipid composition, as a mechanism against proton penetration, contribute to a cross-protection against antibiotics of bacterial cells [9,13] (Figure 2). Moreover, the proton-pumping ATPase (H^+-ATPase) is necessary for pumping protons out of bacterial cells and even H^+-ATPase inhibitors are known to enhance antibiotic activity against several Gram-negative bacteria [51–53] (Figure 2). Additionally, multidrug efflux pumps, which contribute to antibiotic tolerance, have been associated with acid stress response systems and are discussed for their role in pH homeostasis [54]. Under acidic conditions, the expression of numerous efflux-encoding and efflux-associated genes is altered. For example, in *E. coli*, the expression of the multidrug locus *mdtEF* is upregulated by pH-induced transcription factors such as GadX–GadW [55] and EvgA–YdeO [56,57] (Figure 2). During the exposure to extreme acidic conditions (pH 2.0), the efflux pump MdtEF–TolC confers a fitness advantage for *E. coli* cells [58]. The expression of *emrKY*, coding for a drug efflux pump, in *Shigella flexneri* is enhanced under acidic pH and high K^+ concentrations to mediate survival during macrophage infection [59]. The expression of *norB*, coding for a drug efflux pump of *Staphylococcus aureus*, is derepressed under moderate acidic conditions via the transcriptional regulator MgrA, leading to a decrease in bacterial killing by the antibiotic moxifloxacin [60].

In recent years, more attention has been paid to the link between intracellular pH and its variability and the bacterial susceptibility to antibiotics. Adaptation to acidic conditions leads to a shift in pH homeostasis toward intracellular acidification that prevents antibiotic killing of bacteria, such as *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*, as well as *M. smegmatis* [61]. For example, when *E. coli*

Figure 2



The interplay between acid stress response and antibiotic tolerance. Examples of the interconnectivity between acid stress and antibiotic tolerance include changes in cell membrane fluidity and composition, altered gene expression, acidification of cytoplasmic pH, size of outer membrane porins, regulation of efflux pumps spanning the inner and outer membranes, and proton efflux by H^+-ATPase (all circled in black boxes). Notably, the pH-responsive transcriptional regulator YdeO is also affected by several antibiotics such as oxacillin, nafcillin, cloxacillin, and erythromycin, leading to upregulation of the *mdtEF* operon (blue), which encodes an efflux pump [56]. TMP influences the intracellular pH and copy number of RpoS, leading to heterogeneous activation of the Gad system [65]. Lactoferrin and transferrin inhibit the H^+-ATPase activity and impair proton efflux [53]. The cell envelope is shown as a gray, partially orange ring. Solid gray arrows indicate transport, dashed arrows indicate influences of compounds and their consequences. The figure is created with BioRender.com and Affinity Designer 1.10.4, and MolView v2.4 was used to draw the chemical structures.

is cultivated under conditions that cause intracellular acidification, its susceptibility to antibiotics, including kanamycin, norfloxacin, and carbenicillin, has been reduced [61]. Furthermore, a reduction in transcripts, all regulated by RpoS and involved in multiple stress response cascades, including acid resistance, multidrug resistance, and osmotic resistance, led to an increase in persistence of *E. coli* [62]. Persister cells survive the antibiotic challenge and allow regrowth after antibiotic removal [63]. *E. coli* cells that become persisters have a more acidic intracellular pH than cells that are either susceptible or viable but nonculturable. The key to this differential pH regulation in persister cells seems to be the enzyme tryptophanase, encoded by *tnaA* [63]. In *Salmonella*, vacuolar acidification and nutritional deprivation induced persistence immediately after uptake by macrophages of the host [64]. Furthermore, acidification of the intracellular pH of *E. coli* cells contributes to the full implementation of resistance to antibiotics, such as mediated via the *marRAB* operon [2].

Mitosch and colleagues [65] analyzed quantitatively at the single-cell level, using fluorescence microscopy, the survival of *E. coli* cells under extreme acid stress (pH 3.0) when the cells were prestressed with antibiotics. Of the tested antibiotics, the folate biosynthesis inhibitor trimethoprim (TMP) activated an acid stress response and thus cross-protection of bacteria from subsequent extreme acid stress (Figure 2). This response to acidity is mediated via the Gad system by the expression of *gadBC*, which in turn is highly variable under acid stress. The highly variable *gadBC* expression correlated with single-cell survival by maintaining higher intracellular pH (see below). It has been hypothesized that the acid stress response to TMP is activated as a downstream effect of the depletion of purine bases [65]. In a subsequent study [66], they showed that the acid stress response caused by TMP in single cells does not have a strict temporal order with the DNA damage repair response, known as SOS response.

In conclusion, the correlation between the degree of intracellular acidification of individual cells and a resulting antibiotic tolerance/resistance of subpopulations needs to be further investigated.

The heterogeneous acid stress response

Under acid stress, bacteria often show heterogeneous behavior to increase the fitness of a population by enabling division of labor. Phenotypic heterogeneity arises from internal factors such as individual physiological state and stochastic gene expression and is strongly influenced by external factors such as physical and chemical stresses, availability of nutrients, and cell density [67,68].

E. coli, for example, possesses the four enzyme-based H^+-consuming acid-resistant systems Gad, Adi, Cad, and Orn. Recently, activation of the Gad, Adi, and Cad systems was quantitatively analyzed using a fluorescent triple *E. coli* reporter strain under consecutively increasing acid stress conditions [69]. The Cad system (activated at pH 5.8 in the presence of lysine) and the Adi system (activated at pH 4.4) were found to be mutually exclusively activated, with a high level of ON–OFF cells. These phenotypic variations are not only due to differences in the pH homeostasis of individual *E. coli* cells [70], but also to the low copy number of the transcriptional regulators CadC and AdiY [69,71,72]. It should be noted that activation of the Cad or Adi system leads to an increase in internal pH based on the consumption of protons in the decarboxylation of lysine to cadaverine or arginine to agmatine, respectively, in individual cells, but the entire population also benefits due to the secretion of the more alkaline cadaverine and agmatine, which raise the external pH [69].

The Gad system (activated at moderate acid stress and essential under extreme acid stress) was activated in all *E. coli* cells, albeit to varying degrees [69]. Activation of two promoters of the Gad system, namely *gadBC* and *gadX*, is highly dynamic and variable, leading to cell-to-cell heterogeneity [65,73] that ensures the survival of at least a subpopulation of *E. coli* under extreme acid stress. A recent study revealed that *gadE*, encoding the master regulator of the Gad system, is heterogeneously expressed even in the absence of acid stress, thereby preemptively generating acid-resistant subpopulations within a clonal *E. coli* population [74]. The heterogeneous activation of these three enzyme-based acid resistance systems enables division of labor in the bacterial population and survival over a wide range of low-pH values.

Vibrio species have only the Cad and Orn systems to counteract acidic conditions. In contrast to *E. coli*, the Cad system was shown to be activated in all cells in *V. campbellii* [72]. Apparently, all cells in the *Vibrio* population need the Cad system to increase their intracellular pH by inducing the proton-consuming lysine decarboxylase.

In *Salmonella*, which resides in acidic vacuoles of macrophages, transcriptional heterogeneity can have functional consequences for host–pathogen interactions [75,76]. This phenotype involves regulation by the transcription factors SsrB and PhoP [77], but further studies are required to understand the exact mechanism.

Phenotypic heterogeneity is certainly inherent in many other neutralophilic bacteria that survive in acidic environments, but has been less documented. In addition, the underlying molecular mechanisms that give rise to it and maintain it evolutionarily need to be further investigated.

Conclusions and perspectives

In the past decades, great progress has been made in understanding the different molecular mechanisms of acid stress response in model bacteria, such as *E. coli*, *Salmonella*, and *Listeria*, which have diverse acid resistance systems. New opportunities are now emerging using next-generation sequencing methods that allow the systemic analysis of acid stress response of various bacteria originating from humans, soil, and food. As described above, many mechanisms of acid stress response, such as differential expression of genes involved in transport, H^+-efflux, and membrane permeability, as well as motility and chemotaxis, but also nucleotide synthesis, and translation, are conserved in phylogenetically distantly related bacteria. Nevertheless, bacterial species differ in terms of additional components and degree of acid tolerance, which in turn reflect optimal adaptation to pH fluctuations in the habitats they colonize.

An exciting new aspect is that the acid stress response, in particular the reduction in membrane fluidity, the change in membrane channel size, proton flux through H^+-ATPases, the activation of other proton pumps, and the differential gene induction due to decreased intracellular pH are associated with antibiotic tolerance/resistance (Figure 2). In the future, proteins involved in acid stress adaptation could be explored as targets to sensitize pathogenic bacteria to antibiotics.

pH plays a crucial role in bacterial interactions [78]. It is important to note that low pH in nature is often caused by weak organic acids such as acetic acid, lactic acid, and propionic acid, also known as short-chain fatty acids. These acids originate from fermentation processes under anaerobic growth conditions and accumulate in high concentrations, for example, in the human large intestine (100 mM) [79] or rumen. Even mild acid stress (pH 6–7) strongly enhances the uptake of acetic acid (pKa value = 4.75), since this acid is membrane-permeable in the protonated form. In the cytoplasm, acetic acid dissociates, leading to anion accumulation but also to dissipation of the electrochemical gradient. In the future, it will be important to focus on the stress response to organic acids in prominent intestinal bacteria belonging, for example, to the phyla Actinobacteria, Firmicutes, Bacteriodetes, and Verrucomicrobiota. These studies will help to better understand the complexity of the human gut microbiota.

Finally, there are already examples of transient acidification of an environment to coordinate effective symbiosis between bacteria and eukaryotes [80], a still underestimated phenomenon with major implications for host–bacterial interactions that needs to be further explored.

In conclusion, acidification and adaptation to acid stress are exciting areas of research with far-reaching implications for human health, food industry, and agriculture.

CRediT authorship contribution statement

Kilian Schumacher: Conceptualization, Writing – original draft, preparation of figures. **Sophie Brameyer**: Conceptualization, Writing – original draft, preparation of figures. **Kirsten Jung:** Conceptualization, Writing – original draft and editing.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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