CHAPTER 18

MICROBIAL STRESS RESPONSES

When supplied with sufficient nutrients and optimal growth temperature, pH, oxygen levels, and solute concentrations, microbes will grow at a maximum growth rate characteristic for the organism. Variations in any of these parameters can affect the maximum growth rate and, thus, can represent an environmental stress for the microbe. The ability of microbes to sense and respond (correctly) to impromptu alterations in the environment is crucial to their survival. In reality, conditions that allow for maximal growth rates outside the laboratory are few and far between. As a result, most bacteria live in a constant state of stress. This chapter covers the physiologic and genetic responses of certain well-characterized bacteria to various stress conditions.

OSMOTIC STRESS AND OSMOREGULATION

The concentration of solutes (e.g., salts, ions, metabolites) plays a critical role in microbial growth. In the laboratory, most microbes exhibit optimal growth in culture media of relatively low osmolarity. For many bacteria, hypertonic or hyperosmotic conditions result in water loss from the cytoplasm, causing the cell to shrink (plasmolysis). Hypotonic or hypoosmotic conditions result in an influx of water into the cytoplasm, which causes the cell to swell (plasmoptysis) and perhaps burst in a process referred to as osmotic lysis. Microbial membranes are freely permeable to water and, therefore, water inside the cell is essentially at equilibrium with the outside of the cell. Microbes with cell walls can and do maintain a high cytoplasmic solute concentration relative to the outside. This translates into lower water activity (A_w) inside the cell.

\[
A_w = x \div (x + c)
\]

\[c = \text{osmolality of solute(s)}; \ x = \text{moles of water per liter (55.6)}\]
The lower cytoplasmic $A_w$ causes water to at least try and flow into the cell. This places significant pressure on the microbial cell wall as the cell volume attempts to increase at the same time the cell wall prevents the cell from swelling. This pressure placed on the cell wall by the cytoplasmic membrane is referred to as turgor pressure or turgor. Turgor is opposed by the tension of the cell envelope. The function of osmoregulatory mechanisms or osmotic stress responses is to maintain turgor within limits, allowing for maintenance of cell viability.

Movement of water occurs by diffusion and, in a much more rapid process, through water-selective channels called aquaporins. The AqpZ channel of *E. coli* has been shown to mediate rapid and large water fluxes in both directions in response to sudden osmotic upshifts or downshifts, although its role in the cell is not essential. Turgor is maintained by regulating the total osmotic solute pool in the cytoplasm and the relative level of solutes in the periplasm (in Gram-negative bacteria) immediately outside the cytoplasmic membrane. In low-osmolality media, cytosolic osmolality is largely due to ionic solutes (e.g., $K^+$ ions); in high-osmolality media it largely involves neutral solutes (e.g., trehalose).

Some of the mechanisms described below are also responsible for maintaining osmotic homeostasis. For this reason, mechanisms of osmoregulation such as $K^+$ ion influx/glutamate biosynthesis–coupled systems are present all the time in the cell.

### High Osmolality

As the osmolality of the surrounding environment increases, turgor pressure drops and growth slows or halts. Macromolecular biosynthesis is inhibited and respiration rates decline. The most rapid response to this osmotic upshock is an increase in $K^+$ ion influx through, at least, three uptake systems in *E. coli*: Trk, Kdp, and Kup. Homologous systems to Trk and Kdp have been identified in *Salmonella enterica* and other bacteria. The Trk and Kdp systems appear to be the major systems for $K^+$ uptake under these conditions, since they can achieve sufficiently high rates of uptake. The Trk system is composed of three components: TrkA (peripheral membrane protein), TrkE (membrane associated), and either TrkH (in *E. coli*; membrane-spanning protein) or TrkG (*E. coli* and other bacteria; membrane-spanning protein). The Trk system binds NAD(H) via TrkA and may regulate $K^+$ ion uptake. The Kdp system (see Chapter 9) is also a three-component system composed of KdpA (membrane-spanning protein), KdpB (integral membrane protein), and KdpC (peripheral membrane protein). KdpB is a P-type ATPase and likely provides the energy to drive $K^+$ ion influx through this system. Kup is a single, large membrane-spanning protein possessing a significant cytoplasmic tail domain. In addition to influx, $K^+$ ion accumulation results from plasmolysis and the closing of stretch-sensitive $K^+$ ion efflux channels.

Concurrent with the increase in $K^+$ ion influx, as a result of high external osmolality, is a decrease in intracellular putrescine levels due to increased excretion. Putrescine is a divalent cationic polyamine; thus, a divalent putrescine is replaced by the monovalent $K^+$ ion, allowing cytoplasmic osmolality to increase with minimal effect on the intracellular ionic strength.

The major anionic compound involved in osmoregulation (osmolyte) is glutamate. Glutamate is synthesized and accumulates quickly following osmotic upshock and is dependent on $K^+$ ion uptake. In *E. coli* and other enteric bacteria, glutamate is synthesized by two enzymes: glutamate dehydrogenase (GDH) and glutamate synthase (GS).
Many microbes also accumulate the **disaccharide trehalose** (composed of two glucose residues) as a **compatible solute** in response to osmotic stress as well as starvation, thermal stress, and desiccation stress. Trehalose is synthesized by the products of the *otsAB* operon. *OtsA* is a trehalose-6-phosphate synthase and *OtsB* is a trehalose-6-phosphate phosphatase. In the absence of exogenous osmoprotectants (see below), *E. coli* can accumulate trehalose to levels that may comprise as much as 20% of the cytoplasmic osmolality under conditions of high osmolality.

Several compounds, when present externally, can stimulate bacterial growth rates under hyperosmotic conditions. These compounds are **osmoprotectants**. They are zwitterionic in nature and resemble glycine betaine and proline.

![Chemical structures: Betaine, l-proline, glycine betaine](image)

Many potential osmoprotectants are found in plants and animals: betaine, betaine aldehyde, proline betaine, choline, choline-O-sulfate, stachydrine, and β-dimethylsulfonopropionate are present in plants, and carnitine is plentiful in animals. In *E. coli*, choline is converted to betaine aldehyde by the membrane-associated O2-dependent BetA choline dehydrogenase, and then to glycine betaine by the soluble NADP-dependent betaine aldehyde dehydrogenase. Ecotoine produced by many halophilic (salt-loving; marine) bacteria is an excellent osmoprotectant for *E. coli* and other bacteria. These compounds are taken up into the cell by two osmotically regulated permeases, ProP and ProU, in *E. coli* and *S. enterica* and homologs are found in other bacteria. ProP has relatively low affinity for proline and appears to function primarily in the uptake of betaine. The ProU permease also has relatively low affinity for proline but is a high-affinity betaine transporter. The major proline transporter is PutP, but it mainly functions in proline utilization and is not involved in osmoprotection (see Chapter 5 for its regulation).

Interestingly, these mechanisms of osmoprotection exhibit a hierarchy of function. Initially, the intracellular level of K⁺ ions rapidly rises, maximizing by 20 minutes. Glutamate accumulation follows somewhat more slowly. The cell recovers from plasmolysis as turgor is restored. Trehalose levels rise and K⁺/glutamate levels decline after about 60 minutes. If an osmoprotectant such as betaine is added externally, trehalose concentrations decline and K⁺ ion levels decrease even further. Restarting of macromolecular biosynthesis corresponds with a rise in trehalose levels.

**Low Osmolality**

Immediately following a significant decrease in osmolality (**hypoosmotic shock**), there is a rapid flux of water into the cell, increasing turgor. This results in stretching of the cell envelope and may lead to “cracks” in the membrane or may stretch existing pores or activate stretch-activated channels. The permeability of the membrane will be increased but only temporarily. Downshifts in osmolality can result in the extrusion of osmolytes and ions as well as the loss of amino acids, nucleotides, and other solutes from the cytoplasm.
Complex sugars, known as membrane-derived oligosaccharides (MDOs) that contain 6 to 12 glucose residues joined via $\beta(1 \rightarrow 2)$ and $\beta(1 \rightarrow 6)$ linkages, forming a branched structure, are found in the periplasm of Gram-negative bacteria. MDOs are substituted with $sn$-1-phosphoglycerol and phosphoethanolamine derived from the membrane phospholipids and also with O-succinyl ester residues. Synthesis of these compounds is induced by growth in conditions of low osmolality.

**Osmotic Control of Gene Expression**

EnvZ/OmpR is a paradigm of two-component systems (see Chapter 5). The outer membrane of *E. coli* K-12 contains several major outer membrane proteins. The relative amounts of two of them, coded by the *ompC* (47 min) and *ompF* (21 min) loci, are mediated by medium osmolarity. *OmpC* (mwt 36,500 mw) and *OmpF* (mwt 37,000) are porin proteins that form aqueous pores in the outer membrane, allowing polar molecules (<600 da) to cross the outer membrane barrier. In media of low osmolality, OmpF protein is present in greater quantities than OmpC protein. In media of high osmolality, the OmpC porin predominates over OmpF. While the quantitative ratios of these proteins vary, their combined levels remain fairly constant.

Regulation of these genes (Fig. 18-1) is mediated by the regulatory *ompB* locus (21 min). The *ompB* region actually is comprised of two genes, *ompR* and *envZ*, arranged in an operon. EnvZ (50,300 Da) is a transmembrane sensor protein that undergoes autophosphorylation at histidine 243 under conditions of high osmolarity. How EnvZ senses environmental change is unclear, since the periplasmic portion of the protein is apparently not required. Once phosphorylated, EnvZ will then transphosphorylate aspartate 55 in the receiver module of the true DNA-binding regulator, OmpR (27,400 Da), thereby increasing the level of OmpR$\sim$P in the cell. Phosphorylation of OmpR is required for the expression of both *ompC* and *ompF*. Presumably, conformational reshaping of OmpR by phosphorylation may increase DNA-binding affinity to the *ompC* and *ompF* promoters and enables interaction of OmpR with the $\alpha$ subunit of RNA polymerase, thereby activating transcription.

If OmpR$\sim$P is required for the transcription of both *ompF* and *ompC*, what could account for their reciprocal control? As shown in Figure 18-1, there are three OmpR-binding sites in front of *ompC* and *ompF*. At low osmolarity, the OmpR$\sim$P present in the cell binds to *ompF* sites F1, F2, and F3, activating *ompF* transcription, but only binds to *ompC* site C1. At high osmolarity, the higher level of OmpR$\sim$P resulting from EnvZ transphosphorylation now binds to C1, C2, and C3, activating *ompC* transcription. It is strange that this level of OmpR$\sim$P still binds at F1, F2, F3 but now represses *ompF*. It is proposed that when OmpR$\sim$P is bound to DNA, changes in osmolarity affect OmpR$\sim$P-OmpR$\sim$P protein interactions, which in turn alter the ability of OmpR to activate transcription at *ompF* (Fig. 18-1). Osmotic downshift — that is, when osmolarity shifts from high back to low — activates an EnvZ phosphatase that removes P from both OmpR and EnvZ, reversing the OmpC/OmpF expression ratio. A second phosphatase, SixA, may also be involved in this process.

A variety of studies indicate that alternative phosphodonors are capable of phosphorylating OmpR in the absence of EnvZ. One proven alternative phosphodonor is acetyl phosphate, although there may be additional cross-talk with other histidine kinases.

An additional control mechanism that appears to regulate the relative amounts of OmpC and OmpF involves a gene (*micF*) adjacent to *ompC*, which is transcribed
Fig. 18-1. Osmoregulation of major outer membrane proteins OmpF and OmpC.
in the opposite direction from ompC. The micF transcript (micRNA, 93 nucleotides) is complementary to the 5′ end region of ompF mRNA. Under conditions of high osmolarity, the micF-ompC region is induced and transcribed divergently from a central promoter. The resulting micRNA (also referred to as antisense RNA) inhibits translation of ompF mRNA by hybridizing to it. This RNA–RNA interaction is proposed to cause premature termination of ompF transcription and/or destabilization of ompF mRNA. The end result is that when more OmpC protein is produced, less OmpF protein is synthesized.

The role of the EnvZ-OmpR system in cellular physiology extends well beyond its role in governing porin expression. Studies have connected OmpR with flagellar expression, cell division, fatty acid transport, microcin synthesis, curli fibers, Salmonella virulence, and acid tolerance. One mechanism by which OmpR may affect virulence is through its involvement in controlling cytotoxicity toward infected macrophages. In addition, OmpR controls the expression of another two-component regulatory system, SsrAB, which controls expression of genes in the Salmonella pathogenicity island SPI-2. Recent evidence indicates that OmpR autoregulates its own expression in response to acid pH, providing a partial explanation for why the SPI-2 genes are induced by low pH.

AEROBIC TO ANAEROBIC TRANSITIONS

Facultative microorganisms such as E. coli and S. enterica are capable of modifying their metabolism to accommodate growth under either aerobic or anaerobic conditions. The transition between aerobic and anaerobic metabolism is accompanied by alterations in the rate, route, and efficiency of pathways of electron flow. Figure 18-2 illustrates the basic pathways utilized by E. coli for aerobic versus anaerobic electron flow. Under anaerobic conditions without alternate electron acceptors, pyruvate is converted to formate, acetate, or ethanol, CO2 and H2 gas (mixed acid fermentation). However, the choices and energy yield become more plentiful when alternate electron acceptors are available. E. coli, even under aerobic conditions, synthesizes two distinct cytochrome oxidases — cytochrome o (cyo operon) and cytochrome d (cyd operon) — produced under high O2 and low O2 conditions, respectively. Under anaerobic conditions, at least five more terminal oxidoreductases can be produced (Table 18-1).

<table>
<thead>
<tr>
<th>Electron Acceptor</th>
<th>ΔG°(in) (kJ/mol)</th>
<th>Terminal Respiratory Enzyme</th>
<th>Operon</th>
<th>Chromosomal Location (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2</td>
<td>−233</td>
<td>Cytochrome o oxidase</td>
<td>cyoABCDE</td>
<td>10</td>
</tr>
<tr>
<td>O2</td>
<td>−233</td>
<td>Cytochrome d oxidase</td>
<td>cydAB</td>
<td>17</td>
</tr>
<tr>
<td>NO3−</td>
<td>−144</td>
<td>Nitratreductase</td>
<td>narGHJI</td>
<td>27</td>
</tr>
<tr>
<td>NO3−</td>
<td>−144</td>
<td>Nitratreductase</td>
<td>narZYWV</td>
<td>33</td>
</tr>
<tr>
<td>DMSO</td>
<td>−92</td>
<td>DMSO/TMAO reductase</td>
<td>dmsABC</td>
<td>20</td>
</tr>
<tr>
<td>TMAO</td>
<td>−87</td>
<td>TMAO reductase</td>
<td>torA</td>
<td>28</td>
</tr>
<tr>
<td>Fumarate</td>
<td>−67</td>
<td>Fumarate reductase</td>
<td>frdABCD</td>
<td>94</td>
</tr>
</tbody>
</table>

*Free energy calculated by using NADH as an electron donor to the indicated electron acceptor.*
Fig. 18-2. Basic aerobic and anaerobic metabolic pathways of *E. coli*. 
E. coli controls the production of the various respiratory pathway enzymes, first, in response to aerobic and anaerobic growth conditions and, second, to the availability of alternate electron acceptors. Clearly there is a hierarchy or preference for substrate use with the following order: oxygen > nitrate > DMSO > TMAO > fumarate. When several e\textsuperscript{-} acceptors are present simultaneously, the more energetically favored acceptor will be used first. An interesting question is how does the cell regulate such a complex system. There are three basic regulators described that sense changes in oxygen level or redox conditions. Their overlapping control circuits are illustrated in Figure 18-3.

**Formate Nitrate Regulation**

The Fnr protein (formate nitrate regulation) regulates over a hundred genes in response to the presence or absence of oxygen. The manner in which Fnr senses oxygen appears to involve an oxygen-sensitive (4Fe-4S) center (Cys-X\textsubscript{2}-Cys-X\textsubscript{2}-Cys-X\textsubscript{2}-Cys). Active Fnr is a dimer and, therefore, contains two such centers. Exposure to oxygen causes partial disassembly of the two (4Fe-4S) centers to form two (2Fe-2S) centers. This disassembly converts Fnr dimers, which bind DNA, to monomers that are not active (Fig. 18-4). Under anaerobic conditions, Fnr represses cyoABCDE, cydAB and narZYWV (Fig. 18-3) but transcriptionally activates sdhCDAB (succinate dehydrogenase), frdABCD (fumarate reductase), dnsABC (DMSO reductase), and narGHIJ (nitrate reductase). Fnr bears significant sequence homology to the CRP protein and, like CRP, alters transcription through DNA bending and direct interactions with the \(\alpha\)-subunit of RNA polymerase. In contrast to CRP, there is no evidence for cAMP involvement with Fnr.

**Nitrate Response**

Nitrate is the preferred electron acceptor for anaerobic cells because of the high midpoint potential of the nitrate/nitrite couple. In addition to fnr, the narXL operon and narQ narP are required to regulate respiratory gene expression in response to nitrate availability. DNA sequence analysis indicates narX narL and narQ narP are two-component regulatory systems that mediate a series of complex transcriptional adjustments in response to a dynamic ratio of two alternate electron acceptors: nitrate, and its reduction product, nitrite. NarQ and NarX are histidine kinases that sense nitrate and transmit a signal to their cognate response regulators NarP and NarL, respectively. This system contributes to the induction of frd, dms, and narGHIJ operons but has no effect on cyo, cyd, or narZYWV.

The control of the nitrite reductase operons nir and nrf by NarP and L offers a glimpse of the complex adjustments that occur in response to the cell’s needs. Nitrate, at low concentration, induces nitrate reductase and the high-affinity Nrf nitrite reductase via the NarXL and NarQP systems. Thus, the cell will derive maximum energy yield from the nitrate present. However, at high nitrate concentrations, nitrate reductase is still expressed, but NarL will actually repress the Nrf nitrite reductase while inducing the low-affinity NirB nitrite reductase system. This differential regulation avoids generating too much proton motive force and energy that would result from using the high-affinity nitrite reductase under high nitrate conditions when plenty of energy is derived from nitrate reductase. Nevertheless, the low-affinity nitrite reductase is still necessary to detoxify the nitrite produced.
Fig. 18-3. Regulatory scheme for the control of aerobic and anaerobic respiratory pathways in *E. coli.*
ArcAB System

The system that controls cyo and cyd includes the arcA and arcB products (arc, meaning anoxic redox control) comprising a two-component regulatory system. The system actually regulates a total of 30 genes. ArcB functions as the membrane-bound sensor/transmitter that communicates with ArcA, the receiver/regulator. ArcB is an
unorthodox histidine kinase in that it contains three phosphotransfer signaling domains (Figs. 18-3 and 18-4). The first domain, the histidine-kinase module, phosphorylates itself at a conserved histidine (H292). The phosphate is subsequently transferred, first to an aspartate residue (D576) in the receiver module and then to a second histidine (H717) in the phosphotransfer domain located at the carboxyl end of ArcB. The phosphate is subsequently transferred to an aspartate residue (D54) within the amino terminus of ArcA.

The Arc system regulates the coordinate synthesis of tricarboxylic acid cycle enzymes. ArcA∼P is generally a repressor of aerobically expressed target genes [e.g., acn (aconitase), cyo, gltA (citrate synthase), sdh (succinate dehydrogenase)] and an activator of anaerobically expressed target genes [cyd, hya (hydrogenase 1), pdu (propanediol degradation)]. The SixA phosphohistidine phosphatase modulates the system by removing phosphate from ArcB residue H717, downregulating phosphotransfer. The nature of the sensed low oxygen signal, how it is sensed, and how the signal is transduced to activate ArcB kinase are unclear. A model in which ArcB senses proton motive force has been refuted, since the only amino acid (H47) with a physiologically relevant pK within the transmembrane region has proven dispensable. Other models invoke the ArcB-sensing redox state of the cell and/or metabolites (e.g., lactate, acetate) generated by anaerobic metabolism. Figure 18-3 presents a model depicting the regulatory interactions of Nar, Fnr, and Arc that govern selected oxygen-regulated genes.

**OXIDATIVE STRESS**

Generation of energy by the electron transport chain is dependent on the catalytic spin pairing of triplet oxygen. During this process, oxygen species that are toxic to DNA, protein, and lipid components of the cell are formed through both enzymatic and spontaneous chemical reactions. The reaction of oxidative enzymes with molecular oxygen can generate superoxide (superoxide anion):

\[ \text{O}_2 + e^- + \text{oxidative enzymes} \rightarrow \text{O}_2^- \]

Superoxide is relatively unreactive with DNA and proteins. However, it may interact in a number of enzymatic as well as spontaneous chemical reactions to produce more highly reactive oxygen derivatives such as hydrogen peroxide and hydroxyl radicals:

\[ \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{OH}^+ + \text{O}_2 \]

Autooxidation of reduced FAD or reduced flavoprotein gives rise to hydrogen peroxide:

\[ \text{FADH}_2 + \text{O}_2 \rightarrow \text{FAD} + \text{H}_2\text{O}_2 \]

The enzyme NADPH oxidase can generate superoxide anion and hydrogen peroxide:

\[ \text{NADPH} + \text{H}^+ + 2\text{O}_2 \rightarrow \text{NADP}^+ + \text{O}_2^- + \text{H}_2\text{O}_2 \]
Superoxide can release \( \text{Fe}^{2+} \) and \( \text{Fe}^{3+} \) from various cellular compounds and ultimately give rise to hydroxyl radicals through the **Fenton reaction**: 

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\cdot + \text{OH}^-
\]

Reaction of superoxide anion with **nitric oxide** forms **peroxynitrite anion**:

\[
\text{O}_2^\cdot^- + \text{NO}^\cdot^- \rightarrow \text{ONOO}^-
\]

Peroxynitrite is highly reactive with various proteins. Methionine, cysteine, tyrosine, and tryptophan residues of proteins are especially vulnerable. Peroxynitrite can also give rise to other toxic derivatives that are highly reactive with biological compounds.

Most aerobic organisms are protected from the toxicity of superoxide and hydrogen peroxide by the enzymes **superoxide dismutase (SOD)** and **catalase**:

\[
\text{O}_2^\cdot^- + \text{O}_2^\cdot^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \text{ (SOD)}
\]

\[
2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \text{ (catalase)}
\]

**Peroxidases** can also catalyze the reduction of hydrogen peroxide by organic reductants such as **glutathione** or **ascorbic acid**:

\[
\text{H}_2\text{O}_2 + 2\text{RH} \rightarrow 2\text{H}_2\text{O} + 2\text{R}_{\text{ox}}
\]

*E. coli* produces a cytoplasmic **Mn-SOD (SodA)** and **Fe-SOD (SodB)** that protect DNA and proteins from oxidation. Mutants deficient in these enzymes display enzyme inactivation, growth deficiencies, and DNA damage. A periplasmic **Cu/Zn-SOD (SodC)** protects the periplasmic and membrane constituents from exogenous superoxide. Several DNA repair systems also aid in recovery from oxidative damage (see Chapter 2).

The presence of SOD and catalase in anaerobic organisms would appear to be self-defeating since oxygen is a product of both reactions. Anaerobes must rely on other mechanisms to eliminate oxygen and prevent the formation of toxic oxygen species. One method used by organisms that do not carry out oxygen-dependent respiration involves a unique flavoprotein, **NADH oxidase**, which catalyzes the direct four-electron reduction of oxygen to water:

\[
\text{NADH} + \text{H}^+ + 0.5 \text{O}_2 \rightarrow \text{NAD}^+ + \text{H}_2\text{O}
\]

This enzyme actually allows streptococci and other lactic acid bacteria to use oxygen directly in the metabolism of carbohydrates without the inherent problems of oxygen toxicity caused by the formation of reactive oxygen derivatives.

A superoxide reductase system that provides protection against oxidative stress is present in the anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris* and in the hyperthermophilic anaerobe *Pyrococcus furiosus*. It has the advantage of eliminating superoxide without the formation of molecular oxygen. This system consists of the
nonheme iron-containing proteins ruberythrin (Rbr) and rubredoxin oxidoreductase (Rbo) that reduce superoxide to hydrogen peroxide without dismutation:

\[
O_2^{-} + e^- + 2H^+ \rightarrow H_2O_2
\]

This system functions in conjunction with an NADH peroxidase that reduces hydrogen peroxide to water:

\[
NADH + H^+ + H_2O_2 \rightarrow NAD^+ + 2H_2O
\]

These and other mechanisms used by anaerobic or facultative organisms to protect against oxygen damage are discussed later in reference to organisms that utilize primarily fermentation pathways in their metabolism.

Regulation of the Oxidative Stress Response

The natural by-products of aerobic metabolism are the reactive compounds superoxide \((O_2^-)\) and hydrogen peroxide. These two species can lead to the generation of hydroxyl radicals \((\text{OH}^+)\), which can damage biological macromolecules. The oxidation stress modulon includes at least 80 proteins induced during exposure to superoxide. About half are also induced by \(H_2O_2\). Research efforts have uncovered two regulons within this stress response, although there are certainly more. The two known systems, OxyR and SoxRS, used by \(E. coli\), are outlined in Figure 18-5.

The OxyR regulon comprises nine of the proteins induced by \(H_2O_2\). All are controlled by the positive regulator OxyR. OxyR is a class I type activator that, once bound to a target DNA sequence, interacts with the \(\alpha\) C-terminal domain of RNA polymerase (see Chapter 5). The protein is activated by oxidation, which results in the formation of a disulfide bond between cysteine residues C199 and C208. Under noninducing conditions these cysteines are reduced by glutaredoxin I. For most OxyR-dependent genes, the oxidized but not the reduced form of OxyR binds target DNA sequences. However, the reduced form of this regulator can bind to the \(oxyR-oxyS\) target operator/promoter region but will not activate transcription until OxyR itself is oxidized. Some of the enzymes whose expression is regulated by OxyR include catalase (\(katG\)), glutathione reductase (\(gorA\)), glutaredoxin I (\(grxA\)), and alkyl hydroperoxide reductase (\(ahpC\) and \(ahpF\)). The likely in vivo function for alkyl hydroperoxide reductase would be the detoxification of lipid and other hydroperoxides produced during oxidative stress.

In \(Salmonella\), reduced-OxyR was found to act as a repressor of the \(narZYWV\) locus, which encodes a stress-inducible aerobically expressed nitrate unresponsive nitrate reductase (\(NR-Z\)). In the presence of \(H_2O_2\), the oxidized-OxyR no longer represses \(narZYWV\) transcription and the operon becomes derepressed to about one-third of its maximal induced level of expression. Other loci have also been shown to be repressed by OxyR. Thus, OxyR, like many other response regulators, can function as both a transcriptional activator and repressor protein, depending on the target gene.

A second oxidative stress regulon comprises nine proteins induced by superoxide but not hydrogen peroxide. This regulon is under positive transcriptional control by the \(soxRS\) loci. Genes under \(soxR\) control include those responsible for Mn\(^{2+}\)-containing superoxide dismutase (\(sodA\)), the DNA repair enzyme endonuclease
The current model for this system is that preexisting SoxR protein senses oxidative stress and then triggers expression of the soxS gene. The SoxS product then activates transcription from the promoters of the other members of the regulon.

As with Fnr, SoxR utilizes an Fe sulfur center to sense oxidative stress conditions in the cytoplasm. SoxR contains two stable (2Fe-2S) centers anchored to four cysteine residues near its carboxy terminus (Fig. 18-4). These (2Fe-2S) centers remain reduced (+1 state) under normal physiological conditions but rapidly oxidize (+2 state) when challenged with oxidative stress. It is believed that iron-sulfur centers are especially sensitive to redox reactions with superoxide. The change in redox state of the protein induces an active conformation of SoxR but does not change binding affinity.

Both oxidized and reduced forms of SoxR bind to the soxS promoter. Thus, oxidation, while not affecting binding, must enable SoxR to interact with RNA polymerase, stimulating open complex formation. There is some evidence that oxidized SoxR is reduced by the NADPH:flavodoxin oxidoreductase/flavodoxin couple.
with the oxidation of NADPH. The genes encoding these enzymes are themselves induced by SoxR, which suggests a mechanism of SoxRS autoregulation—that is, as the system is induced by oxidized SoxR (e.g., aerobic growth), the levels of the oxidoreductase/flavoredoxin couple increase, which will reduce SoxR and downregulate the system.

**pH STRESS AND ACID TOLERANCE**

Microbes can grow over a wide range of hydrogen ion concentrations (pH). For example, *acidophilic bacteria* will grow in acidic sulfur springs where a pH of 1 is common. In contrast, *alkalophilic bacteria* prefer to grow in environments such as soda lakes where pH conditions rise as high as pH 11. Most bacteria in the human sphere, however, prefer to grow at pH values closer to neutral and are called *neutralophilic*. They generally grow over a range of pH 5 to pH 9 and include *E. coli*, *S. enterica*, *Streptococcus lactis*, *Bacillus subtilis*, and many others. Although the neutralophiles do not generally grow under conditions of extreme acid or base, they can survive these exposures to various degrees if they are allowed to go through an adaptive transition whereby pH gradually changes.

It is well established that one way microbes respond to an acidifying pH is by producing enzymes that can convert acidic metabolites to neutral ones or neutral metabolites to alkaline products. Good examples of these types of enzymes are glutamate decarboxylase, lysine decarboxylase, and arginine decarboxylase of *E. coli*, all of which exhibit increased expression at external acidic pH. The concept of pH homeostasis or the ability of the microbe to control its internal pH is discussed in Chapter 9. It has been demonstrated that the major mechanisms typically used by Gram-negative organisms to control internal pH during growth involves the modulation of the primary proton pumps as well as the K⁺/H⁺ and Na⁺/H⁺ antiporters. However, additional adaptive mechanisms are engaged to survive under pH conditions outside the growth range.

Exposures of *S. enterica* and *E. coli* to a nonlethal acidic pH between pH 5 and 6 results in the induction of sets of genes whose products can protect the cell when exposed to potentially lethal pH conditions from pH 4 to pH 2. These inducible systems protect the cell at external pH values where the nonadaptive pH homeostasis mechanisms fail. The genetic and physiologic changes that occur in the cell are referred to as the acid tolerance response (ATR). Full induction of the ATR results in the increased expression of at least 50 newly synthesized or existing proteins called acid shock proteins, some of which are also heat shock induced.

One system important to acid tolerance in some organisms is the Mg²⁺-dependent proton translocating ATPase (see “Energy Production” in Chapter 9). In Gram-positive organisms such as *Enterococcus faecalis*, the ATPase, which normally harnesses proton motive force to generate ATP, can work in reverse, hydrolyzing ATP to extrude a proton from the cell. As opposed to Gram-negative organisms that struggle to keep internal pH near 7.8, the streptococci are more flexible. Their only requirement is to maintain a pH of 0.5 to 1 unit—a goal the ATPase alone could accomplish.

The superior acid resistance of *E. coli* and *Shigella* over that of *S. enterica* is of particular interest from a medical view, since it helps explain the differences in infectious dose required by these organisms to overcome the gastric acid barrier and
cause disease (about 10 organisms for *E. coli* and *Shigella* vs 10,000 for *Salmonella*). The primary reason for this difference is the production of glutamate decarboxylase, an enzyme missing from *S. enterica*. Besides the enzyme proper, this system includes a specific antiporter that links export of $\gamma$-amino butyric acid (the decarboxylation product of glutamate) to the import of more glutamate. The pH optimum for this enzyme is around pH 5 and thus it does not play a role until internal pH falls to that level. Although initially thought to have a part in consuming and extruding intracellular protons, how this system really helps the cell survive pH 2 environments is not clear. Another organism that uses this system is the Gram-positive *Lactococcus lactis*.

In addition to glutamate decarboxylase, *Lactococcus* uses an arginine deiminase (ADI) pathway to generate ATP during acid stress. ADI converts arginine to citrulline and ammonia, ornithine transcarbamylase converts the citrulline to ornithine and carbamyl phosphate, and carbamate kinase cleaves carbamyl phosphate to ammonia and CO$_2$, generating an ATP in the process. The system also requires an arginine/ornithine antiporter to rid the cell of the end-product ornithine while importing new arginine substrate. The ammonia generated can alkalinize the environment, but since this system also helps survival of dilute cultures, the ATP generated must substantially aid in survival.

*Helicobacter pylori*, an important cause of gastric ulcers and cancer, utilizes another potent acid resistance system to survive in the stomach. The organism constitutively produces a powerful urease, a nickel-containing metalloenzyme that converts urea to carbon dioxide and ammonia. Contrary to what would be expected, the pH optimum of urease is around 7.5, raising the question of why cells grown at pH 7.5 have very little urease activity. There are two reasons. Although the urease is constitutively produced, the degradation of transcripts encoding nickel-incorporating enzymes appear subject to pH control. In addition, UreI is a membrane transporter of urea that only becomes activated at acid pH. Therefore, urea cannot gain access to intracellular urease except at low pH. Like the glutamate decarboxylase system, it is not entirely clear how urease provides acid resistance. The ammonia produced does not seem to be sufficient protection, since the system works even at low cell density—a condition where the small amount of ammonia produced will not change external pH. *Yersinia enterocolitica* is another Gram-negative enteropathogen that uses urease to gain safe passage through the stomach.

Much remains to be learned regarding how different bacteria cope with acid or alkaline threats to survival. What is clear is that the microbial world has taken full advantage of what is available in the environment to counter this form of stress and that successful strategies have been shared among species.

**THERMAL STRESS AND THE HEAT SHOCK RESPONSE**

As with pH, microbes exhibit a wide range of temperatures at which they can grow (Table 1-4). For example, bacteria can be isolated from hot springs where temperatures can reach as high as 90°C or so (thermophilic bacteria) and from frozen tundra or polar caps where temperatures are below 0°C (psychrophilic bacteria). Most bacteria, however, prefer to grow at milder temperatures such as 20°C to 40°C (mesophilic bacteria). This latter group includes *E. coli* and *S. enterica* serovars as well as other animal and human pathogens.
Upon a shift from 30° to 42°C, E. coli and other bacteria transiently increase the rate of synthesis of a set of proteins called heat shock proteins (HSPs). Many of these HSPs are required for cell growth or survival at more elevated temperatures (thermotolerance). Among the induced proteins are DnaJ and DnaK, the RNA polymerase σ70 subunit (rpoD), GroES, GroEL (see “Protein folding and Chaperones” in Chapter 2), Lon protease (see “Proteolytic Control” in Chapter 2), and LysU. There are nearly 50 heat shock–inducible proteins identified in E. coli. They can be subdivided into those regulated by alternative σ factors and two component regulatory systems.

The σH regulon provides protection against cytoplasmic thermal stress. The E. coli rpoH locus (formerly called htpR) encodes a 32 kDa σ factor, alternatively called σ32, which redirects promoter specificity of RNA polymerase. The σH protein regulates the expression of 34 heat shock genes. The simple explanation for how heat shock increases expression of the σH regulon is that heat shock first causes an elevation in σH levels, which in turn increases expression of the σH target genes.

Although the principle is simple, the controls governing σH production are complex. First, a temperature upshift from 30° to 42°C results in the increased translation of rpoH message. Cis-acting mRNA sites within the 5′ region of rpoH message form temperature-sensitive secondary structures that sequester the ribosome-binding site. At higher temperatures, these secondary structures melt, thereby enabling more efficient translation of the rpoH message. In addition to the increased translation of rpoH message, the σH protein itself becomes more stable, at least transiently. The mechanism regulating proteolysis centers on whether σH associates with RNA polymerase. During growth at 30°C, σH can be degraded by several proteases including FtsH, HslVU, and ClpAP. However, if σH is bound to RNAP, σH is protected from degradation.

The cell uses the DnaK-DnaJ-GrpE chaperone team to interact with σH at low temperature, sequestering σH from RNA polymerase (Fig. 18-6). Failure to bind RNAP facilitates degradation of the σH factor. Upon heat shock, there is an increase in the number of other unfolded or denatured proteins that can bind to DnaK or DnaJ. This reduces the level of free DnaK/DnaJ molecules available to bind σH, allowing σH to bind RNAP, which protects σH from degradation. As the cell reaches the adaptation phase following heat shock, the levels of DnaK and DnaJ rise (both are induced by σH) and can again bind σH, redirecting it toward degradation. Nevertheless, even though σH degradation resumes, translation of rpoH remains high at the elevated temperature and σH continues to accumulate, although at a slower rate.

In addition to translational and proteolytic controls, production of σH is regulated at the transcriptional level via a feedback mechanism. There are four promoters driving rpoH expression, three of which are dependent on σ70, the housekeeping σ factor. The gene encoding σ70, rpoD, is also a heat shock gene induced by σH. So increased production of σH increases σ70, which increases transcription of rpoH. The fourth rpoH promoter is recognized by another σ factor, σE, encoded by rpoE. The heat shock response is also triggered by a variety of environmental agents such as ethanol, UV irradiation, and agents that inhibit DNA gyrase. Induction by all of these stimuli occurs through σH. How can all of these seemingly diverse stresses activate rpoH? The only explanation that appears reasonable is the accumulation of denatured or incomplete peptides. There is a potential alarmone that has been implicated in signaling expression of this global network. The molecule is diadenosine 5′, 5″′-P1, P4-tetraphosphate.
Fig. 18-6. Modulation of the heat shock response.
MICROBIAL STRESS RESPONSES

(AppppA), which is made by some aminoacyl–tRNA synthetases (e.g., lysU) at low tRNA concentrations. How this may influence the response is not known.

The σE regulon provides protection against extracytoplasmic stress. The hallmark of the Gram-negative cell is the existence of two membrane-bound subcellular compartments: the cytoplasm and the periplasm. Conditions in each of these compartments differ markedly. The cytoplasm is energy rich, reducing (low redox potential), and osmotically stable, whereas the periplasm lacks ATP, is oxidizing, and is in contact with the external milieu. Since optimal cell growth requires that the cell senses and responds to changes in these disparate subcellular compartments, it is not surprising that the stress responses in E. coli and S. typhimurium are compartmentalized into cytoplasmic and extracytoplasmic responses. The extracytoplasmic response pathways involve two partially overlapping signal transduction cascades: the σE and Cpx systems. These pathways are induced following the accumulation of misfolded proteins in the periplasm as a result of stresses such as high temperature, pH extremes, or carbon/energy starvation.

σE is a member of the extracytoplasmic function (ECF) subfamily of σ factors. In E. coli, σE is responsible for the transcription of up to 300 genes, including rpoH (σH), degP (htrA) encoding a periplasmic protease for the degradation of misfolded proteins, fkpA encoding a periplasmic peptidyl prolyl isomerase, and rpoE rseABC operon (see below).

The gene encoding σE, rpoE, is the first member of an operon followed by the genes rseA, B, and C (rse, meaning regulators of σE). RseA is a transmembrane protein whose cytoplasmic C-terminal domain interacts with σE, acting as an anti-σ factor. The periplasmic face of RseA binds to the periplasmic RseB. Extracellular stress in some way increases proteolysis of RseA by the periplasmic protease DegS, thus relieving the anti-σ effect of RseA on σE. It has been proposed that RseB and perhaps other periplasmic proteins involved in protein folding protect RseA from degradation by binding to the RseA periplasmic domain, capping the target site of DegS. Stress-induced misfolding of periplasmic proteins would titrate the RseA cap proteins off of RseA, rendering the anti-σ factor vulnerable to attack by DegS. The result would be increased activity of σE leading to increased levels of σE protein and RseA anti-σ (since they form an operon). The increased amount of σE will drive further expression of genes whose products handle the periplasmic damage while the increased level of RseA will enable the cell to downregulate the system once the capping proteins are again free to bind and protect RseA from DegS degradation.

A second system dedicated to protecting the periplasmic perimeter of the cell is the CpxRA two-component system (see Chapter 5) with CpxA playing the role of membrane-localized sensor histidine kinase and CpxR as the cytoplasmic response regulator. CpxA responds to envelope stress by autophosphorylation followed by phosphotransfer to CpxR. CpxR∼P activates expression of dsbA (disulfide oxidoreductase), ppiA and ppiD encoding peptidyl-prolyl isomerases, and, in E. coli, in conjunction with σE, degP (htrA). In addition, CpxR∼P activates transcription of cpxP encoding a small protein that negatively regulates the CpxAR regulon, probably by binding to a periplasmic domain of CpxA. The ability to autoactivate and then repress (via CpxP) enables a temporary amplification of the Cpx response that may be important to rescue cells from transitory stresses. PrpA and PrpB are type I serine/threonine phosphatases that also participate in the ECF pathway at least in part by affecting the phosphorylation level of CpxR.
B. subtilis has four classes of heat shock genes. All of them are not described here, but one is particularly interesting because of the mechanism used to control the genes. **Class I heat shock genes** include the major chaperones DnaK-DnaJ-GrpE and GroEL-GroES. Their transcription requires the housekeeping $\sigma$ factor, $\sigmaA$ ($\sigma^{70}$) and is negatively controlled by a repressor called HrcA. HrcA binds to a class I gene operator, a well-conserved 9 bp inverted repeat with a 9 bp spacer, called CIRCE. The CIRCE/HrcA regulon is normally repressed by the HrcA repressor but can be heat induced by inactivating the repressor. The molecular switch involves the chaperone GroE. Unlike $\sigma^{32}$ in E. coli, the GroE chaperones bind to and facilitate folding of HrcA and thereby modulate repressor function. Titration of GroE by stress-induced misfolded proteins results in lower HrcA repressor activity.

**NUTRIENT STRESS AND THE STARVATION — STRESS RESPONSE**

Nutrient starvation and other environmental stresses are routine occurrences for most bacteria. Situations of true “feast” or nonstress conditions are few and far between for microbes outside the laboratory. Thus, microbes are most frequently found in a state of nutrient starvation or stress-induced slow growth or nongrowth. Fortunately this is the case, because given bacterial growth rates achieved under nonlimiting conditions in the laboratory, if microbial growth were nonlimiting in natural or animal host environments, the consequences would be deadly.

**Starvation-Stress Response**

When E. coli, Salmonella, and many other nondifferentiating microbes are starved for an essential nutrient such as a carbon-energy (C) source, they respond by inducing the expression of up to 50 or so new proteins or preexisting proteins. The genetic and physiologic reprogramming that occurs is the starvation-stress response (SSR). The function of the SSR is to allow for the long-term starvation survival of the bacteria and to provide a general cross-resistance to a variety of other environmental stresses including extremes in temperature, pH, and osmolarity as well as exposure to reactive oxygen and nitrogen species and antimicrobial peptides/proteins.

It should be noted that a distinction must be made between starved cells and so-called stationary-phase cells. Typically, stationary-phase cells populate cultures that have stopped growing following exponential growth in rich or nonlimiting media, in contrast to starved cells, which populate cultures that have ceased growing in response to exhaustion of one or more defined nutrients. For stationary-phase cells, the condition that limits growth is not necessarily defined nor is it typically limited to a single stress; for starved cells, the limitation/stress that restricts growth is defined. Another key difference is that stationary-phase cultures normally achieve a much higher cell density compared with starved cultures, which can have a significant effect on overall cellular responses and long-term survival. Furthermore the genes/proteins expressed in stationary-phase cells may or may not overlap with those expressed in starved cells. The SSR refers specifically to the response of starved cells.

In general, for Gram-negative bacteria, starved cells are morphologically and physiologically very different from log-phase cells. The initial response to carbon-energy source limitation is to try and avoid the stress by increasing expression or expressing
new uptake or scavenging systems to be able to utilize any nutrients that may become available. Persistence of the starvation-stress eventually results in a cell that is smaller, much more hardy, and metabolically efficient. This is mediated by the accumulation of at least two cellular nucleotides: cyclic 3′, 5′ adenosine monophosphate (cAMP; see “Catabolite Control” in Chapter 5) and guanosine 3′, 5′-bis(diphosphate) (ppGpp; see “Stringent Control” in this chapter). In addition, at least two alternative σ factors, σS (or σ38; see Chapters 2 and 5) and σE (see above under heat shock response), encoded by the rpoS and rpoE genes, respectively, are key SSR regulators. These regulators are general factors controlling responses to starvation stress in a more global manner. However, this is not sufficient for the regulation of some stress-responsive genes. Thus, additional regulators may be involved in the regulation of specific genes, indicating a discriminating complexity for the SSR and other stress responses.

Examples of some regulatory proteins include Fis (factor for inversion stimulation first characterized for its role in flagellar phase variation; see “Phase Variation” in Chapter 5), FadR (regulator of fatty acid metabolism, which binds to medium/long-chain fatty acyl-CoA molecules and represses fatty acid biosynthetic genes and activates fatty acid degradation genes; see Chapter 13), Lrp (leucine-responsive protein, which controls certain aspects of amino acid metabolism; see “Serine/Glycine Family” in Chapter 15), OxyR (see above), SpvA/SpvR (regulators of the Salmonella plasmid virulence or spv genes; see “Paradigms of Bacterial Pathogenesis” in Chapter 20), PhoP (response regulator that controls virulence factors; see “Paradigms of Bacterial Pathogenesis” in Chapter 20), IHF (integration host factor; see Chapter 5), and H-NS (“Nucleoid Structure” in Chapter 2).

Some of the physiologic changes that occur during SSR include the expression of new or higher-affinity nutrient utilization systems to scavenge the environment for carbon-energy sources and other nutrients; the degradation of cellular RNA, proteins, and fatty acids; the reduction in the number of ribosomes; altering of the amounts and types of lipid components in the cytoplasmic membrane; an increase in the relative amounts of lipopolysaccharide or LPS in the outer membrane of Gram-negative bacteria; and the condensation of chromosomal DNA in order to protect it from damage.

**Stringent Control**

When bacteria experience conditions that limit the availability of one or more amino acids (shift from a rich medium to a minimal medium) or exhaust their primary carbon source, growth stops temporarily and rapid adjustments in metabolism are made. These include decreasing the rates of RNA accumulation (particularly stable RNAs such as rRNA and tRNA) and DNA replication, as well as reducing the biosynthesis of carbohydrates, lipids, nucleotides, peptidoglycan, and glycolytic intermediates. The transport of many macromolecular precursors into the cell is also shut down. This set of responses, characterized best as a response to amino acid starvation, is referred to as the **stringent response** or **stringent control**. The stringent response collectively enhances cellular viability during periods of amino acid or energy limitation and allows rapid recovery and reinitiation of growth when conditions improve.

For rapidly growing cells, a major amount of the available energy is used for ribosome synthesis. Therefore, blocking ribosome synthesis under amino acid starvation
conditions is a major mode of energy conservation. Conditions causing a stringent response lead to an abrupt change in the rate of ribosome synthesis caused by inhibiting the transcription of genes encoding ribosome-associated components such as tRNA, tRNA, and mRNA for ribosomal proteins. When starved for amino acids, bacterial cells rapidly accumulate millimolar concentrations of two unusual nucleotides: guanosine pentaphosphate (guanosine 5′-triphosphate-3′-diphosphate, ppGpp) and guanosine tetraphosphate (guanosine 5′-diphosphate-3′-diphosphate, ppGpp). These nucleotides, first referred to as Magic Spot II and Magic Spot I, respectively, accumulate in E. coli and Salmonella as well as other bacteria during amino acid limitation.

Starvation for a specific amino acid will lead to an increase in the corresponding uncharged tRNA species. As the ratio of charged to uncharged tRNA falls, ribosomes stall on mRNAs when encountering the codon for that amino acid. Synthesis of ppGpp is triggered by the repeated binding of the uncharged tRNA molecules to the stalled ribosome. On the ribosome, the product of the relA gene (RelA, stringent factor), a ribosome-bound pyrophosphotransferase (pppGpp synthetase I) present on about 1% of ribosomes, catalyzes the formation of ppGpp from GTP and ATP, as shown in Figure 18-7. A second, ribosome-independent route to ppGpp also exists involving the spoT product (pppGpp synthetase II). SpoT is responsible for basal levels of (p)ppGpp. The enzyme ppGpp phosphohydrolase (gpp) is the major pppGpp hydrolase, degrading pppGpp to ppGpp. SpoT appears to have two functions: one in the synthesis of pppGpp and one in the degradation of ppGpp to GDP plus pyrophosphate.

A major result of the stringent response is a reduction in the rate of stable RNA accumulation—a response strongly correlated with the rise in (p)ppGpp concentration in the cell. Several studies suggest that RNA polymerase is a target of ppGpp action and that the regulated process during stringent control is transcription itself. The probable target is the β-subunit of RNA polymerase (see Fig. 2-16c in Chapter 2). Both transcription initiation and polymerase pausing (important in certain regulatory mechanisms and transcription termination) are postulated as the major targets of ppGpp action. Thus, ppGpp would reduce the affinity of RNA polymerase for rRNA promoters or inhibit elongation of polymerase. Either effect will obviously lower the amount of rRNA available for ribosome synthesis, which can subsequently affect the synthesis of ribosomal proteins. On the other hand, ppGpp has also been found to stimulate transcription of several amino acid biosynthetic operons (e.g., his) and possibly other genes. Thus, it appears that ppGpp can act either as a negative effector or a positive effector depending on the target gene.

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**Fig. 18-7. Stringent control.** (a) Ribosome-dependent and -independent pathways for the biosynthesis of guanosine 5′-diphosphate-3′-diphosphate. ppG, guanosine 5′-diphosphate; ppG, guanosine 5′-triphosphate; ppA, adenosine 5′-triphosphate; adenosine 5′-monophosphate; ppGpp, guanosine 5′-diphosphate-3′-diphosphate; ppGpp, guanosine 5′-triphosphate-3′-phosphate; pp, inorganic pyrophosphate. Ndk, nucleoside 5′-diphosphate kinase; Gpp, ppGpp 5′-phosphohydrolase. (b) Role of (p)ppGpp in maintaining an efficiently balanced pool of amino acids. In this example, histidine levels have fallen below optimum. The ensuing accumulation of ppGpp stimulates expression of the his operon while slowing translation and ribosome synthesis. As growth slows, the other amino acids accumulate and repress their own synthesis. Restoration of histidine levels shut off (p)ppGpp synthesis, allowing the resumption of normal ribosome synthesis rates.
MICROBIAL STRESS RESPONSES

(a) ppGpp

pppGpp

ppGpp

ppA

pppG

RIBOSOME DEPENDENT

RelA

RIBOSOME INDEPENDENT

SpoT

Gpp

pppA

pppGpp

ppG

Ndk

(b) his BIOSYNTHETIC GENES

STIMULATES EXPRESSION

[Low HIS]

High Uncharged tRNA level

TRIGGER ppGpp SYNTHESIS

OTHER AMINO ACID BIOSYNTHETIC GENES

REPRESSION

[Other Amino Acids]

HIGH Levels

Other Effects

Slows rRNA Synthesis
In vivo, the synthesis of (p)ppGpp on the ribosome is controlled by the charging of total tRNA species as a function of intracellular concentrations of the 20 amino acids. The ratio of charged to uncharged tRNA appears to be of central importance rather than the overall level of charged tRNA. It would appear that ppGpp is a component of a sensing mechanism that functions to adjust the synthesis, for example, of histidine biosynthetic enzymes with respect to the need for histidine relative to the total amino acid concentration of the cell as well as in the growth medium. Thus, along with the operon-specific attenuator mechanism that responds to the need for histidine, specifically altering the level of ppGpp enables the organism to sense how the supply of histidine, in this example, compares with the availability of all the amino acids in the cell—a kind of fine-tuning mechanism that maintains the correct relative levels of each amino acid.

EXTREMOPHILES

Although, the stress responses described above are important for the survival of most bacteria during exposure to the various stresses, some bacteria view stresses such as extremes in pH or temperature as a lifestyle choice. These bacteria survive and thrive in conditions that would send other microbes to their maker. Acidophiles typically survive and grow at an external pH (pH₀) below 4.0; alkalophiles grow only at a pH₀ of 8.0 or above. Similarly, thermophiles grow at temperatures generally around 50° to 65°C and psychrophiles grow at temperatures as low as 5° to 10°C.

Despite growing at pH 2–4, acidophilic bacteria maintain their cytoplasmic pH at 6.0 or higher. Consequently, there has been considerable interest in determining the mechanism whereby this large pH differential (∆pH) is maintained. It is generally agreed that maintenance of this large transmembrane ∆pH is energy dependent and that its maintenance requires a transmembrane electrical potential (∆Ψ) that is positive inside (the reverse of that found in neutralophilic bacteria). A reverse transmembrane potential has been observed in both Bacillus acidocaldarius and Thiobacillus acidophilus. If this transmembrane potential is abolished, the ∆pH collapses. Despite the large ∆pH, the cytoplasmic pH is extremely stable. The cytoplasmic buffering capacity of T. acidophilus is responsible for the cytoplasmic pH homeostasis in metabolically comprised cells. When a large influx of H⁺ occurs, the cytoplasmic buffering capacity prevents drastic changes in pH. In addition, the resultant increase in positive membrane potential due to this influx of H⁺ eventually leads to cessation of further H⁺ influx.

Studies with a heterotrophic, mesophilic, obligate acidophile provide additional insights into the nature of changes in membrane potential under active and inactive conditions (Fig. 18-8). Starving cells of this acidophile continue to show a ∆pH of about 1.7 but exhibit changes in membrane potential (∆Ψ) and proton motive force (∆ϕ) that are just the opposite of those seen under conditions of optimal nutrition. The linkage of the transient H⁺ influx with the rise of ∆Ψ and the cytoplasmic buffering capacity play central roles in acidophily. It is considered that the same impermeant cellular macromolecules can account for both. Thus, the ∆Ψ represents a Donnan potential (electrochemical potential across a membrane at electrochemical steady state) that is offset in active cells by energy-dependent H⁺ extrusion.

Many bacteria display optimal growth in alkaline media. Although a number of these are Bacillus species, alkalophilic strains of Micrococcus, Pseudomonas, Clostridium,
Fig. 18-8. Model for bioenergetic parameters in an obligately acidophilic bacterium at an outside pH (pH₀) of 4.0 in inactive (a) and respiring (b) cells. Left and right arrows indicate the forces impelling the H⁺ ion flux into and out of the cells and are drawn to scale. There is no H⁺ ion flux in inactive cells. In active cells H⁺ ion influx equals H⁺ ion efflux. (Source: From Goulborne et al., 1986.)

The photosynthetic bacterium *Ectothiorhodospiras*, and many others have been reported. These alkalophiles can grow only at pH levels of 8.0 to 11.5. A true alkalophile maintains a cytoplasmic pH of 9.0 or lower even at an external pH of 11.0, thus growing optimally under conditions in which the cytoplasm is more acidic than the external medium. The alkalophiles *B. firmus* and *B. alcalophilus* both depend on the activity of a Na⁺/H⁺ antiporter to achieve acidification of the cytoplasm relative to the exterior. A nonalkalophilic strain of *B. firmus* isolated after mutagenesis of the alkalophilic strain lacks the Na⁺/H⁺ antiporter. The inability of *B. firmus* to grow at neutral pH is not due to excessive acidification but is related to a failure of respiratory activity to generate a transmembrane electrical potential that is high enough to maintain certain cellular functions such as Na⁺/solute symport and motility. Nonalkalophic mutant strains of *B. alcalophilus* exhibit loss of Na⁺/H⁺ antiporter activity and Na⁺ coupling of solute transport as well as lower amounts of membrane-bound cytochromes and a membrane-bound chromophore.

**Thermophilic organisms** have been studied intensively in an effort to determine the mechanisms whereby these organisms not only survive but also prefer higher temperatures for growth. Chemical differences in the lipids found in thermophiles, higher metabolic rates facilitating rapid resynthesis of heat-denatured cellular components, and macromolecules with higher thermostability have all been suggested as mechanisms for greater heat tolerance in thermophiles. Thermophilic organisms contain lipids with higher melting points than those found in mesophiles, suggesting that the temperature at which the major lipid components of the cell melt may establish the upper limit for growth. In yeast, a direct correlation has been established between the growth temperature limits and the degree of unsaturation in mitochondrial lipids.
The lower the temperature limit of growth, the greater the degree of lipid unsaturation. The membrane lipid compositions of thermophilic yeasts are distinguished by the higher percentage (30–40%) of saturated fatty acids as compared with mesophilic and psychrophilic species. Psychrophilic yeasts contain approximately 90% unsaturated fatty acids, 55% of which is linolenic acid ($\text{C}_3\alpha_{18:3}$).

Certain lines of evidence indicate that thermophilic organisms possess an intrinsic thermostability that is independent of any transferable, stabilizing factors. Membranes with greater heat stability, more rapid metabolic and growth rates, and factors that impart structural stability and greater inherent heat stability to individual protein macromolecules have been implicated as contributing factors. The greater heat stability of protein molecules from thermophilic organisms appears to reside in their ability to bind certain ions more tightly, thereby enhancing the establishment of more stable conformations. Proteins from thermophiles also contain increased levels of hydrophobic amino acids as compared to those from mesophiles. Although macromolecules from thermophiles are more thermostable, they are, for the most part, physicochemically similar to their mesophilic counterparts with regard to molecular weight, subunit composition, allosteric effectors, amino acid composition, and major amino acid sequences.

Certain attributes of protein thermostability are due to subtle changes in structure and to alterations in hydrogen bonding, hydrophobic interactions, and other noncovalent activities. For example, the thermostability of the tryptophan synthetase of *E. coli* is increased by amino acid substitutions that alter the hydrophobicity of the molecule in the absence of gross changes in conformation. In the thermophile *B. stea rothermophilus*, a marked increase in thermostability is engendered by the presence of lysine in place of threonine in the plasmid-encoded enzyme that inactivates kanamycin. The nucleotide sequence of the plasmid encoding this enzyme in *B. stearothermophilus* differs by only one base from the plasmid coding for the identical enzyme in the mesophilic *S. aureus*. The lysine substitution permits increased electrostatic bridging with little significant change in the three-dimensional structure.

Isolation of thermophilic mutants of *B. subtilis* and *B. pumilus* and transformation of the thermophilic trait to mesophilic strains provides direct genetic evidence that the thermophilic trait is a phenotypic consequence of at least two unlinked genes. Additional studies of this type should provide information that could shed light on the specific nature of thermophily.

**Psychrophilic organisms**—those that exhibit an optimal temperature for growth at 15°C or lower and a maximum temperature for growth at 20°C—are defined as obligate psychrophiles to differentiate them from organisms that can grow at low temperatures but are actually mesophiles in terms of their optimum growth temperatures. Although some confusion still exists with regard to the proper nomenclature and cardinal temperatures that differentiate psychrophiles from organisms that are psychrotolerant or psychrotropic, those organisms that grow well at temperatures at or below the freezing point of water are considered to be true psychrophiles.

The psychrophilic yeast *Leucosporidium* grows well at $-1$ °C. In this organism there is a positive correlation between the growth temperature and the unsaturated fatty acid composition of the cell lipids. At subzero temperatures ($-1$ °C) with ethanol as the substrate, 90% of the total fatty acid is unsaturated, with linolenic acid (35–50%) and linoleic acid (25–30%) predominating. At temperatures close to the maximum for growth, linolenic acid accounted for less than 20% of the total fatty acid, and oleic acid
(20–40%) and linolenic acid (30–50%) were the major components. Marked changes also occurred in the cytochrome composition of the cell, \(a + a_3 : b : c\) were 1:1:2.9 at 8°C with glucose as the substrate, whereas at 19°C they were 1:2.3:16.7. Thus, it can be concluded that changes in membrane structure and composition are fundamental to temperature adaptation in psychrophilic yeasts.

The psychrophilic bacterium *Micrococcus cryophilus* also undergoes alteration in its lipid composition with changes in growth temperature. Cultures of this organism continue to grow without a lag following a sudden increase in temperature from 0°C to 20°C (upshift) or a reciprocal decrease (downshift). The growth rate changes gradually to that typical of cultures grown isothermally at the final temperature. After a temperature downshift, the phospholipid acyl chain length begins to change immediately, whereas there is a delay following upshift. However, the final fatty acid composition is attained within the same number of cell division times after an upshift or downshift. It appears, therefore, that this psychrophile is more stressed by a sudden increase in growth temperature than by a sudden decrease.

Studies on the viscosity and phase transition temperatures of lipids isolated from psychrophilic, psychrotropic, and mesophilic organisms are able to adjust their lipid-phase transition temperature to the growth temperature. By comparison, a psychrophilic *Clostridium* synthesizes lipids that have the same phase transition temperature after growth at different temperatures. This lack of growth temperature–inducible regulation of lipid-phase transition temperatures appears to be a molecular determinant for psychrophily in this organism. Comparisons of the properties of triosephosphate isomerase purified from psychrophilic, mesophilic, and thermophilic clostridia indicate that the purified enzymes have the same molecular weight, subunit molecular weight, and susceptibility to the active site-directed inhibitor, glycidol phosphate. However, their temperature and pH optima, as well as stabilities to heat, urea, and sodium dodecyl sulfate (SDS), differ markedly.

**SUMMARY**

Our understanding of stress responses has made great strides over the last several years, especially in the responses of microbes other than *E. coli* and *Salmonella*. However, the ever-increasing realization of the complexity and interrelationships of stress responses clearly indicates that we still have a great deal to uncover. Since most microbes spend the vast majority of their life under environmental stress, further knowledge of stress responses is critical to the complete understanding of microbial physiology. Information obtained may contribute to the development of new vaccines, new disease therapies, new agricultural strategies, new food safety procedures, and new antimicrobial agents. Thus, the study of stress responses continues to be an exciting area of basic and applied biomedical and biological research.

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Aerobic to Anaerobic Transitions


Oxidative Stress


610 MICROBIAL STRESS RESPONSES


**pH Stress and Acid Tolerance**


**Thermal Stress and the Heat Shock Response**


Nutrient Stress and the Starvation Stress Response


Stringent Control


Extremophiles


